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Implications for breeding

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Phenotypic and genetic diversity in wild and domesticated timothy and related *Phleum* species: implications for breeding

Abstract

Timothy (*Phleum pratense* L.) is a perennial grass widely cultivated for forage production and grazing in cold temperate regions. Higher temperatures and longer growing seasons caused by climate change require new adapted timothy cultivars. To broaden the gene pool for breeding, the overall aim was to understand the phenotypic and genetic diversity in the hexaploid timothy and the related diploid species *P. nodosum* and the tetraploid *P. alpinum*. In total 246 wild and domesticated accessions of different geographical origins were evaluated as individual plants in field and greenhouse. Large diversity among species and groups of accessions was found for biomass, development and production of the different tiller types; vegetative, generative and non-flowering elongated tillers. Within timothy, no significant difference was found in biomass between wild and cultivar groups of accessions. However, cultivars showed later emergence and tillering, but reached stem elongation and heading earlier than wild accessions, showing that an overall faster development has been favored in cultivars. Timothy cultivars had a higher percentage of generative tillers than wild accessions, which had more vegetative tillers that increased with latitude of accession origin. *P. nodosum* cultivars produced the highest number of non-flowering elongated tillers of all accessions and species, while no such tillers were found in *P. alpinum*. Thirty-three of the accessions were evaluated for genetic diversity, population differentiation, and signature of selection using whole genome resequencing. The genomic analyses revealed a considerably higher number of single nucleotide polymorphisms (SNPs) in timothy and *P. nodosum* compared to *P. alpinum*. A more extensive pattern of linkage disequilibrium, a lower rate of observed heterozygosity, and a higher rate of inbreeding were also found in *P. alpinum*, indicative of partial or complete self-pollination in this species. In timothy and *P. nodosum*, low relative divergence (F_{ST}) was observed when comparing cultivars with wild accessions, indicating that there has been little genetic divergence from the wild populations due to domestication. However, the genomic pattern of F_{ST} and haplotype genome scan tests showed that some genomic regions were highly differentiated, thus indicating regions affected by positive selection. These regions are involved in crucial biological processes, exemplified by the coding genes *g22404*, *g39371*, and *g35607*, which play key roles in biosynthesis of structural polymers like lignin and its precursors, which affect forage nutritive value and digestibility. The identified phenotypic diversity and genomic resources can be integrated in future breeding.

Keywords: accessions, breeding, forage, grass, genetic resources, perennial, population differentiation, signature of selection

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Fenotypisk och genetisk diversitet hos vilda och domesticerade accessioner av timotej och besläktade arter: betydelse för växtförädling

Abstrakt

Timotej (*Phleum pratense* L.) är ett flerårigt gräs som odlas för foderproduktion och bete i tempererade områden. Högre temperaturer och längre växtsäsonger till följd av klimatförändringarna kräver nya anpassade timotejsorter. För att bredda genpoolen för växtförädling var det övergripande syftet med denna avhandling att förstå den fenotypiska och genetiska diversiteten hos den hexaploida timotejen och de besläktade arterna *P. nodosum* som är diploid och *P. alpinum* som är tetraploid. Totalt 246 vilda och domesticerade accessioner av olika geografiskt ursprung utvärderades i fält och växthus. Stor diversitet bland arter och grupper av accessioner upptäcktes för biomassa-produktion, utveckling och bildandet vegetativa skott, icke-blommande långsträckta strån och reproduktiva strån. Gruppen av vilda accessioner och gruppen av sorter hos timotej visade ingen signifikant skillnad i biomassa. Timotej-sorterna hade dock längre stadier för uppkomst och tidig tillväxt än de vilda accessionerna men nådde stråskjutning och blomning tidigare. Detta visar att en snabbare utveckling och tillväxt har gynnats hos de växtförädlade sorterna. Timotej-sorterna bildade även en högre andel reproduktiva strån än de vilda accessionerna. De vilda accessionerna hade en större andel vegetativa skott som ökade med breddgraden för deras ursprungliga växtplats. Sorter av *P. nodosum* bildade det högsta antalet av icke-blommande långsträckta strån jämfört med alla accessionsgrupper och arter medan denna typ av strån saknades hos *P. alpinum*. Trettiotre av accessionerna utvärderades med avseende på genetisk diversitet, populations-differentiering och selektions-signatur med hjälp av hel-genom-resekvensering. De genomiska analyserna avslöjade ett betydligt högre antal nukleotidpolymorfismer (SNP) hos timotej och *P. nodosum* jämfört med hos *P. alpinum*. Ett mer omfattande mönster av kopplings-ojämnvikt mellan gener, en lägre grad av observerad heterozygoti och en högre grad av inavel hittades också i *P. alpinum*, vilket tyder på partiell eller fullständig självpollinering hos denna art. Hos timotej och *P. nodosum* observerades låg genomisk relativ divergens (F_{ST}) när vilda accessioner och sorter jämfördes, vilket tyder på att domesticeringen har lett till en låg genetisk divergens från de vilda populationerna. Det genomiska mönstret för F_{ST} och haplotyp-genomscannings-tester visade dock att vissa genomiska regioner var mycket differentierade, vilket tyder på att dessa regioner har påverkats av positiv selektion. Dessa genomiska regioner är involverade i viktiga biologiska processer, vilket exemplifieras av de kodande generna *g22404*, *g39371* och *g35607*. Dessa gener har nyckelroller i biosyntesen av strukturella polymerer som lignin och dess intermediärer, vilket påverkar näringsvärdet och smältbarheten hos foder. Kunskapen om den fenotypiska diversiteten och de identifierade genomiska resurserna kan integreras i framtida växtförädling.

Nyckelord: accessioner, foder, genetiska resurser, gräs, perenn, populations-differentiering, selektions-signatur, växtförädling

Preface

It is a moment of joy, excitement, and gratitude as I reflect on the wonderful opportunity I have had to contribute to this project. Along the way, I have been fortunate to meet great people, including my supervisors, fellow project members and people at the department who have provided me with invaluable support and inspiration. These four years have been full of memorable experiences, from setting up the trials and to working in the laboratory, analysing data and the art of scientific writing. Each step has not only contributed to the progress of my research but has also allowed me to find out new aspects of my scientific identity. This thesis is evidence of the commitment and teamwork of several individuals. I sincerely hope that you will find inspiration and enlightening information as you read through its contents.

Warm regards,
Yousef Rahimi

Dedication

To my wonderful wife Hiva, who has always been there for me through every stage of our shared life.

To my loving mother, who encouraged her son to follow his dreams.

To my caring father, who devoted his life to creating a beautiful family.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Rahimi, Y.**, Bedada, G., Moreno, S., Gustavsson, A. M., Ingvarsson, P. K., Westerbergh, A. (2023). Phenotypic diversity in domesticated and wild timothy grass, and closely related species for forage breeding. *Plants*, 12 (19), 3494-3514. doi.org/10.3390/plants12193494

II. **Rahimi, Y.**, Bedada, G., Gustavsson, A. M., Ingvarsson, P. K., Lundquist, P. O., Westerbergh, A. Development and tiller formation in wild and domesticated accessions of timothy (*Phleum pratense*) and its relatives *P. nodosum* and *P. alpinum*. (manuscript)

III. **Rahimi, Y.**, Bedada, G., Westerbergh, A., Ingvarsson, P. K. Genetic diversity, population structure, and linkage disequilibrium in three *Phleum* species. (manuscript)

IV. **Rahimi, Y.**, Bedada, G., Westerbergh, A., Ingvarsson, P. K. Genomic landscape of diversity, recombination rate, and signature of selection in *Phleum* species with a focus on forage grass timothy. (manuscript)

Related Publications

I. Moreno, S., Bedada, G., **Rahimi, Y.**, Ingvarsson, P. K., Westerbergh, A., Lundquist, P. O. (2023). Response to waterlogging stress in wild and domesticated accessions of timothy (*Phleum pratense*) and its relatives *P. alpinum* and *P. nodosum*. Plants, 12 (23), 4033-4053. doi.org/10.3390/plants12234033

The contribution of Yousef Rahimi to the papers included in this thesis was as follows:

I. Planned the study together with the co-authors, conducted most of the experimental work, highly involved in the analysis of the data, wrote the original draft, and highly involved in the editing before publication.

II. Planned the study together with the co-authors, conducted the experimental work, highly involved in the analysis, and contributed to the writing of the manuscript.

III. Planned the study together with the co-authors, conducted the experimental work, highly involved in the analysis of the data, and wrote the original draft.

IV. Planned the study together with the co-authors, conducted the experimental work, highly involved in the analysis of the data, and wrote the original draft.

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Abbreviations

DTB	Days to booting
DTE	Days to emergence
DTH	Days to heading
DTS	Days to stem elongation
DTT	Days to tillering
DW	Dry weight
EHH	Extended Haplotype Homozygosity
ELONG	Vegetative elongated tillers
F_{ST}	Fixation index
FW	Fresh weight
GCA	General combining ability
GEBVs	Genomic breeding values
GEN	Generative tillers
GS	Genomic selection
GWAS	Genome-wide association studies
LD	Linkage disequilibrium
MAS	Marker-assisted selection
PH	Plant height
QTL	Quantitative trait loci
SCA	Specific combining ability
SNP	Single nucleotide polymorphism
TTN	Total tiller number
VEG	Vegetative tillers
WGS	Whole-genome resequencing
Δ	Time between developmental stages
π	Nucleotide diversity

1. Introduction

1.1 Challenges in food production

"Zero Hunger" is one of the UN Sustainable Development Goals, which aims to end hunger, achieve food security, improve nutrition, and advance sustainable agriculture globally. This goal is, however, challenged by a growing world population. By 2050, the world population is expected to have reached to a total of 9 billion people (Cohen 2001; Kingston-Smith et al. 2013). The size of the food demand is debated, but a doubling is required by 2050 according to the agroindustry (Von Lampe et al. 2014; Steensland and Zeigler 2021), or 40-55% according to the FAO (FAO 2019). Moreover, climate change poses challenges to agricultural productivity and food security, as rising temperatures and unpredicted weather patterns disrupt optimal conditions for crop cultivation (Steensland and Zeigler 2021). On the other hand, modern agriculture with high inputs of fertilizers, pesticides and herbicides introduces negative impacts on the climate and environment such as increased soil erosion, nutrient leaching and greenhouse gas emission (Lichtenberg 2002; Clark and Tilman 2017; Tudi et al. 2021).

The food supply is dependent on the livestock sector, which is supported by the production of meat and dairy products. Ruminants, in particular, can efficiently convert non-human-consumable plant material into high-quality protein (Kingston-Smith et al. 2013). With a growing human population, the demand for global milk production is predicted to increase from 580 in 1999/2001 to 1043 million tonnes per year in 2050, and the amount of meat produced from 200 to 470 million tonnes per year in 2050 (FAO 2006). Thus, the grazing intensities are expected to increase with 53.13% from 0.064 TLU (Tropical Livestock Units) per ha in 2000 to 0.098 TLU per ha in 2050.

Today, forage crops are grown on a significant part of agricultural land worldwide. For instance, in the European Union, 63% of agricultural land is used for forage production (Ahamed et al. 2023), and in Sweden, the corresponding figure is 40% (The Swedish Board of Agriculture 2023). The perennial grass timothy (*Phleum pratense* L. subsp. *pratense*) is the most cultivated forage crop in Sweden and other regions with cold temperate climates due to its winter hardiness, high forage yield and forage quality (Østrem et al. 2013; Helgadóttir et al. 2016). It can be grown in mixture (Figure 1) with other forage grasses such as tall fescue (*Festuca arundinacea* Schreb.), perennial ryegrass (*Lolium perenne* L.) and legumes like red clover (*Trifolium pratense*) (Swift 1977; McBratney 1981; Frame and Harkess 1987; Kunelius et al. 2006).

Figure 1. Mixture of timothy, red and white clover, and *Lotus corniculatus*. Photo by Anna Westerbergh.

In eastern Canada, timothy is the primary forage grass species grown alongside alfalfa (*Medicago sativa* L.) (Pomerleau-Lacasse et al. 2019). Moreover, in the establishment year of a perennial legume-grass pasture such as timothy-alfalfa, spring cereals (oats, barley, wheat) can be grown as companion crops (Spaner and AG Todd 2004).

Forage crop production in Sweden and other Nordic countries is restricted by short growing seasons, recurrent frosts during spring and fall, and low temperatures during winter (Helgadóttir et al. 2016). Global warming on the other hand is expected to increase crop productivity in these regions by extending the growing seasons and by increasing the number of frost-free intervals (Olesen et al. 2011; Höglind et al. 2013). Höglind et al. (2013), however, predicted an increased potential for frost damage, particularly during winter, in certain eastern Baltic Sea regions for timothy, and in a greater number of locations both east and west of the Baltic Sea for perennial ryegrass. The predicted weather fluctuations are also expected to bring about changes in precipitation, potentially leading to drought conditions for crops (Rapacz et al. 2014; Helgadóttir et al. 2016).

Developing new cropping and cultivating practices to cope with changes in the climate will be important. However, most of our crops are not adapted to ongoing changes in precipitation and temperature and the subsequent abiotic and biotic stresses. Development of new stress tolerant crops and cultivars are therefore needed. Breeding programs are dependent on efficient integration of genetic resources with traits of importance for improved productivity and adaptation to climate changes. These genetic resources are identified by evaluation of diverse collections in pre-breeding research projects.

Active transfer of knowledge from research into practical forage crop improvement is crucial for successful breeding programs (Helgadóttir 2014; Helgadóttir et al. 2016). The first National Food Strategy for Sweden was launched by the Swedish government in 2017 (Bucht 2016). The overall goal is to develop a competitive food supply chain that increases food production while the national environmental objectives are achieved. In one of the three strategic areas of the Food Strategy the development of knowledge and innovation systems is supported to increase productivity, innovation and sustainable food production and consumption. The establishment of SLU Grogrund–Center for Plant Breeding of Food Crops (https://www.slu.se/centrumbildningar-och-projekt/grogrund/) is part of this effort.

This thesis is a project within SLU Grogrund and focuses on the forage crop timothy and the related species *P. nodosum* and *P. alpinum*. The overall aims are to contribute with knowledge to further pre-breeding studies and to identify potential genetic resources of use for breeders in the development of new improved timothy cultivars for cultivation at northern latitudes. To achieve this aim we have investigated the phenotypic and genetic diversity in domesticated and wild accessions of timothy and the two related *Phleum* species from different geographical areas. Moreover, by studying the genetic differentiation, population structure, linkage disequilibrium and signatures of selection, we have further increased our knowledge of the diversity among the *Phleum* accessions and species.

1.2 Opportunities of perennial forage crops

While the perennial characteristics of timothy and other perennial forage crops may pose challenges in the breeding due to longer selection cycles, the cultivation of these perennial crops presents environmental advantages that can contribute to sustainability efforts in agriculture. With their perennial growth, the soil will be covered year around, reducing nutrient leaching and soil erosion and the binding and storage of carbon in the soil will increase (Persson et al. 2008; Culman et al. 2013; Poeplau et al. 2015; Rasche et al. 2017; Rui et al. 2022). Moreover, with less tillage of the soil, and less inputs of energy, fossil fuels and fertilizers needed in perennial cropping systems, the emission of greenhouse gases will be reduced. The organic matter content and beneficial microbial activity will also increase, which will improve soil health. Since timothy and other perennial forage crops are widely grown at northern latitudes, these perennial cropping systems can contribute to a more sustainable agriculture. Due to the potential advantages, the desire to include perennial crops in agriculture has increased (Crain et al. 2021).

1.3 Timothy and related *Phleum* species

1.3.1 Usage, cultivation and morphology

Timothy is one of the most important forage crops in cool temperate and boreal regions. It is an outcrossing, short-lived perennial with shallow roots and, due to its winter hardiness, it is the preferred species for forage grass cultivation at higher latitudes in Northern Europe, North America and East Asia (Berg et al. 1996). The optimal time for planting timothy is in the early spring or late summer and biomass production is highest in the second year of establishment. Biomass production is determined by the tiller formation. The tillers emerge from buds in leaf sheaths at the swollen base of the stem (corms) and can grow to a height of 80 cm to140 cm. The leaves are flat, linear and pointed. The inflorescence (head) is formed of closely spaced spikelets that forms a cylindrical structure which resembles a spike (Figure 2). Due to their small size and fine texture, the seeds are easily dispersed by wind.

Figure 2. Timothy plant with tillers at booting and heading (left), plant with tillers at heading (middle) grown in a field north of Uppsala, Sweden (latitude 60ºN), timothy inflorescences (heads) with and without anthers (right). Photos by Yousef Rahimi.

Timothy grows quickly, which allows for two to four harvests per season depending on the length of growing season. However, cattle grazing and trampling damage the buds and corms in the crown, reducing tiller production. Another constraint on its persistence, whether in pure stands or mixed with perennial legumes and other forage grasses, is its shallow root system and susceptibility to drought (Medl et al. 2018).

Compared to other forage grasses, timothy has a high nutritive value and digestibility (Helgadóttir et al. 2014). Timothy forage quality is determined by the plant's developmental stage. Even though plants have reached flowering, the formation of vegetative tillers continues during spring growth. Most tillers develop into reproductive tillers, while some remain in the vegetative stage. In addition to vegetative tillers and generative tillers, that will form an inflorescence, timothy can also develop non-flowering elongated tillers (Virkajärvi et al. 2012). These tillers are arrested in the development and form leaves at the shoot apex instead of floral organs (Seppänen et al. 2010; Virkajärvi et al. 2012). The growth of these tillers is stimulated by a longer photoperiod, but not by low temperatures, and are formed both during spring growth and regrowth after harvest (Jokela et al. 2014; Jokela et al. 2015). The proportion of the three tiller types on a plant is likely to affect biomass production and forage quality since their proportions change during the season.

The vegetative tillers have a higher content of crude protein and a lower fiber content (cellulose, hemicellulose, pectins and lignin) than elongated and flowering tillers (Bélanger et al. 2001; Kobayashi et al. 2005). The quality and the digestibility are therefore usually highest during the early growth of the plant (Figure 3) (Bélanger et al. 2001; Gustavsson and Martinsson 2004).

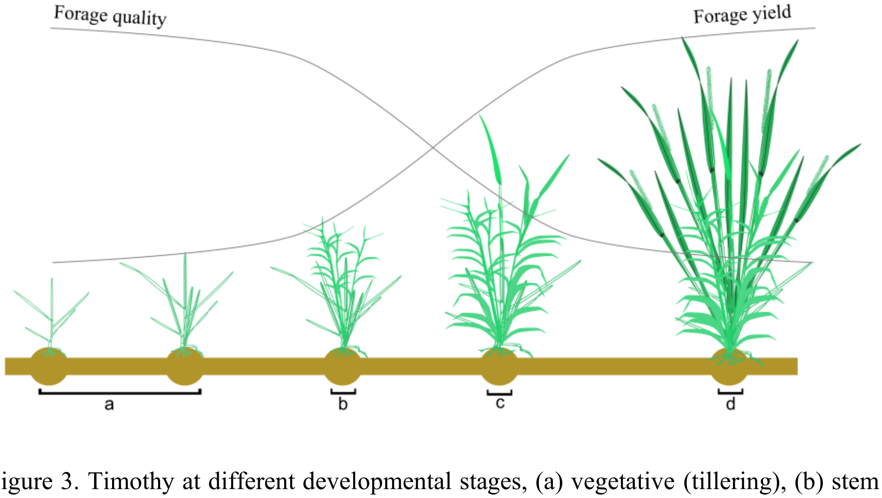


Figure 3. Timothy at different developmental stages, (a) vegetative (tillering), (b) stem elongation, (c) booting and (d) heading. The figure also shows the relationship between forage yield and forage quality, where the forage quality decreases with development and growth.

However, biomass production increases with age as the tillers elongate and reach the flowering stage. In contrast to other forage crops such as *Lolium perenne* and *Festuca arundinacea*, timothy can form elongated and heading tillers without vernalization (cold treatment) (Heide 1994; Seppänen et al. 2010). However, flowering is stimulated by vernalization, especially in northern accessions (Seppänen et al. 2010; Fiil et al. 2011a; Jokela et al. 2014; Jokela et al. 2015). Flowering and stem elongation are also stimulated by a longer photoperiod (Heide 1982; Heide 1994; Jokela et al. 2014; Jokela et al. 2015).

1.3.2 Taxonomy, origin and evolution

Timothy belongs to the Poaceae family and the genus *Phleum* (Tzvelev 1989; Clark et al. 1995; Group et al. 2001). The genus *Phleum* consists of 8 annual and 6 perennial species with varying ploidy levels from diploid to octaploid. *Phleum* is the third most significant forage genus in temperate regions after *Lolium* and *Festuca* (Stewart et al. 2005; Stewart et al. 2010). The genus *Phleum* is divided in four sections including: (a) *Phleum*, (b) *Chilochloa* (Beauv.) Dum., (c) *Achnodon* (Nees) Griseb., (d) *Maillea* (Parl.) Horn af Rantzien, and timothy belongs to the section *Phleum* (Stewart et al. 2010). The most widely grown species in this genus is timothy. Timothy is hexaploid (2n=6x=42).

The genetic relationship between different *Phleum* species and the origin of hexaploid timothy have puzzled scientists over the years and is still not fully understood. Research have suggested that the genus *Phleum* originated from an Asian ancestor that is closely related to or identical with the diploid *P. alpinum* subsp. *rhaeticum* Humphries (2n=2x=14) (Doğan 1991; Stewart et al. 2005). This ancestor migrated into Europe and underwent significant differentiation during the glacial eras and subsequent warmer periods and new *Phleum* species evolved locally in Europe (Stewart et al. 2010). Speciation has been facilitated by environmental dynamics as populations adapted to varied habitats, from lowlands to the Alps. Allotetraploid species emerged through hybridization between diploid forms and subsequent polyploidization events. Hexaploid timothy is suggested to have evolved through hybridization between an allotetraploid pratense (formed from progenies of *P. alpinum* subsp. *rhaeticum*) and the diploid *P. nodosum* (syn.

P. pratense L. subsp. *bertolonii* (DC.) Bornm. and *P. bertolonii* (DC.) Bornm., 2n=2x=14) in the Italian Alps (Stewart et al. 2010).

Based on genetic analysis, the similarity between hexaploid timothy and the *P. nodosum* was found in chloroplast trnL intron DNA sequences (Stewart et al. 2010). Genetic similarities were also found between timothy and *P. alpinum* subsp. *rhaeticum*. Moreover, cytological studies suggest the presence of two genomes of *P. nodosum* in hexaploid timothy (Cai and Bullen 1994; Joachimiak 2005). The genetic relationship between the three *Phleum* species including timothy, *P. nodosum* and a tetraploid *P. alpinum* are under investigation in our group at SLU, Uppsala, Sweden.

1.3.3 Distribution and habitats

Timothy spread to Northern Europe and was among the first *Phleum* species that were domesticated (Cai et al. 2013). Subsequent cultivation of domesticated timothy took place in North America during the early 1700s, initiated by European colonists and further promoted by John Herd and the Swedish immigrant Timothy Hansen (Tamaki et al. 2010), from whom the species name of timothy was inherited. This domestication history facilitated the widespread distribution of timothy across Europe, North America and further in Asia, Australia, and New Zealand (Figure 4a). *P. nodosum* can be found in Northern Europe, Spain, Portugal, Greece, and the Balkans (Figure 4b) and tetraploid *P. alpinum* can be found in Northern hemisphere and in mountain regions in Europe, America, and Asia (Figure 4c).

Wild populations of the different *Phleum* species are adapted to various habitats, including grasslands and meadows, and they originate from different geographical regions. Wild populations of timothy can be found throughout the Nordic countries and grow at low elevations on meadows and in human-impacted and disturbed soils. Wild populations of *P. nodosum* are commonly found on meadows at low elevations. At northern latitudes, it has a more southern distribution than *P. alpinum*. Wild populations of *P. alpinum* usually grow on meadows, riverbanks, roadsides and in birch forests at high elevations. At northern latitudes *P. alpinum* is found in Iceland, Norway, northern Sweden, and northern Finland.

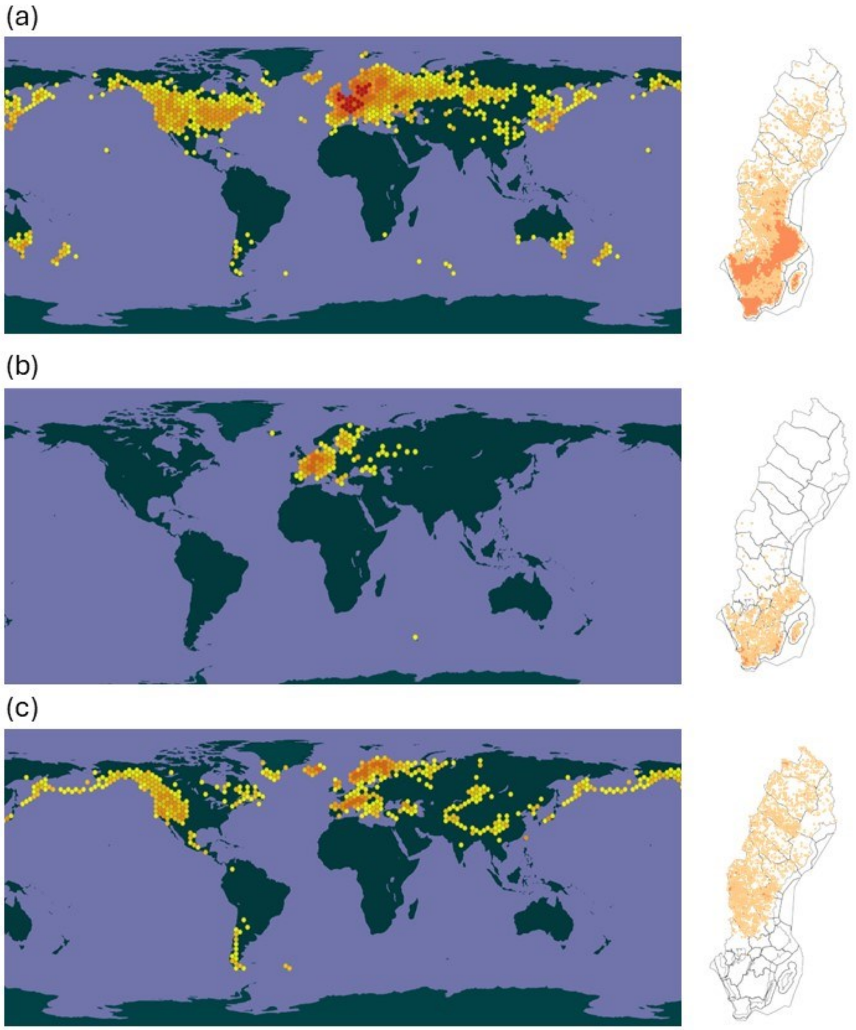


Figure 4. Distribution range of the three *Phleum* species across the world (left) and in Sweden (right). (a) *P. pratense* (timothy), (b) *P. nodosum*, (c) *P. alpinum*. Worldwide distribution range is accessed via https://www.gbif.org, where yellow to dark orange represents low to high density of the observed *Phleum* populations. Distribution range in Sweden is accessed via Artportalen, where light orange to dark orange represents low to high density of the observed *Phleum* species. Source: SLU Artdatabanken (2024). Artportalen. https:/www.artportalen.se [2023-11-01]

1.3.4 Genetic resources for breeding

Due to strong human selection during crop domestication, species may have undergone large bottlenecks, leaving many of the traits and genetic variation behind in wild populations (Gepts 2004; Doebley et al. 2006; Olsen and Wendel 2013). These traits may be of large interest for the improvement of crops (Dempewolf et al. 2017). Wild populations of timothy and the related *Phleum* species from different geographical areas and habitats are therefore potential genetic resources for the development of new high-yielding timothy cultivars adapted to a changing climate. Potential genetic resources for breeding of timothy are divided into four gene pools; a primary gene pool, a secondary gene pool, a tertiary gene pool, and a quaternary gene pool (Figure 5, (Stewart et al. 2010)).

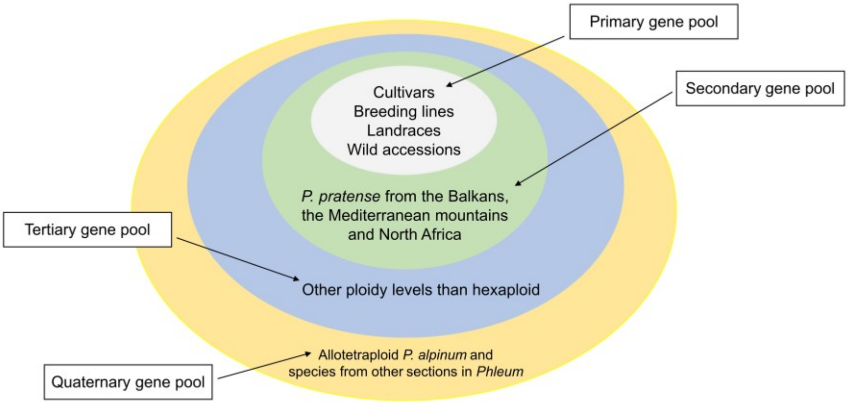


Figure 5. Gene pools with genetic resources for timothy breeding.

These gene pools are based on how easily the genetic resources within each pool can hybridize with timothy. The primary gene pool consists of domesticated cultivars, breeding lines and landraces (locally adapted varieties), and wild accessions of hexaploid timothy that can easily be crossed and give fertile offspring with timothy. Larsen and Honne (2001) found that local populations of timothy can be valuable resources for breeding in the northern climate. However, introducing exotic germplasm into breeding programs can be successful. Germplasm of timothy mainly from cooler regions in Northern Europe, North America and Northern Asia, and to a lesser extent from New Zealand are considered to belong to the primary gene pool (GUO et al. 2003; Stewart et al. 2010). This plant material originates from cooler regions and is winter hardy. However, germplasm of hexaploid timothy originating from warmer regions in the Balkans/Italy, the Mediterranean mountains, and North Africa belongs to the secondary gene pool (Stewart et al. 2010). Even though this plant material may not be winter hardy it may harbour genetic diversity for other traits important for adaptation and cultivation.

The tertiary gene pool contains germplasm of other taxa than hexaploid timothy and with other ploidy levels. These taxa belong to the section *Phleum* and all are derived from *P. alpinum* subsp. *rhaeticum* (Stewart et al. 2010). Traits and genes may also be transferred through wide hybridization between species of different ploidy levels. Nordenskiöld (1937) found that crosses between diploid *P. nodosum* and hexaploid timothy, and between hexaploid timothy and tetraploid *P. alpinum*, could give fertile hybrids. However, allotetraploid *P. alpinum* from the section *Phleum* (the same section as timothy) and species like *P. phleoides* and *P. hirsutum* from the section *Chilochloa* and *P. subulatum* from the section *Achnodon* are found in the quaternary gene pool, and difficult to cross with hexaploid timothy and with species from other sections. Compared to the secondary and tertiary gene pools, the quaternary gene pool presents less opportunity for breeders (Stewart et al. 2010).

Germplasm of the different gene pools are stored *ex situ* as seed accessions in different genebanks (https://eurisco.ipk-gatersleben.de/) such as NordGen, Alnarp, Sweden, External Branch North of the Department Genebank, IPK, Oil Plants and Fodder Crops in Malchow, Malchow/Poel, Germany, Plant Breeding and Acclimatization Institute, Blonie, Poland and N.I. Vavilov Research Institute of Plant Industry, St. Petersburg, Russian Federation. An accession consists of genetically related seeds that are collected at one time from a specific location often from one or several plants of a single species. The core collection of timothy accessions from the Nordic countries are kept at NordGen, Alnarp, Sweden. A core collection is an attempt to represent the genetic diversity of a species within a geographical area with a limited number of genetically different accessions. Besides the collection of accessions from the Nordic countries there are also accessions from Poland (2868 accessions), Russia (1473 accessions), Germany (989 accessions) and other European countries (1057 accessions).

NordGen holds in total 803 timothy accessions with 66 cultivars, 45 breeding/research material, 149 landraces, 108 semi-wild accessions (from populations nearby cultivated fields with potential gene flow between populations), and 435 wild accessions. Accessions of related *Phleum* species including *P. alpinum* and *P. nodosum* are also kept at NordGen. A smaller subset of the timothy collection at NordGen has been involved in previous studies (Fjellheim et al. 2007; Fiil et al. 2011a; Fjellheim et al. 2015; Jokela et al. 2015; Tanhuanpää et al. 2016b). This thesis focuses on a larger set of timothy accessions including 212 domesticated and wild accessions from the Nordic countries, UK, Germany, the Netherlands, and Russia. In addition, 14 accessions of *P. nodosum* and 20 accessions of tetraploid *P. alpinum* are evaluated in this thesis.

1.3.5 Breeding of timothy

Breeding of timothy began in the early part of the twentieth century (Wilkins and Humphreys 2003; Vogel and Hendrickson 2019). Traditional breeding methods based on phenotypic selection and crossing have been mainly used to date (Table 1). Breeding is challenged by the biological nature of the species. The high outcrossing rate and high ploidy level in timothy lead to high genetic heterogeneity within plants and high genetic and phenotypic diversity in populations and cultivars. The high phenotypic and genetic diversity and the perennialty of timothy make selection of genotypes with favourable traits more difficult and therefore result in low genetic gain per selection (breeding) cycle.

However, new breeding methods such as genomic selection (GS) can shorten the selection cycles and increase the precision of selection (Hayes et al. 2013; Lin et al. 2014; Simeão Resende et al. 2014). In breeding programs of timothy, the focus has traditionally been on improving dry matter yield, nutritional value, persistence of the stands and resistance to diseases such as leaf spot and snow moulds (Larsen and Marum 2006; Boller et al. 2010; Tamaki et al. 2010; Tanaka et al. 2018).

Table 1. Conventional breeding methods of timothy. Adapted from Tamaki et al. (2010).

Method	Procedure
Mass selection	Individual plants are selected visually based on desired traits and bulked seed from previous generation will be used for the next generations. This method is more efficient when screening for high heritable traits.
Synthetic variety method	Potential parents are isolated from a developed breeding population, and parents with higher general combining ability (GCA) will form next generation.
Maternal line selection	Seeds from elite parents are kept and the progeny will be planted as ear-to-row in maize. Progenies are half-sib family and due to open pollinating system, only the maternal parent is known. Further individual selection and polycross* progeny test can be performed coincide.
Clone and Strain Synthesis (CSS)	In this method both GCA and specific combining ability (SCA) can be exploited, and the risk of inbreeding depression is reduced (Tamaki et al. 2009). The procedure is similar to the synthetic variety method, where two seed parental clones (SPs) will be crossed with a common pollen parental strain and seeds from both maternal parents will be harvested (Syn1) and evenly mixed to produce Syn2 or subsequent generations. Potential of Syn2 to produce more yield relied on SCA between two SPs and SPs with pollen parental strain.

* Naturally inter-crossing of several plants in an isolated crossing block (Nyquist and Santini 2007).

The challenges in breeding timothy is exemplified by the slight increase in dry matter yield in modern Scandinavian cultivars (e.g. Lidar) compared to older cultivars (e.g. Grindstad) (Tamaki et al. 2010). Also, recurrent phenotypic selection over three generations based on sward and spaced plant evaluation resulted in no improvement of dry matter yield in Canadian cultivars (Suter et al. 2008). However, a steady increase by 0.32% per year in dry matter yield has been achieved between 1969 to 2004 in Japanese breeding programs. This progress has mainly relied on improving regrowth vigour (Tamaki 2005; Tamaki et al. 2010). Tanaka et al. (2018) showed that, paternal selection would potentially increase genetic gain for timothy forage yield in Japan. The negative relationship between forage yield and forage quality (Figure 3) also suggests a trade-off between agronomically important traits and is yet another example of challenges faced by timothy breeders.

1.3.6 Genetic and genomic tools for breeding

To diminish challenges in timothy breeding, traditional phenotypic selection methods could be complemented by genetic and genomic breeding tools that require the development of genomic resources. Over the last decades emerging high-throughput sequencing and new genomic technologies have provided great opportunities to make plant breeding programs more efficient (Taylor 2008; Brazauskas et al. 2018). High-throughput sequencing allows for the acquisition of genomic data, which can be utilized for various purposes such as reference genome assembly, identifying molecular markers such as single nucleotide polymorphisms (SNPs), population genomics, studying diversity among and within populations, identification of marker-trait associations through genome-wide association studies (GWAS), and ultimately facilitating the prediction of genomic breeding values (GEBVs) through the development of genomic selection models (Narum et al. 2013; Chung et al. 2017).

In timothy, the first marker development effort was reported in 2003, where Cai et al. (2003) developed several hundred SSR markers. In 2009, more than 800 SSR markers were developed using the same screening panel (Yamada et al. 2009). Around 80% of these markers were also amplified in tetraploid and diploid *Phleum* accessions. By using some of these markers, Tanhuanpää and Manninen (2012) studied the genetic diversity of 88 Nordic timothy accessions and 8 accessions from other European countries. The first quantitative trait loci (QTL) mapping study in timothy was then performed in 2016, where Tanhuanpää et al. (2016b) identified six retrotransposon-based markers associated with resistance to the fungal pathogen *Typhula ishikariensis*.

However, using high density and genome-wide markers like SNPs can further assist in the identification of genomic regions associated with agriculturally important traits. This would provide crucial insights for breeding programs aimed at enhancing crop productivity and resilience. There are examples of using SNP markers for GWAS in different forage crops leading to the identification of regions associated with forage quality traits. Niu et al. (2020) identified 9 QTL that were associated with lignin content in sorghum, and which harboured a total of 184 genes. In another study on sorghum, 42 SNPs were found to be associated with forage quality traits such as neutral detergent fibre and acid detergent fibre concentrations, crude protein, cellulose and hemicellulose contents (Li and Xie 2018). Lin et al. (2021) found 28 SNP markers associated with 16 quality traits that were mainly associated with fibre digestibility and protein content in alfalfa.

Genomic data and knowledge obtained from GWAS can be further used in marker-assisted selection (MAS) or in the development of genomic selection (GS) models to identify superior accessions/populations to be used in crosses or selection cycles. Previous studies have shown the efficiency of GS models in predicting GEBVs in well-known forage crops such as perennial ryegrass (Lin et al. 2016; Pembleton et al. 2018; Malmberg et al. 2023) and GS is fast becoming a crucial component of many contemporary plant breeding initiatives (Voss-Fels et al. 2019). However, before reaching this stage in timothy, the development of genomic resources including the assembly of high-quality reference genome, obtaining reliable genomic and phenotypic data collected from genetic resources like wild and domesticated accessions/populations is necessary. These types of information can be used in picturing the diversity within different groups of accessions, understanding the structure of populations, assessing the population differentiation and in identification of selection signatures in the genome of timothy as important pre-breeding practices.

Understanding the genetic diversity within timothy populations can help us select diverse parents for crossing, thereby increasing the genetic variability in the progeny. Understanding the genomic differences between wild and domesticated accessions provides insights into the genetic basis of domestication and adaptive traits that have been important during this process. Breeders can use this information to introgress desirable alleles into cultivars to enhance their adaptability, stress tolerance and other key agronomic traits. Therefore, in this thesis I will initially evaluate phenotypic diversity among genebank accessions including cultivars, breeding lines, landraces, semi-wild and wild accessions of timothy and the related *Phleum* species *P. nodosum* and *P. alpinum*. I will further extend our understanding of diversity and population genomic variation in timothy using whole genome resequencing data in a sub-selection of the genebank accessions. The data and results produced in this thesis can then be used to perform GWAS and to develop GS models.

2. Overall aim of the study

Commercial timothy cultivars have been developed through different selection processes and crosses using classical breeding methods, such as mass selection, creation of synthetic varieties, and maternal line selection. Despite their historical effectiveness, these methods are constrained by a number of factors. These includes lack of detailed genetic information, high costs, labor intensity, and inefficiency in enhancing polygenic traits, such as forage yield and forage quality.

The advent of climate change and rapid change in growing conditions, have created a pressing need for development of new timothy cultivars using more advanced breeding approaches that are supported by new genomic resources and methods. These resources and methods are important for developing highly productive and high-quality cultivars that can meet the demand for forage production, particularly in the Nordic countries like Sweden.

The overall goal of my PhD thesis was to deepen the knowledge of the phenotypic and genetic diversity of timothy and the closely related species *P. nodosum* and *P. alpinum*. The identified diversity can be used as resources in the breeding of new cultivars. A large set of wild and domesticated accessions from a genebank collection of timothy and the related species were investigated for diversity in biomass production, tiller formation and development during greenhouse and field conditions. A subset of the accessions was further studied for identifying selection signatures within the genomes of timothy and *P. nodosum* to identify genomic regions with adaptive genetic variation. In addition, genome-wide patterns of nucleotide diversity and recombination rates were investigated to gain further insights into the evolutionary processes that have shaped the species genomes.
During the studies I have addressed the following questions:

- Do the three *Phleum* species differ in biomass production, development and the formation of different tiller types?

- Do wild and domesticated accessions differ in the mentioned traits?

- Do the studied accessions show traits of interest for further pre-breeding studies and as genetic resources for breeding?

- Do the three *Phleum* species differ in pattern of genomic diversity?

- Are wild and domesticated accessions genetically differentiated?

- Have some genomic regions been under selection during domestication and breeding?

3. Materials and Methods

3.1 Phenotypic studies

3.1.1 Climate chamber, greenhouse, and field trials

A total of 246 accessions including 212 timothy, 14 *P. nodosum* and 20 *P. alpinum* accessions were evaluated (Table 2, Figure 6). The accessions were categorized as wild, semi-wild (from populations nearby cultivated fields with potential gene flow between populations), landrace (a cultivated variety adapted to a specific eco-geographical area), breeding line (group of related genotypes under evaluation in a breeding program) or cultivar based on information from the Nordic Baltic Genebanks Information System. The seeds were provided by the NordGen genebank, Alnarp, Sweden. More detailed information such as geographical origin (latitude and longitude coordinates) and collecting year are given in paper I and II.

Table 2. Number of accessions studied in each accessions group.

Species	Cultivar	Breeding line	Landrace	Semi-wild	Wild	Total number of accessions
P. pratense	56	10	55	30	61	212
P. nodosum	7	-	-	-	7	14
P. alpinum	-	-	-	-	20	20

P. pratense (6x) *P. nodosum* (2x) *P. alpinum* (4x)

Figure 6. The studied *Phleum* species; hexaploid timothy (*P. pratense*), diploid *P. nodosum* and tetraploid *P. alpinum*.

Most of the accessions originated from the Nordic countries, others from countries close by such as Russia, UK, Germany, and the Netherlands (Table 3).

Table 3. Country of origin of the studied *Phleum* accessions.

Origin	Number of accessions
Denmark	21
Finland	76
Germany	1
Iceland	19
the Netherlands	1
Norway	43
Russia	10
Sweden	74
the UK	1
Total	246

3.1.2 Cloning process

In April 2020, eight seeds of each accession were planted in a climate chamber at the Plant Cultivation Facility, BioCenter, Swedish University of Agricultural Sciences, Uppsala, Sweden, with 16/8 h day/night photoperiod (350 µmol m−2 s−1 light), 22/17 °C day/night temperature and 65% humidity. After two months of growth, four randomly selected plants (genotypes) of each accession were cloned (Figure 7). Since the *Phleum* species are outcrossing, we considered each of the four plants within an accession genetically different. Vegetative tillers were gently separated from each other, and six of the tillers (clones) from each plant were individually transplanted into separate pots.

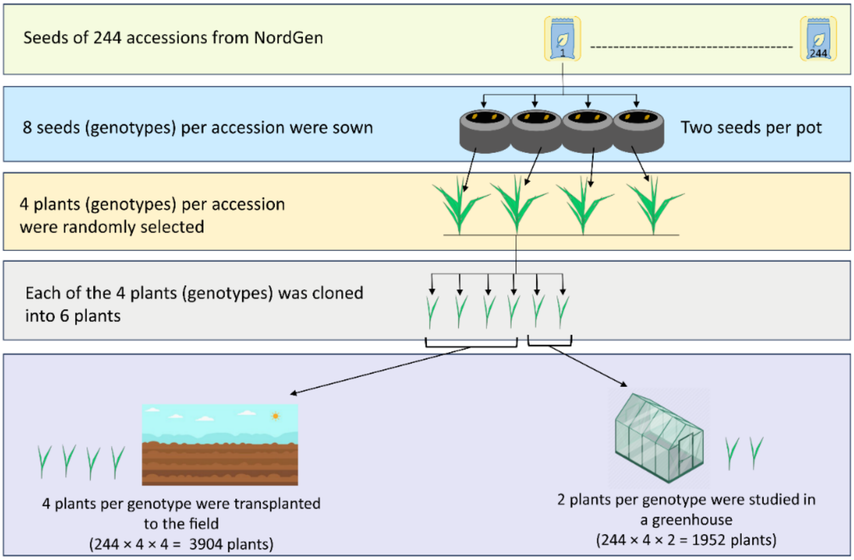


Figure 7. Cloning of *Phleum* accessions planted in the field and in the greenhouse.

Two of the cloned plants from each genotype were grown in the greenhouse for two weeks and then given a vernalization treatment for six weeks at 4 °C under an 8 h photoperiod at 100 µmol m−2 s−1, to stimulate flowering. After the vernalization, the plants were transferred back to the greenhouse (16/8 h; 22/17 °C) and placed in two blocks with one replicate of each genotype randomized within each block. In July 2020, the remaining four clones of each genotype were transplanted to a farmer's field north of

Uppsala, Central Sweden (60°00′ N, 17°42′ E). In total, 246 *Phleum* accessions with 4 genotypes of each accession and 4 plants of each genotype were planted in a randomized complete block design with one plant of each genotype in each of the 4 blocks. The distance between the plants was 1 m within and between rows.

3.1.3 Evaluated traits

The same accessions were studied both in the field and in the greenhouse. In paper I, plants were individually evaluated for the same growth and developmental traits in the field (growing seasons 2021 and 2022) and in the greenhouse (between Sep 2020 and March 2021). The growth traits included fresh weight (FW), dry weight (DW) and plant height (PH), and the developmental traits included number of days from emergence of the coleoptile to stem elongation (DTS), number of days from emergence to booting (DTB, and number of days from emergence to heading (DTH). The winter survival rate was also recorded in the field.

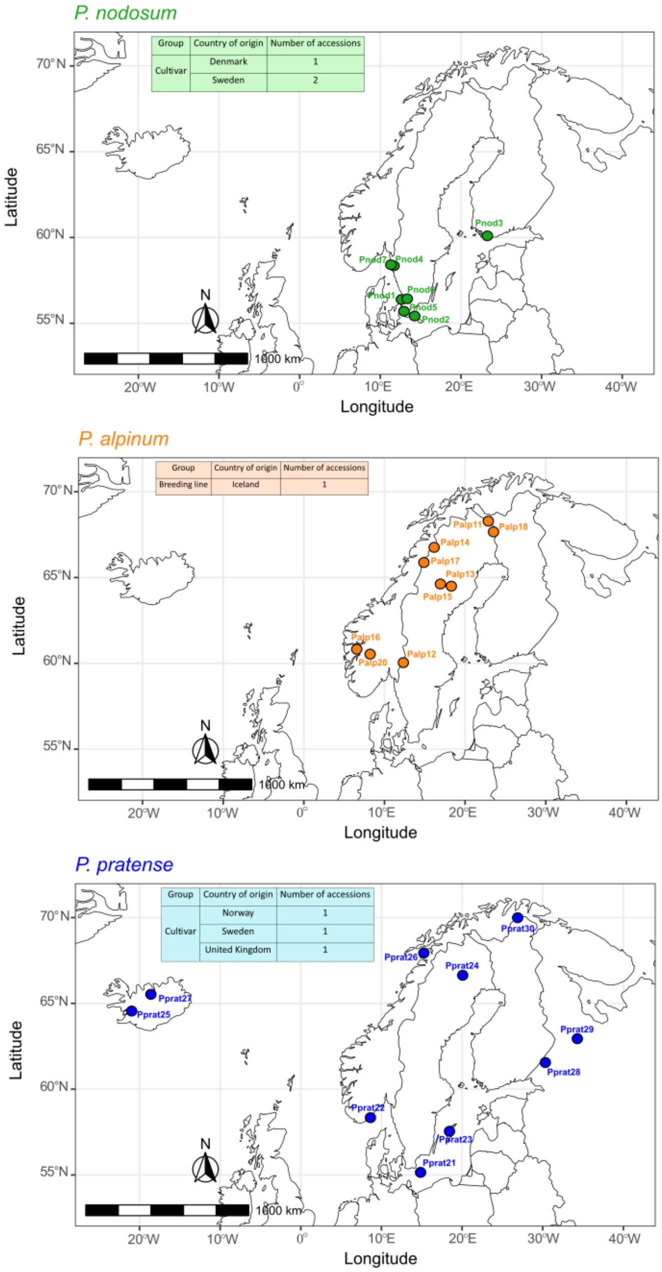
In paper II, the plant development and tiller formation were studied in more detail by evaluating the length of the developmental stages from emergence to heading and the formation of different tiller types, as well as the correlation between these traits. Paper II is based on further analysis of data collected during the greenhouse study in paper I, and additional data from studies conducted in the climate chamber. In the climate chamber, the number of days from seed planting to emergence of coleoptile (DTE) and the number of days from seed planting to development of the first tiller (ΔDTT) were recorded for each plant. In the greenhouse, the plants were individually studied for number of days between the start of tillering to stem elongation (ΔDTS) including 42 days of vernalization, between stem elongation to booting (ΔDTB), and between booting to heading (ΔDTH). When the peduncle on the first spikes of each plant turned yellow, the tillers were cut at the base. Then the number of the three tiller types, vegetative tillers (VEG), generative tillers (GEN) and vegetative elongated tillers (ELONG) were counted and the weight of each of the three types of tillers were recorded.

3.2 Genomic analysis

A subset of the 246 accessions were further evaluated for genetic diversity. These accessions included 13 accessions of timothy, 10 accessions of *P. nodosum* and 10 accessions of *P. alpinum.* These accessions were selected to represent the geographical distribution of the species (Figure 8). Timothy accessions included 7 wild and 3 semi-wild accessions, and 3 commercial cultivars (Aberystwyth, Grinstad and Tryggve). Seven wild accessions and 3 commercial cultivars (Evergreen, Parant and Teno) were selected for *P. nodosum* and 9 wild accessions and 1 breeding line were selected for *P. alpinum.*

The genomes of these accessions were analyzed using the whole genome resequencing (WGS) approach and short-read NGS data. WGS data of the three species were then used for reference-based SNP calling. The reference genomes for the tetraploid *P. alpinum* and diploid *P. nodosum* had already been developed in a separate subproject in the group (Bedada et al., unpublished). At the time of the analysis of the 33 accessions, the hexaploid timothy genome assembly was still in progress. Consequently, the reference genomes of *P. nodosum* and *P. alpinum* were used to reconstruct and test different synthetic genomes for timothy. The synthetic genome RefPn:7Chrs (Subg-Pn) + RefPaA:7Chrs (Subg-PaA), also known as genome RefPnPaA, produced the most dependable result in terms of mapping coverage and thus served as a reference in all downstream analyses of timothy samples.

Three reference genomes were then indexed with BWA and samtools, and a GATK sequence dictionary was created. Reads were aligned using BWA-MEM with read group information. Qualimap was used to assess mapping quality. GATK v4.2.0.0 pipeline followed best practices for SNV calling. HaplotypeCaller generated 'g.vcf' files for individual samples, merged with CombineGVCFs, and transformed to 'VCF' format with GenotypeGVCFs. High-quality SNVs were obtained via "hard filtering" using the "Variant Filtration" method. SNPs sets of approximately ~17 M, ~1.7 M, and ~36 M markers were called in the diploid, tetraploid, and hexaploid species, respectively. The downstream genetic analyses included different genetic diversity measures. Observed heterozygosity (H_O), nucleotide diversity (π), inbreeding coefficient, linkage disequilibrium, population structure, and population differentiation were studied in paper III, and recombination rate and identification of signatures of selection were discussed in paper IV.



Group	Country of origin	Number of accessions
Cultivar	Denmark	1
	Sweden	2

Group	Country of origin	Number of accessions
Breeding line	Iceland	1

Group	Country of origin	Number of accessions
Cultivar	Norway	1
	Sweden	1
	United Kingdom	1

Figure 8. Geographical origin of wild accessions and number of cultivars and breeding lines of the three *Phleum* species selected for whole genome sequencing.

4. Results and Discussion

4.1 The three *Phleum* species differed in growth, development and formation of different tiller types

At the species level timothy, *P nodosum* and *P. alpinum* differed significantly in growth and development under both field and greenhouse conditions (Tables S1 and S2, Paper I). Timothy had the highest fresh weight (FW), dry weight (DW), and plant height (PH) in the field (Table S3, Paper I), while *P. nodosum* had the highest means of FW, DW, and PH in the greenhouse (Table S4, Paper I). Timothy reached booting and heading later than *P. nodosum* and *P. alpinum* in the field, while *P. nodosum* and *P. alpinum* did not differ significantly in the number of days to booting (DTB) and heading (DTH) (Table S3, Paper I). Also, in the greenhouse timothy flowered later than *P. nodosum* (Table S4, Paper I), while most of the *P. alpinum* remained in the vegetative stage. When looking in more detail at plant development and the transition between different developmental stages, timothy showed earlier emergence than the other species, while *P. alpinum* had the highest number of days between seed planting and coleoptile emergence (DTE) in the greenhouse (Table 2, Paper II). Timothy also produced its first tiller (ΔDTT) earlier than *P. nodosum* and *P. alpinum* but had the highest number of days between the formation of the first tiller and stem elongation (ΔDTS). However, no significant differences were observed in number of days between tillering and booting (ΔDTB), or between booting and heading (ΔDTH) between timothy and *P. nodosum* or between these two species and the three accessions of *P. alpinum* that developed reproductive tillers (Table 2, Paper II). The species also showed variation in tiller formation in the greenhouse. The total tiller number (TTN) was significantly highest in *P. nodosum*, while the production of TTN in *P. alpinum* was only 20-25% of that observed in the other species. Most of the tillers formed were in the vegetative stage (VEG-TN) in all three species, and the percentage of non-flowering elongated tillers (ELONG-TN%) was higher than the percentage of generative tillers (GEN-TN%) in timothy and *P. nodosum* (Table 2, Paper II). *P. nodosum* cultivars produced the highest number of ELONG, while no such tillers were found in *P. alpinum*.

The higher FW and DW found in timothy compared to the other species grown in the field was expected as timothy is one of the most important forage crops in cold temperate climates and is grown for its high biomass yield. Timothy, as a species, had a longer development time until stem elongation, booting and heading, which allowed a longer growing season in the field (Paper I).

The lower FW and DW and the high formation of vegetative tillers found in all the species in the greenhouse were likely caused by differences in the cultivation conditions between the greenhouse and field, including differences in temperature, length of photoperiod, and the availability of nutrients. A longer photoperiod has been shown to stimulate growth and biomass production in *P. alpinum* (Heide and Solhaug 2001), and a longer photoperiod stimulates flowering in timothy (Seppänen et al. 2010; Jokela et al. 2014; Jokela et al. 2015). Also, the difference in length of vernalization between the six-week cold treatment in the greenhouse and the longer cold period in the field may have affected the development and ability to flower. Plants differ in their need for vernalization to flower, and winter-type plants of annual cereals and some perennial grasses such as ryegrass and tall fescue require vernalization(Heide 1994; MacMillan et al. 2005; Fjellheim et al. 2014). Some genotypes of timothy, particularly plants growing at lower latitudes has been found to not require vernalization for flowering (Fiil et al. 2011a; Jokela et al. 2014; Jokela et al. 2015). *P. nodosum* was, however, less affected by the different growing conditions in the field and greenhouse compared to timothy and *P. alpinum*. The different performance of the species in the two trials is likely due to that they are adapted to different habitats. Both timothy and *P. nodosum* are mainly growing in lowland meadows. While timothy is found throughout the Nordic countries, *P. nodosum* has a more southern distribution. *P. alpinum* is found at high altitudes and is adapted to harsh mountainous environments (Hultén and Fries 1986). The different geographical and habitat origin may be seen in the different growth rate among the species. In addition to the high TTN found in *P. nodosum* it also had the highest growth rate and the largest number of leaves, while *P. alpinum* had the lowest growth rate and produced few leaves in other greenhouse studies conducted by our research group (Moreno et al 2023; Moreno et al. unpublished).

4.2 Accessions showed large variation in growth, development and formation of different tiller types

The different groups of accession (cultivar, breeding line, landrace, semi-wild, and wild), showed large diversity within and between groups in growth, development, and tiller formation (Figure 1 and 2, Paper I; Table 3 Paper II). Interestingly, in timothy, cultivars and wild accessions did not show significant differences in FW and DW, and had the highest values compared to other accession groups both in the greenhouse and in the field (Figure 1a-e, Paper I). The wild accessions together with the semi-wild accessions and landraces showed lower PH compared to the timothy cultivars in both trials (Figure 1c-d, Paper I). Also, in *P. nodosum*, the cultivars and wild accessions did not differ in FW and DW under field conditions. They also showed similar PH in both trials, while they differed in DW in the greenhouse (Figure 1a-b, d-e, Paper I). In the field the PH were about one-third higher in all accession groups of timothy compared to *P. nodosum* cultivars and wild accessions, and more than twice the height of *P. alpinum* (Figure 1c, Paper I). In the greenhouse, *P. nodosum* and timothy accessions showed similar PH (Figure 1d, Paper I).

A comparison of the development among accession groups revealed a somewhat deviant pattern of variation when compared to the differences in growth. When wild accessions and cultivars showed high similarity in FW and DW in both timothy and *P. nodosum* these two accession groups differed in development. In timothy, the cultivars reached booting and heading earlier than the other accession groups under both field and greenhouse conditions (Figure 2a–h, Paper I). Also, in *P. nodosum*, the cultivars showed an earlier development with lower DTS, DTB and DTH in the field (Figure 2a–c, Paper I). However, a reverse pattern was observed in the greenhouse where the wild accession of *P. nodosum* reached booting and heading earlier than the cultivars (Figure 2d–f, Paper I).

The length of the different developmental stages from emergence to heading varied also among accession groups (Table 3, Paper II). In timothy, the domesticated cultivar group and the breeding line group had similar DTE and ΔDTT, and showed later emergence and tillering compared to the other groups. Landraces had the lowest ΔDTT. The cultivars had the lowest number of days between tillering and stem elongation (ΔDTS) compared to all other accession groups, except the breeding lines. ΔDTB was similar in all accession groups, while cultivars had significantly lower ΔDTH compared to landraces, but not significantly different from other accessions. Differences were also found between the cultivar and wild accession groups in *P. nodosum*, where the cultivars had a higher DTE, ΔDTB, and a lower ΔDTT, ΔDTS and ΔDTH than the wild accessions (Table 3, Paper II).

While most of the timothy accession groups had similar TTN in the greenhouse, they differed in the formation of the different tiller types (Table 3, Paper II). Vegetative tillers accounted for the highest proportion of tillers, ranging from 82% to 92% in all accession groups, but contributed less to total dry weight, ranging from 62% to 82%. The timothy cultivars had the significantly lowest percentage of vegetative tillers (VEG-TN%) and the highest percentage of generative tillers (GEN-TN%). Additionally, cultivars showed higher percentage of non-flowering elongated tillers (ELONG-TN%) and a higher percentage of the elongated tiller dry weight (ELONG-DW%) compared to landraces and semi-wild accessions (Table 3, Paper II). VEG-TN% increased with the latitude of origin of the timothy and the *P. nosoum* wild accessions, while the ELONG-TN% decreased (Figure 2, Paper II). Weak to strong correlations were found between developmental stages and formation of different tiller types (Figure 1a, Paper II). ΔDTS correlated positively with VEG-TN% (r = 0.51) and VEG-DW% (r = 0.45), but negatively with ELONG-TN% (r = −0.36) and GEN-TN% (r = −0.49) as well as with GEN-DW% (r = −0.43). ΔDTT showed weak positive correlations with GEN-TN, GEN-TN% and GEN-DW% (r =0.34-0.38) and a weak negative correlation with VEG-TN% (r = −0.35).

Interestingly, a majority of the tillers in the *P. nodosum* cultivars where non-flowering elongated tillers. The cultivars produced significantly higher ELONG-TN% and ELONG-DW% and significantly lower VEG-TN% and VEG-DW% compared to all other accession groups and species (Table 3, paper II). In *P. nodosum* moderate to strong correlations were found between developmental stages and formation of different tiller types (Figure 1b, Paper

II). DTE, ΔDTB and ΔDTH correlated positively with ELONG-TN% and ELONG-DW% (r = 0.48 to 0.75). In contrast, ΔDTS correlated positively with VEG-TN, VEG-TN%, VEG-DW and VEG-DW% (r = 0.49 to 0.59), and negatively with GEN-TN% (r = −0.45) and GEN-DW% (r = −0.48). ΔDTT had a negative correlation with ELONG-TN (r = −0.49).

In timothy, studied in field plots, the tiller types contributed differently to leaf biomass in primary growth with GEN being the dominant type (Virkajärvi et al. 2012). During primary growth, the ELONG had a higher leaf to stem biomass that was maintained throughout the season, while GEN had a significantly lower leaf to stem biomass that decreased during growth. In the regrowth after harvest, vegetative tillers and elongated tillers with vegetative apices were the dominant tiller types, with proportion of elongated tillers increasing during the season (Virkajärvi et al. 2012). During stem elongation, lignification increases, resulting in lower digestibility and nutritional value of the plant (Gustavsson and Martinsson 2004). As leaves have a higher digestibility than stems and leaf sheaths (Gustavsson and Martinsson 2004; Karkonen et al. 2014), a plant with a high proportion of leaves can be advantageous for a harvest with high digestibility. Considering the different types of tillers in timothy and related species, plants with a high proportion of ELONG may be advantageous, which could become a breeding target to achieve both high digestibility and high yield.

4.3 Breeding has favoured faster development

The diversity found in the development and tiller traits between accession groups of timothy made it possible to discuss if the selection during domestication and breeding has influenced some of the traits of timothy. Populations in agroecosystems and in natural habitats are exposed to different selection pressures. The differences found between the cultivars and wild accessions may reflect their different histories. In timothy, the lengths of different developmental stages and the proportions of tiller types were highly similar between cultivars and breeding lines, that both are accessions from breeding programs. Landraces, locally adapted varieties in farmers' fields, showed similarities with the breeding lines, but differed from the cultivars in many developmental and tiller traits. The wild accessions showed similarities in developmental traits with the landraces but not with the cultivars. The wild accessions differed from the semi-wild accessions in the proportions of tiller types. The differences between cultivars and landraces may reflect a stronger directed selection that has favoured agronomic traits in the cultivars, while wild accessions are exposed to diverse natural selection that fluctuates in direction over time. According to our findings, timothy cultivars may have been selected for overall faster development. This was seen in the field where the group of cultivars showed lower total number of days to booting and to heading compared to the wild accession group (Paper I). However, the early developmental stages DTE and ΔDTT were longer, while the later stages ΔDTS and ΔDTH were shorter for the cultivars than for the group of wild accessions in the greenhouse (Paper II). Even though cultivars and wild accessions of timothy did not differ in TTN and DW, the lengths of developmental stages seemed to have favoured the formation of higher production of ELONG and GEN in cultivars. Also, in *P. nodosum* the cultivars showed earlier stem elongation, booting, and heading than the wild accession group under field conditions. Rapid development and rapid growth allow early harvest and possibility for several harvests per season. Early harvests give higher digestibility and nutritional value (Bélanger et al. 2001; Gustavsson and Martinsson 2004).

4.4 The large phenotypic diversity identified provides knowledge for further pre-breeding studies

The large diversity found in growth, development and tiller formation will be essential for further studies of traits favouring forage yield and forage quality. The identified diversity and the knowledge of the relationships between developmental traits and tiller traits will serve as resources for selecting accessions with different combinations of traits for further studies under greenhouse and field conditions. Accessions with early development and high production of non-flowering elongated tillers (ELONG) may serve as genetic resources in future breeding. The positive correlation found between the latitude of origin of the wild accessions and the proportion of different tiller types formed should also be considered when selecting germplasm for breeding of cultivars adapted to different day-lengths. In other studies, conducted by the research group, using a subset of the accessions, large diversity in response to drought and waterlogging stress was found (Moreno et al 2023; Moreno et al. unpublished). The tolerant accessions identified will be of great value for future breeding of stress tolerant timothy cultivars. Also, the high TTN and DW in the studied *P. nodosum* accessions and the considerably higher proportion of ELONG tillers found in the *P. nodosum* cultivars compared to all other groups of accessions make this species interesting for further breeding and development of a new forage crop. Favourable traits and genes may also be transferred from *P. nodoum* into timothy cultivars. Although wide hybridization between related species of different ploidy level may be challenging, crosses between *P. nodosum* and timothy have resulted in fertile hybrids (Nordenskiöld 1937).

4.5 Whole genome resequencing and variant discovery

Based on the whole genome re-sequencing of the 33 selected accessions more than 97% of the sequence reads could be mapped to the existing reference genomes of *P. nodosum* and *P. alpinum* or to the synthetic genome for timothy (Table S1-S3, Paper III). Variant discovery showed that diploid *P. nodosum* had a high number of single nucleotide variants (SNVs, 17 M), with variant density being associated with chromosome lengths, while tetraploid *P. alpinum* had significantly fewer SNPs (1.7 M) and a lower SNP density (Figure 2a-d, Paper III). Timothy showed the highest SNVs number (36 M) (Figure 2e-f, Paper III). The number of SNVs on the *P. nodosum* subgenome (Subg-Pn) was nearly twice of the SNVs found on the *P. alpinum* subgenome (Subg-PaA) in the synthetic genome.

4.6 Differential pattern of genetic diversity

Different measures of diversity, such as observed heterozygosity (H_O) and inbreeding coefficient was used to understand differences both between the three species and between accession groups. The observed heterozygosity (H_O) in timothy and *P. nodosum* was comparable (0.25 and 0.23, respectively) and higher than in the tetraploid *P. alpinum* (0.12, Figure 3a, Paper III). *P. alpinum* had significantly higher inbreeding coefficients (0.51) than the other two species, *P. nodosum* (0.14) and timothy (0.05) (Figure 3b, Paper III).

In *P. nodosum*, the average H_O in the cultivar group was identical to that of the wild accession group (0.23) (Figure 3c, Paper III). When comparing the wild accession group of *P. alpinum* to the single breeding line of *P. alpinum* and other groups of accessions in the other two species, a low level of heterozygosity was observed (Figure 3c, Paper III). In timothy, the cultivar group had greater heterozygosity (0.27) compared to wild (0.25) and semi-wild accessions (0.23) (Figure 3c, Paper III). Additionally, in *P. nodosum*, average inbreeding coefficient of the wild accessions was marginally higher than that of the cultivars (Figure 3d, Paper III). Cultivars in *P. pratense* had the lowest inbreeding coefficient (roughly 0), whereas semi-wild accessions had the highest (0.13, Figure 3d, Paper III).

The findings suggest that *P. alpinum* has high levels of selfing, as evidenced by low number of SNVs, high inbreeding coefficient and low heterozygosity in contrast to the other two species that appear to be largely or completely outcrossing. In outcrossing plants, inbreeding coefficients are generally lower than in selfing plants because outcrossing introduces genetic diversity. Furthermore, geographical barriers and smaller population sizes in plants that are acclimated to harsh environments, such as *P. alpinum*, can eventually lead to reduced heterozygosity and gene flow. On the other hand, outcrossing has contributed to higher heterozygosity and lower inbreeding coefficient in hexaploid timothy and diploid *P. nodosum*. Excessive inbreeding may have detrimental effects on population fitness and might lead to reduction in genetic diversity (Reed and Frankham 2003). Thus, maintaining heterozygosity in forage crops like timothy seems to be essential to allow the populations to adapt to different growing conditions and prevent a decline in fitness.

4.7 Genome-wide nucleotide diversity and recombination

The three *Phleum* species differed in average nucleotide diversity (Figure 5a, Paper III). In comparison to hexaploidy timothy (π = 0.00066) and tetraploid alpinum (π =0.00018) species, it was found that the diploid species *P. nodosum* had a substantially higher π-value (π =0.0031). The nucleotide diversity in *P. nodosum* was comparable between wild and cultivar samples (Figure 5b, Paper III). The average nucleotide diversity of the wild accessions in *P. alpinum* was π=0.00017 (Figure 5b, Paper III). The three groups of timothy had varying levels of nucleotide diversity, with an average

π of 0.00075 in the cultivar group, 0.00070 in the wild group, and 0.00064 in the semi-wild group (Figure 5b, Paper III).

In a study of Italian ryegrass (Yu et al. 2022), the average π was around 0.0008 among 11 cultivars, which is lower than what we have found among timothy accessions. Fiil et al. (2011b) also evaluated nucleotide diversity in nine floral control genes of perennial ryegrass (*Lolium perenne* L.) and reported a range of π=0.00042 to π=0.02160 and a lower diversity in coding than non-coding regions. Compared to an outcrossing species like maize (*Zea mays* L.) with $\pi = 0.0066$ (Gore et al. 2009), the average observed nucleotide diversity is relatively lower in the *Phleum* species.

Population-scaled recombination rates were also estimated on a per-chromosome basis in the three species (Figure 1c and d, Paper IV). A higher rate of recombination was observed in timothy (0.050 ρ/bp) than in *P. nodosum* (0.027 ρ/bp) and *P. alpinum* (0.004 ρ/bp). Based on regression models 0.10-4.41% of the nucleotide variation could be explained by recombination rate variation in timothy (Figure 2, Paper IV). In *P. nodosum*, recombination explained 0-0.40% of nucleotide diversity across chromosomes and in *P. alpinum*, the range was 0–6.76%.

The relationship between recombination and nucleotide diversity varies between species; for example, in maize, a significantly high association has been reported (Tenaillon et al. 2004), whereas in wild tomatoes (Solanum section Lycopersicon), Roselius et al. (2005) found weak correlations. Nucleotide diversity can also be influenced by background selection, recombination, demographic processes (such as population division), differences in neutral mutation rates, and/or selective constraints between loci (Roselius et al. 2005). These factors could help explain the weak correlations observed between nucleotide variation and recombination in *P. nodosum* and *P. alpinum*, in particular.

4.8 Distinct pattern of linkage disequilibrium

P. alpinum showed different linkage disequilibrium (LD) decay patterns than *P. nodosum* and timothy (Figure 6, Paper III). The LD decay distance was roughly 10 kilobases (kb) in *P. nodosum* and timothy. In contrast, *P. alpinum* has a distinct LD profile with persistently high levels of LD, suggesting extended haplotype blocks and high homozygosity. This is another clear sign of high selfing levels in *P. alpinum*.

According to earlier research, there are differences in LD between selfing and outcrossing species. For instance, in rice (highly selfing) , LD decays in between 100 and ≥ 500 kb (Mather et al. 2007; Huang et al. 2010), in *Arabidopsis thaliana* (highly selfing) there are variations between 10 kb and 250 kb (Nordborg et al. 2002; Kim et al. 2007), or less than 174 kb in perennial ryegrass cultivar (highly outcrossing) originating from 336 parents (Auzanneau et al. 2007).

4.9 Genetic differentiation among wild and domesticated accessions of timothy and *P. nodosum*

Relative divergence (F_{ST}) was estimated to assess differentiation between groups of accessions and clusters derived from phylogeny and PCA (Figure 8, Paper III). In *P. nodosum*, a F_{ST} of 0.04 was calculated for the comparison of cultivar and wild groups (Figure 8a). Since there was only one breeding line available for *P. alpinum* in the study, a comparison between accession groups was avoided in favour of a comparison of the two clusters found by PCA and phylogeny (Figure S6, Paper III). Three groups of accession including cultivars, wild and semi-wild, and clusters (from PCA) were compared in timothy. Since all F_{ST} values were less than 0.03, the results indicated either no differentiation at all or very little differentiation among the accession groups of timothy (Figure 8c, Paper). Nevertheless, a greater degree of differentiation was observed when clusters were compared (Figure 8d, Paper III).

According to Casler (2001), most perennial forage crops have experienced few selection cycles (less than 10 generations) from their wild ancestors compared to most seed crops, which could explain the low differentiation between cultivated and wild accessions in timothy. Through the use of SSR and REMAP markers, previous research have also demonstrated a lack of genetic divergence between the accessions of timothy (Tanhuanpää and Manninen 2012; Tanhuanpää et al. 2016a). Moreover, the low F_{ST} value in the cultivar versus wild comparison could mean that there has been recent gene flow between the populations, maintaining genetic similarity. It could also suggest that the domestication process has not led to significant genetic divergence from wild populations. This may have implications for plant breeding, as traits from wild populations could be introduced into cultivars through hybridization or introgression as there is still a reservoir of genetic diversity in the wild population that could be utilized to improve cultivars.

Breeding strategies could involve genetic material from wild populations to enhance traits like yield potential, disease resistance and stress tolerance. This may also have conservation implications, since low genetic differentiation could mean that efforts to conserve genetic diversity should focus on both wild and cultivated populations, as they contribute to the overall genetic diversity of the species. However, genomic patterns of F_{ST} obtained from the comparison of accession groups in both timothy and *P. nodosum* showed that some genomic regions were highly differentiated (Figure 3 and 4, Paper IV), indicative of natural or artificial selection.

4.10 Signatures of selection in timothy and *P. nodosum*

To complement the F_{ST} tests, EHH (Extended Haplotype Homozygosity) tests were run in the cross-population mode (XP-EHH) to compare cultivated and wild accessions of timothy and *P. nodosum* (Figure 6 and 7, Paper IV). The idea was to identify signatures of selection in these two species that could help explain differences between wild and cultivated accessions. EHH works by identifying genomic regions with unusually levels of local haplotype homozygosity (Sabeti et al., 2002).

In timothy and *P. nodosum*, 479 and 261 outliers (corresponding to the top 1% of F_{ST} values) were identified across different chromosomes (Table S1 and S2, respectively, Paper IV). Using the XP-EHH, the top 1000 hits (corresponding to ca. 0.01% of the total variants) were initially identified based on their p-values for timothy (Table S3, Paper IV) and *P. nodosum* (Table S4, Paper IV).

In both species, variants subjected to positive selection were found to be enriched within coding genes that are involved in various crucial biological processes (Table 3 and 4, Paper IV). In timothy for example, SNPs that are under selection in the coding genes *g22404* and *g39371*, which both encode cytochrome P450s (CYPse), were found. These proteins are involved in the biosynthesis of structural polymers, defence against pathogen infections, communication with other organisms, hormonal signaling, herbicide resistance, and tolerance to abiotic stress (Minerdi et al. 2023). CYPs are found to be critical in the biosynthesis of lignin in plants (Schuler and Werck-Reichhart 2003; Alber and Ehlting 2012; Chakraborty et al. 2023) which is an important factor determining digestibility and nutritive value in forage crops (Oliveira et al. 2020; Sulc et al. 2021). Moreover, SNPs were also found within the gene *g35607* encoding a jasmonate O-methyltransferase. This protein is crucial for the methylation of different classes of flavonoid compounds that results in phytoalexins or caffeic acid and/or 5-hydroxylferulic acid, leading to lignin precursors (Ye et al. 2001; Zhao et al. 2002; Rastogi and Dwivedi 2008).

Other biological processes enriched in this study included metabolism, signaling and membrane transport, RNA splicing, DNA repair, and response to abiotic stressors. Of those, C2H2-type zinc finger, small heat shock protein (HSP20), RWP-RK domain, AAA ATPase family, potassium transporter, phosphatidylinositol phosphate kinases, strictosidine synthase activity, Aux IAA proteins, lipoxygenase, and beta-galactosidase can be mentioned. Some of the identified variants in both timothy and *P. nodosum* were also annotated within transposable elements (TEs) such as Gypsy_LTR_retrotransposon and LTR_retrotransposon. TEs are DNA sequences that can move within the genome of plants through various self-replicating mechanisms. Recent studies highlight the role of TEs in modulating gene expression and underlying adaptive evolution in many plant species (Chuong et al. 2017; Quadrana et al. 2019; Akakpo et al. 2020).

5. Summary of findings

This thesis provides new insights into the phenotypic and genetic diversity of the important forage crop timothy and two related species and will serve as a basis for future research on traits related to yield, quality and resilience, and development of genomic-based breeding strategies. The main findings are:

- The three *Phleum* species evaluated in this study showed differences in developmental stages, growth and formation of different tiller types under both greenhouse and field conditions. Timothy had high biomass production in the field and formed more generative tillers and fewer vegetative tillers under greenhouse conditions than the other species. At the species level, timothy had a longer overall development and reached booting and heading later. *P. nodosum* showed earlier development with high proportions of non-flowering elongated tillers and has interesting traits for further development as a forage crop. In timothy, high leaf to stem ratio in non-flowering elongated tillers corresponds to low lignification and high digestibility and nutritional value of the harvest.
- The studied accessions and accession groups (wild, semi-wild, landraces, breeding lines, and cultivars) showed large phenotypic diversity. The proportion of non-flowering elongated tillers was higher in timothy cultivars than in landraces and semi-wilds. The cultivars had longer early developmental stages (emergence and tillering) but reached stem elongation and heading earlier than the other accession groups. Although the wild accessions and cultivars did not differ in total biomass production, the cultivars

had higher production of generative tillers and lower production of vegetative tillers, which may have been favoured by the earlier plant development.

- Faster development seen both in the cultivars of timothy and *P. nodosum* under field conditions may have been directly or indirectly selected for in the breeding of these species.
- The differences found between the cultivars and wild accessions may reflect their different histories. In timothy, cultivars and breeding lines showed similar development and tiller formation, while landraces showed similarities with the breeding lines, but differed from the cultivars. The wild accessions showed similarities with the landraces but not with the cultivars. The differences between cultivars and landraces may reflect a stronger directed selection that has favoured agronomic traits in the cultivars, while wild accessions are exposed to diverse natural selection that fluctuates in direction over time.
- The genomic analysis showed distinct pattern of inbreeding, heterozygosity, and LD in *P. alpinum* compared to timothy and *P. nodosum*, suggesting that this species is not a complete outcrosser and might be even a predominantly selfing species.
- The three *Phleum* species differed in genome-wide nucleotide diversity as highlighted by higher values in *P. nodosum* and timothy compared to *P. alpinum*. Higher rates of recombination were also found in timothy and *P. nodosum* compared to in *P. alpinum*. However, weak correlations between nucleotide diversity and recombination rate suggests that other factors such as demographic history, selection pressures, or mutation rates may also be influencing the genome-wide distribution of genetic diversity within these species.
- For both timothy and *P. nodosum*, the low F_{ST} found when comparing cultivars to the wild accessions indicates that there may not have been much genetic divergence from wild populations as a result of domestication or due to possible gene flow between populations. However, genomic patterns of F_{ST} suggested that some genomic regions were highly differentiated and could be indicative of positive selection.

- Selective regions were found to harbour genes involved in various crucial biological processes. In timothy, the coding genes *g22404* and *g39371* were found to be under selection. These genes encode cytochrome P450s (CYPs) that play key role in the biosynthesis of structural polymers like lignin that is important in forage nutritive value and digestibility. Gene *g35607* that encodes jasmonate O-methyltransferase, was also found to be under selection. This protein is crucial for the methylation of different classes of flavonoid compounds that results in phytoalexins or caffeic acid and/or 5-hydroxylferulic acid, which leads to lignin precursors. Some of the other genes under selection were involved in metabolism, signaling and membrane transport and response to abiotic stressors, and some SNPs were enriched within TEs regions that may be important for the species adaptation.

6. Future perspectives

The identified large phenotypic diversity among the studied *Phleum* species and accessions including wild and domesticated groups offer opportunities for developing more efficient breeding strategies. Using accessions with desirable tiller type proportions, rapid development, and with adaptation to diverse growing conditions into breeding programs could serve as important genetic resources to introduce new combinations of traits and alleles to improve productivity and resilience in new cultivars. Considering the need for sustainable forage production in a changing climate, breeding efforts should prioritize development of cultivars resilient to environmental stresses, such as drought, waterlogging and high temperature.

Further evaluation of selected genotypes under adverse conditions and at different geographical locations will enable identification of resilient genetic resources and to understand genotype by environment interactions. Furthermore, as indicated by the selection signatures found in this study, the identification of genomic regions linked to adaptive traits can direct breeding efforts to improve stress tolerance in *Phleum* species. The findings regarding genome-wide nucleotide diversity, population structure, and linkage disequilibrium provide essential insights into the evolutionary history and genetic architecture of these three *Phleum* species. Future research could focus deeper on understanding the genetic basis of traits affecting forage yield, digestibility and nutritional content, as well as resilience to stresses.

Genome-wide association mapping and functional genomics approaches such as gene expression studies and gene editing technologies could be fruitful approaches. Moreover, to increase the genetic gain and shortening the time of breeding programs for timothy through conventional breeding, it is necessary to start the development of genomic selection models. By combining genomic data with conventional breeding techniques, it will be possible to develop high-yielding cultivars that are more resilient to climate change. The results generated and accessions identified in this thesis can serve as foundation in this regard.

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Popular science summary

Timothy is one of the most important forage crops in Sweden and in other countries in Northern Europe. It is grown in mixtures with other plant species, such as red clover, for grazing or as feed for livestock. Timothy has been cultivated in Sweden since the beginning of the 18th century. It is tolerant to cold winters and can grow productively for some years without having to be re-seeded, as it is a perennial crop. Therefore, in addition to its importance as a forage crop, it can also benefit the environment by reducing soil erosion and improving soil quality through less tillage.

However, the production of timothy in Sweden and other Nordic countries is limited by short growing seasons and unpredictable changes in weather due to climate changes. To cope with the adverse conditions and to enable the production of sufficient and high-quality timothy fodder, we need to develop new cultivars that are adapted to the changing climate. An important step towards the development of new cultivars is to evaluate the diversity in traits affecting yield and quality such as plant development, tiller formation, and growth within timothy and related species. Wild populations of timothy are native to Sweden and Northern Europe and are adapted to different habitats and climates. They may harbour diversity and different combinations of traits not seen in cultivars and may be used as donors and genetic resources of favourable traits and genes for improvement of timothy cultivars.

In this thesis a diverse collection of 246 accessions of timothy and its two related species turf timothy (*Phleum nodosum*) and alpine timothy (*Phleum alpinum*) have been studied. These species have different geographical distributions. In Sweden, timothy grows at low altitudes throughout the country, while turf timothy has a southern distribution and alpine timothy is restricted to high altitudes in northern Sweden. The studied accessions are from different habitats and locations in Northern Europe and include wild accessions from natural populations, semi-wild accessions collected nearby cultivated fields, landraces (local farmers' varieties), and breeding lines and cultivars from breeding programs. An accession can be described as a collection of seeds from a specific site. Plants of all accessions have been individually evaluated for biomass production, lengths of different developmental stages (from emergence to heading) and production of different tiller types (vegetative, reproductive and non-flowering elongated tillers) under greenhouse and field conditions.

The greenhouse and field studies showed that these three species differed in their development, tiller formation and growth, and large variation was found within each species. Interestingly, in timothy the group of cultivars and the group of wild accessions did not differ in biomass production. However, they differed in development, where the cultivars showed a faster development and reached stem elongation and flowering earlier than the wild accessions. This suggests that the breeding of timothy has favoured fast growing plants. The cultivars also formed a larger portion of reproductive tillers, while the wild accessions had more vegetative tillers, which increased by latitude. In turf timothy, cultivars formed the highest proportion of non-flowering elongated tillers among all accessions and species, while no such tillers were produced by alpine timothy. Leaves have higher digestibility than stems by cows, and if the non-flowering elongated tillers has a higher fraction of leaves compared to other plant parts, it is possible that this tiller type can contribute with a more digestible feed. Some wild accessions and landraces showed interesting combinations of development and tiller type formation and should be further investigated as potential genetic resources. In addition, turf timothy accessions with favourable traits may be crossed with timothy or further domesticated for development of a new forage grass. Thirty-tree of the accessions from the three species were further studied by whole genome resequencing to investigate how the domestication process and breeding have affected the genomes of the species. A different pattern of genetic diversity was found in timothy and turf timothy compared to alpine timothy, showing higher genetic similarity in alpine timothy. This suggests that alpine timothy has a different mating system with higher degree of self-pollination. Moreover, no clear genetic differentiation was found between cultivated and wild accessions in both timothy and turf timothy. However, some genomic regions were highly differentiated, which indicates that these regions have been under selection during domestication and breeding. These genomic regions are associated with different biological processes such as synthesis of lignin. Lignin production increases in stems and leaves of grasses during growth and decreases forage digestibility and quality. In addition, other genomic regions were found to be associated with metabolism, signalling and the response to abiotic stresses which are likely to influence the adaptation of these species. The identified phenotypic and genetic diversity, as well as accessions with different combinations of traits are important resources for further studies in greenhouse and in field, and for developing genomic-based methods in timothy breeding.

Populärvetenskaplig sammanfattning

Timotej är en av de viktigaste fodergrödorna i Sverige och i andra länder i norra Europa. Den odlas ofta tillsammans med andra växtarter, t.ex. rödklöver, för bete eller som foder till boskap. Timotej har odlats i Sverige sedan början av 1700-talet. Den tål kalla vintrar och kan växa under några år utan att behöva sås varje år eftersom det är en flerårig gröda. Eftersom fleråriga odlingssystem inte kräver samma omfattning av jordbearbetning som ettåriga odlingssystem kan timotej utöver dess betydelse som fodergröda också gynna miljön genom minskad jorderosionen och förbättrad jordhälsa.

Produktionen av timotej i Sverige och andra nordiska länder begränsas dock av korta växtsäsonger och oförutsägbara förändringar i odlingsförhållanden till följd av klimatförändringarna. För att möjliggöra hög produktion av högkvalitativt timotejfoder måste vi utveckla nya sorter som är anpassade till det föränderliga klimatet. Ett viktigt steg mot utvecklingen av nya sorter är att undersöka mångfalden i egenskaper som påverkar avkastning och kvalitet, såsom växtens olika utvecklingsstadier, och förmågan att bilda olika typer av strån (vegetativa skott, långsträckta icke-blommande och reproduktiva strån) hos timotej och besläktade arter. Vilda populationer av timotej är anpassade till olika livsmiljöer och klimat. De kan uppvisa stor mångfald och olika kombinationer av egenskaper som inte finns hos odlad timotej. De kan användas som donatorer och genetiska resurser av gynnsamma egenskaper och gener för förbättring av timotejsorter.

I denna avhandling har en samling av 246 accessioner av timotej och dess två besläktade arter vildtimotej (*Phleum nodosum*) och alpintimotej (*Phleum alpinum*) studerats. Dessa arter har olika geografisk utbredning. I Sverige växer timotej i hela landet, medan vildtimotej har en sydlig utbredning och alpintimotej växer i högfjällsområden i norra Sverige. De studerade accessionerna kommer från olika habitat och platser i norra Europa och inkluderar vilda accessioner från naturliga populationer, "halv-vilda" accessioner insamlade i närheten av odlade fält, lantraser (lokala sorter) samt förädlingslinjer och sorter från förädlingsprogram. En accession kan beskrivas som en samling frön som har insamlats från en specifik plats. Växter från alla accessioner har utvärderats individuellt för produktion av biomassa, längden på olika utvecklingsstadier (från uppkomst till blomning) och produktion av olika typer av strån under växthus- och fältförhållanden.

Växthus- och fältförsöken visade att dessa tre arter skiljde sig åt i utveckling, tllväxt och bildandet av vegetativa skott, långsträckta icke-blommande och reproduktiva strån, och att det fanns stora variationer inom varje art. Intressant nog skiljde sig inte gruppen av sorter och gruppen av vilda accessioner hos timotej åt i biomassaproduktion. De skiljde sig dock åt i utveckling, där sorterna visade en snabbare utveckling och nådde stråskjutning och blomning tidigare än de vilda accessionerna. Detta tyder på att växtförädlingen av timotej har gynnat snabbväxande plantor. Sorterna bildade också en större andel reproduktiva strån, medan de vilda accessionerna hade fler vegetativa skott, som ökade med breddgraden för deras ursprungliga växtplats. I vildtimotej bildade sorterna den högsta andelen icke-blommande långsträckta strån bland alla accessioner och arter, medan denna typ av strån inte bildades hos alpintimotej. Blad har högre smältbarhet än strå för kor, och om de icke-blommande långsträckta stråna har en högre andel blad jämfört med andra växtdelar, är det möjligt att denna stråtyp kan bidra med ett mer smältbart foder. Vissa vilda accessioner och lantraser visade intressanta kombinationer av egenskaper. Dessa accessioner bör studeras vidare för deras användning som potentiella genetiska resurser. Dessutom kan accessioner av arten vildtimotej som har gynnsamma egenskaper korsas med odlad timotej eller domesticeras vidare för utveckling av ett nytt fodergräs.

Trettiotre av accessionerna från de tre arterna studerades vidare genom helgenom-resekvensering för att undersöka hur domesticeringsprocessen och växtförädlingen har påverkat arternas genom. Ett annorlunda mönster av genetisk mångfald hittades i timotej och vildtimotej jämfört med alpintimotej, vilket visar större genetisk likhet inom alpintimotej. Detta tyder på att denna art har ett annorlunda reproduktionssystem med högre grad av självpollinering. Dessutom hittades ingen tydlig genetisk differentiering mellan odlade och vilda accessioner i både timotej och vildtimotej. Stor differentiering upptäcktes dock i vissa genomiska regioner, vilket tyder på att dessa regioner har varit påverkade av urval under domesticeringen och växtförädlingen. Dessa genomiska regioner är associerade med olika biologiska processer såsom syntes av lignin. Ligninproduktionen ökar i gräsets stjälkar och blad under tillväxten och minskar fodrets smältbarhet och kvalitet. Andra genomiska regioner var associerade med metabolism, signalering och respons på abiotisk stress som sannolikt påverkar arternas anpassning. Den identifierade fenotypiska och genetiska mångfalden, liksom de identifierade accessionerna med olika kombinationer av egenskaper är viktiga resurser för vidare studier i växthus och i fält, och för utveckling av genomik-baserade metoder i timotejförädlingen.

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Article

Phenotypic Diversity in Domesticated and Wild Timothy Grass, and Closely Related Species for Forage Breeding

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Abstract: Timothy grass (*Phleum pratense* L.) is one of the most important forage crops in temperate regions. Forage production, however, faces many challenges, and new cultivars adapted to a changing climate are needed. Wild populations and relatives of timothy may serve as valuable genetic resources in the breeding of improved cultivars. The aim of our study is to provide knowledge about the phenotypic diversity in domesticated (cultivars, breeding lines and landraces) and wild timothy and two closely related species, *P. nodosum* (lowland species) and *P. alpinum*, (high altitude species) to identify potential genetic resources. A total of 244 accessions of timothy and the two related species were studied for growth (plant height, fresh and dry weight) and plant development (days to stem elongation, days to booting and days to heading) in the field and in a greenhouse. We found a large diversity in development and growth between the three *Phleum* species, as well as between the accessions within each species. Timothy showed the highest growth, but no significant difference was found between wild accessions and cultivars of timothy in fresh and dry weight. However, these two groups of accessions showed significant differences in plant development, where timothy cultivars as a group reached flowering earlier than the wild accessions. This suggests that there has not been a strong directional selection towards increased yield during the domestication and breeding of timothy; rather, timothy has been changed for other traits such as earlier heading. Principal component analysis and cluster analysis based on all traits revealed distinct clusters. Accessions falling within the same cluster showed similarities in the development and growth rather than the type of accession. The large diversity found in this study shows the potential of using timothy accessions as genetic resources in crosses with existing cultivars. Also, accessions of *P. nodosum* with favorable traits can be candidates for the domestication of a novel forage crop, and the high-altitude relative *P. alpinum* may be a source of genes for the development of more cold and stresstolerant cultivars.

Keywords: biomass; development; forage crop; genetic resources; perennial; *Phleum alpinum*; *Phleum nodosum*; *Phleum pratense*; wild relatives

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1. Introduction

Timothy (*Phleum pratense* L. subsp. *pratense*) is one of the most important forage crops in temperate regions. It is an outcrossing, short-lived perennial with shallow roots and, due to its winter hardiness, it is the preferred species for forage grass cultivation at higher latitudes in Northern Europe, East Asia and North America [1]. Timothy is a leafy and tall-growing grass with high biomass production, as well as high nutritive value and digestibility compared to many other forage grasses [2]. The forage quality of timothy is regulated by the developmental stage of the plant and is usually highest during the early growth of the crop when most of the tillers are in the vegetative stage [3,4].

Biomass production increases with age when the tillers elongate and reach the flowering stage. In contrast to some other forage crops such as *Lolium perenne* L. and *Festuca arundinacea* Schreb., timothy can form elongated and heading tillers without vernalization (cold treatment) [5,6]. However, flowering is stimulated by vernalization, especially in northern accessions [6–9]. Flowering and stem elongation are also stimulated by a longer photoperiod [5,8–10].

New tillers are formed from buds in leaf sheaths at the base of the stem. The stem bases (corms) are swollen and can serve as energy reserves [11]. With its rapid growth and formation of tillers, timothy can be harvested two to four times per season. However, the buds and corms in the crown are sensitive to trampling and grazing by cattle, which decreases the production of tillers. Another limitation for its persistence in pure stands or in mixtures with perennial legumes and other forage grasses is its shallow root system and its sensitivity to drought [12].

Timothy, together with fourteen perennial and annual species as well as several subspecies, form the genus *Phleum* [13,14]. Besides timothy, several other of these species are domesticated and cultivated as forage crops. The *Phleum* species vary in ploidy level from diploid to octoploid [13,14]. Even different ploidy forms are described within the same taxa. The polyploid forms are presumed to be the result of hybridization events and subsequent polyploidizations. The most common form of timothy is hexaploid, with 42 chromosomes (2n = 6x = 42). Hexaploid timothy is native to Europe, except for the Mediterranean areas, and the cultivated crop was domesticated from hexaploid wild populations in Northern Europe. Wild timothy populations grow in lowland areas throughout Europe.

The genetic relationship between the *Phleum* species and the origin of hexaploid timothy have puzzled scientists over the years and are still not fully understood. Based on genetic analysis, the similarity between hexaploid timothy and the diploid relative *P. nodosum* (syn. *P. pratense* L. subsp. *bertolonii (DC.) Bornm.* and *P. bertolonii (DC.) Bornm.*, 2n = 2x = 14) was found in chloroplast trnL intron DNA sequences [14]. Genetic similarities were also found between hexaploid timothy and the diploid *P. alpinum* subsp. *rhaeticum* Humphries (2n = 2x = 14). A hybridization is suggested to have occurred between the two diploid species *P. nodosum* and *P. alpinum* subsp. *rhaeticum* in the Italian Alps, which resulted in an allotetraploid *Phleum*. Moreover, cytological studies suggest the presence of two genomes of *P. nodosum* in hexaploid timothy [15,16]. Thus, the allotetraploid may have hybridized with northern European populations of *P. nodosum*, and with subsequent polyploidization, formed the hexaploid timothy, *P. pratense* subsp. *pratense*.

Wild populations of the different *Phleum* species originate from various geographical regions and are adapted to different habitats such as meadows and grasslands. Hybridization and polyploidy events in the evolution of *Phleum* are likely to have played an important role in the geographical and ecological patterns as well as in the diversification of the species. Also, variation in the direction and intensity of natural selection may have resulted in locally adapted populations within the species. Due to the strong human selection during crop domestication, the species may have undergone large bottlenecks, leaving much of the traits and genes behind in the wild populations [17–19]. These traits may be of large interest for the improvement of crops [20]. The wild populations of timothy and the related *Phleum* species are therefore potential genetic resources for the development of new high-yielding timothy cultivars adapted to a changing climate.

In this study, we investigated the development and growth in domesticated and wild hexaploid timothy and the two closely related species, the diploid *P. nodosum* and the tetraploid *P. alpinum* (2n = 4x = 28), growing in the Nordic countries. Taxonomically, the tetraploid *P. alpinum* belongs to the same species as the diploid *P. alpinum* subsp. *rhaeticum* [13,14]. The wild populations of *P. alpinum* commonly grow on meadows, riverbanks, roadsides and in birch forests at high elevations. At northern latitudes *P. alpinum* is found in Iceland, Norway, northern Sweden and northern Finland. Wild populations of *P. nodosum* are commonly found on meadows at low elevations. At northern latitudes, it has a more southern distribution than *P. alpinum*. Wild populations of timothy are found throughout the Nordic countries and grow at low elevations on meadows and in human-impacted and disturbed soils. The wild and domesticated accessions were studied both in the greenhouse and in the field to address the following questions: Do the three *Phleum* species differ in growth and development? Does the pattern of phenotypic diversity vary within and between the *Phleum* species? Do the wild and domesticated accessions of timothy differ in growth and development? Do some accessions show traits of interest for future breeding, and are therefore important genetic resources?

2. Results

2.1. Survival in the Field

The survival after the first winter was high in all groups of timothy accessions comprising wild, semi-wild, landraces, breeding lines and cultivars (100% of the accessions and from 93% to 96% of the plants; Table 1). The survival was also high in the cultivars and in the wild accessions of *P. nodosum* (100% of the accessions and from 92% to 96% of the plants). However, in *P. alpinum,* only 67% of the wild accessions and 66% of the plants survived the first winter.

Table 1. Number of accessions and number of plants planted in the field and the survival of accessions and plants after the first winter for different groups of accessions.

Category	Species	Group	Planted (Number)	Survived (Number)	Survival Rate (%)
Accessions	*P. pratense*	Cultivar	56	56	100.0
		Breeding line	10	10	100.0
		Landrace	55	55	100.0
		Wild	61	61	100.0
		Semi-wild	30	30	100.0
	P. nodosum	Cultivar	7	7	100.0
		Wild	7	7	100.0
	P. alpinum	Wild	18	12	66.6
Plants	*P. pratense*	Cultivar	823	769	93.4
		Breeding line	151	145	96.0
		Landrace	779	741	95.1
		Wild	928	876	94.4
		Semi-wild	440	415	94.3
	P. nodosum	Cultivar	90	86	95.6
		Wild	106	97	91.5
	P. alpinum	Wild	29	19	65.5

2.2. Phenotypic Differences between Species

The ANOVA results demonstrated significant differences between the three *Phleum* species for all the studied growth traits and developmental stages under both field and greenhouse conditions (Tables S1 and S2). In the field, we observed significant block effects but failed to detect such effects in the greenhouse experiment. *P. nodosum* and *P. alpinum* showed similar days to booting (DTB) and days to heading (DTH) in the field, while timothy reached booting and heading later (Tukey HSD, $p < 0.05$; Table S3). However, in the greenhouse, *P. alpinum* and timothy showed similar development and reached booting and heading later than *P. nodosum* (Tukey HSD, $p < 0.05$; Table S4). In the field, the highest fresh weight (FW), dry weight (DW) and plant height (PH) were found in timothy (Tukey HSD, $p < 0.05$; Table S3), while in the greenhouse *P. nodosum* had the highest means of FW, DW and PH (Tukey HSD, $p < 0.05$; Table S4).

2.3. Variation in Growth Traits among Accessions

2.3.1. Field Trial

The mean FW and DW were about one-third higher in almost all groups of accessions of timothy compared to the cultivars and wild accessions of *P. nodosum,* and almost twice as high compared to the wild accessions of *P. alpinum* (Figure 1a,b). Interestingly, no significant difference was found between cultivars and wild accessions of timothy in FW or in DW (Tukey HSD, $p > 0.05$), while the semi-wild accessions had a significantly lower FW and DW. Similarly, no significant difference was observed between cultivars and wild accessions of *P. nodosum* (Student's *t*-test, $p > 0.05$).

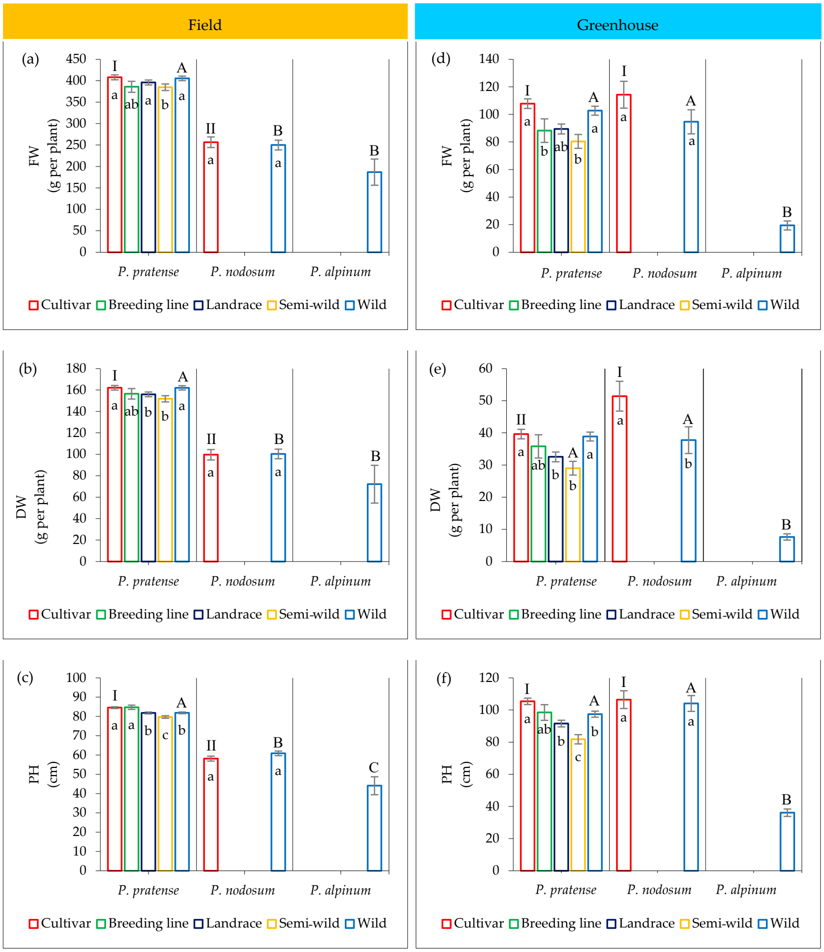


Figure 1. Mean and SD of fresh weight (FW), dry weight (DW) and plant height (PH) in different groups of accessions of *P. pratense*, *P. nodosum* and *P. alpinum* in the field (**a**–**c**) and in the greenhouse (**d**–**f**). Mean values that do not share the same letter are significantly different among accessions according to Student's *t*-test, $p < 0.05$ for comparing two groups of accessions, and Tukey HSD, $p < 0.05$ for comparing more than two groups. Letters inside the bars show comparisons between groups of accessions within each species and capital letters outside the bars show comparisons between the groups of wild accessions of the three species, and Roman letters show comparisons between groups of cultivars of *P. pratense* and *P. nodosum*.

A similar pattern was also found for PH. All groups of timothy accessions showed about one-third higher mean than the *P. nodosum* cultivars and wild accessions, and more than twice the mean of *P. alpinum* (Figure 1c). There was no significant difference in PH between wild and cultivated accessions of *P. nodosum* (Student's *t*-test, $p > 0.05$). However, within timothy the wild and semi-wild accessions as well as the landraces showed significantly lower PH than the breeding lines and cultivars (Tukey HSD, $p < 0.05$).

2.3.2. Greenhouse Trial

The mean FW and DW of all groups of accessions in all species were significantly lower in the greenhouse than in the field (Figure 1d,e). For timothy, the FW and DW were about one-fourth of the yield in the field, and for *P. alpinum* they were only one-fifth of the yield in the field. However, in *P. nodosum* the yield was only reduced by half in the greenhouse compared to the field.

Even though the FW and DW were lower in the greenhouse, the pattern of variation among groups of timothy accessions was similar to the pattern found in the field, where no significant difference was found between the cultivars and the wild accessions (Tukey HSD, $p > 0.05$). In contrast to the field trial, the DW was significantly higher in the cultivars than in the wild accessions of *P. nodosum* (Student's *t*-test, $p < 0.05$).

The mean PH was higher in the greenhouse than in the field for all groups of timothy accessions, except for the semi-wilds (Figure 1f), but the pattern of variation was similar to that found in the field.

2.4. Variation in Development among Accessions

2.4.1. Field Trial

A partially deviant pattern of variation was found when comparing the development within and between the three *Phleum* species compared to the pattern of variation for the growth traits. In timothy, the mean of DTB and DTH was significantly lower in the cultivars compared to the other groups of accessions, while for days to stem elongation (DTS) it was only significantly different from the landraces (Tukey HSD, $p < 0.05$; Figure 2a–c). In *P. nodosum*, the cultivars showed a significantly lower mean than the wild accessions for all developmental stages. In other words, the cultivars of both timothy and *P. nodosum* reached the three developmental stages earlier than their wild accessions did. Moreover, the *P. nodosum* cultivars reached these developmental stages earlier than the timothy cultivars. The wild accessions of timothy and *P. nodosum* did not differ significantly in DTB or DTH, while the wild accessions of *P. alpinum* reached booting earlier than timothy and *P. nodosum*, and reached heading earlier than the wild timothy accessions.

2.4.2. Greenhouse Trial

In timothy, two wild accessions, four semi-wild accessions, four landraces, one breeding line and two cultivars only formed vegetative tillers at the end of the greenhouse trial about six months after the vernalization treatment. In addition, twelve wild accessions, nine semi-wild accessions, nine landraces, two breeding lines and four cultivars remained in the elongated stage at the end of the trial. For the plants that reached heading, the results from the greenhouse trial showed a pattern similar to what was found in the field for the developmental traits (Figure 2d,f), where cultivars had a significantly lower mean of DTS compared to the other groups of timothy accessions (Tukey HSD, $p < 0.05$). The cultivars also showed a significantly lower DTB and DTH compared to the landraces and the wild accessions of timothy (Tukey HSD, $p < 0.05$).

The pattern of variation was, however, different for *P. nodosum*. In contrast to the field trial, the cultivars reached booting and heading later than the wild accessions. Moreover, the development of the wild accessions of *P. alpinum* was rather different in the greenhouse than in the field since 12 out of the 18 accessions (86%) did not form elongated or heading tillers in the greenhouse.

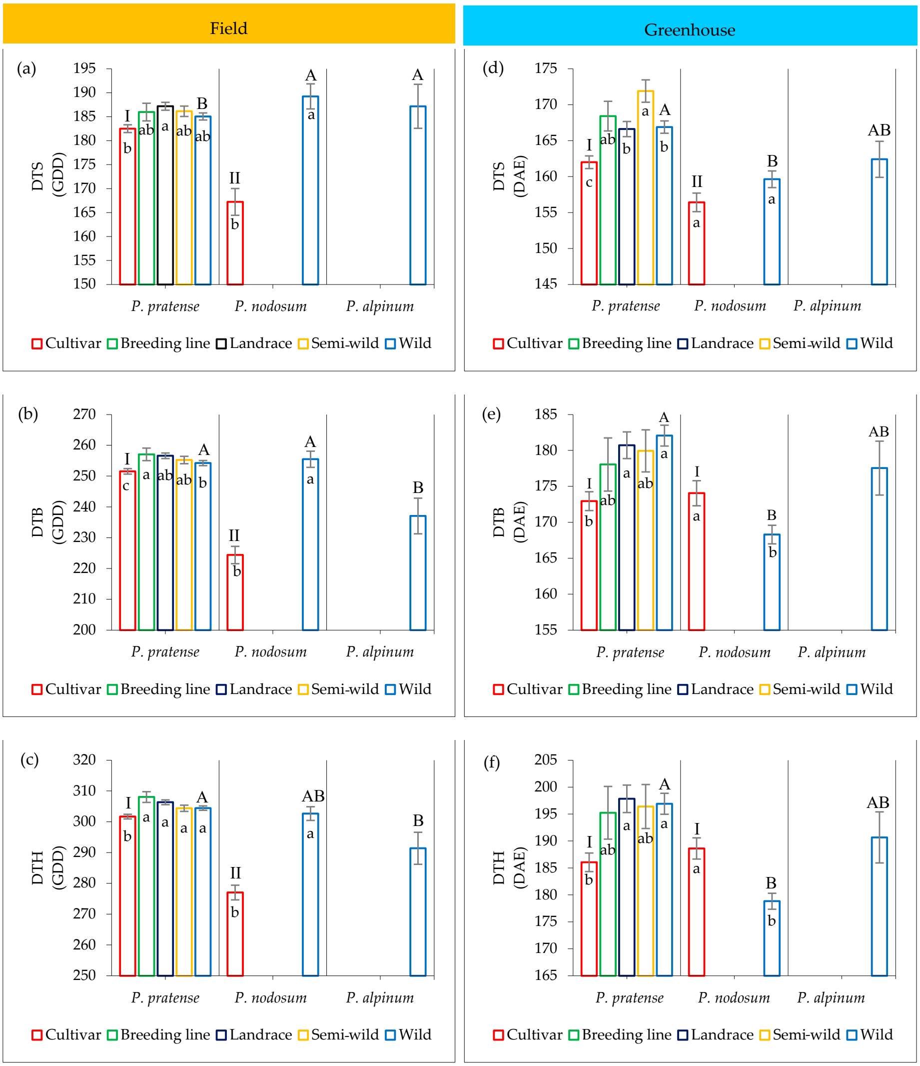


Figure 2. Mean and SD of days to stem elongation (DTS), days to booting (DTB) and days to heading (DTH) in different groups of accessions of *P. pratense*, *P. nodosum* and *P. alpinum* in the field based on growing degree days (GDD, **a**–**c**) and in the greenhouse based on days after emergence (DAE, **d**–**f**). Mean values that do not share the same letter are significantly different among accessions according to Student's *t*-test, $p < 0.05$ for comparing two groups of accessions, and Tukey HSD, $p < 0.05$ for comparing more than two groups. Letters inside the bars show comparisons between groups of accessions within each species and capital letters outside the bars show comparisons between the groups of wild accessions of three species, and Roman letters show comparisons between groups of cultivars of *P. pratense* and *P. nodosum*.

2.5. Correlation between Traits

2.5.1. Field Trial

A strong positive correlation was found between the variation in all developmental stages (DTS, DTB and DTH) in all three species in the field (Figure 3a–c). FW and DW were also strongly correlated in all three species, while FW and PH, as well as DW and PH, showed a weaker correlation in both timothy and *P. nodosum*. The variation in the growth traits was not strongly correlated with the variation in DTS, DTB and DTH in all three species. In fact, we found no correlation between most of the growth and developmental traits in *P. nodosum* and *P. alpinum*. In timothy, no correlations or weak correlations were found between the two types of traits.

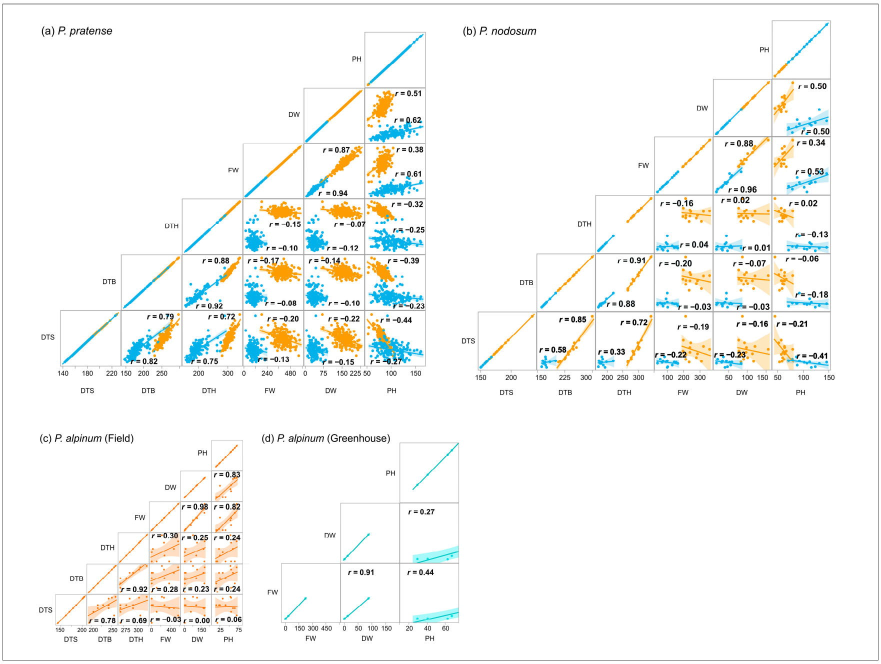


Figure 3. Correlation analysis between the studied traits, days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the field (brown) and in the greenhouse (blue). (**a**) *P. pratense*, (**b**) *P. nodosum* and (**c**) *P. alpinum* in the field and (**d**) *P. alpinum* in the greenhouse.

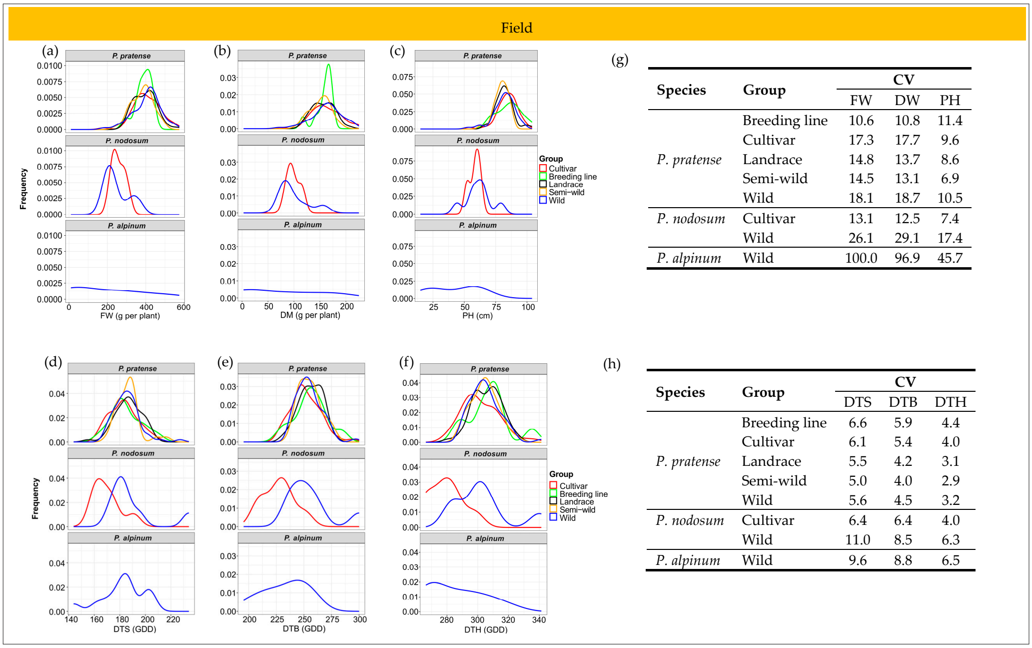
2.5.2. Greenhouse Trial

Only the accessions that developed stem elongation, booting or heading in the greenhouse trial were included in the correlation studies. In timothy, a similar pattern of correlation between traits as seen in the field was also found in the greenhouse (Figure 3a). This was also true for *P. nodosum*, except for the weaker correlation between DTS and DTB, and between DTS and DTH, and the stronger correlation between FW and PH found in the greenhouse (Figure 3b). Moreover, *P. alpinum* plants showed a weaker correlation between FW and PH, and between DW and PH in the greenhouse (Figure 3d). Due to the low number of *P. alpinum* plants that formed elongated and flowering tillers, we did not perform a correlation test between the three developmental stages or between them and the growth traits.

2.6. Patterns of Phenotypic Diversity

2.6.1. Field Trial

The distribution of the studied traits varied between species and between groups of accessions within the species (Figure 4a–f). Coefficient of variation (CV) was calculated for each trait to evaluate the phenotypic diversity among the species and groups of accessions. *P. alpinum* showed a much higher CV than timothy and *P. nodosum* for all growth traits in the field (Figure 4g). In timothy, the wild accessions and cultivars showed a similar diversity and the highest CV for FW and DW, while the breeding lines had the highest CV for PH. However, in *P. nodosum*, the wild accessions had a much higher CV than the cultivars for all growth traits. The highest diversity in the developmental traits was found in *P. alpinum* and the wild accessions of *P. nodosum* (Figure 4h). In timothy, the cultivars and breeding lines showed larger diversity in DTS, DTB and DTH than the other groups of accessions.



Species	Group	CV		
		FW	DW	PH
P. pratense	Breeding line	10.6	10.8	11.4
	Cultivar	17.3	17.7	9.6
	Landrace	14.8	13.7	8.6
	Semi-wild	14.5	13.1	6.9
	Wild	18.1	18.7	10.5
P. nodosum	Cultivar	13.1	12.5	7.4
	Wild	26.1	29.1	17.4
P. alpinum	Wild	100.0	96.9	45.7

Species	Group	CV		
		DTS	DTB	DTH
P. pratense	Breeding line	6.6	5.9	4.4
	Cultivar	6.1	5.4	4.0
	Landrace	5.5	4.2	3.1
	Semi-wild	5.0	4.0	2.9
	Wild	5.6	4.5	3.2
P. nodosum	Cultivar	6.4	6.4	4.0
	Wild	11.0	8.5	6.3
P. alpinum	Wild	9.6	8.8	6.5

Figure 4. Distribution of studied traits in different groups of accessions of *P. pratense*, *P. nodosum* and *P. alpinum* in the field. (**a**) Fresh weight (FW), (**b**) dry weight (DW), (**c**) plant height (PH), (**d**) days to stem elongation (DTS), (**e**) days to booting (DTB), (**f**) days to heading (DTH), (**g**) coefficient of variation (CV) of growth traits, (**h**) CV of developmental stages.

A principal component analysis (PCA) based on the variation in all growth traits and the developmental stages studied in the field showed a large phenotypic diversity among timothy accessions and formed six clusters (Figure 5a). In general, the accessions did not cluster according to the type of accessions (cultivar, breeding line, landrace, semi-wild and wild).

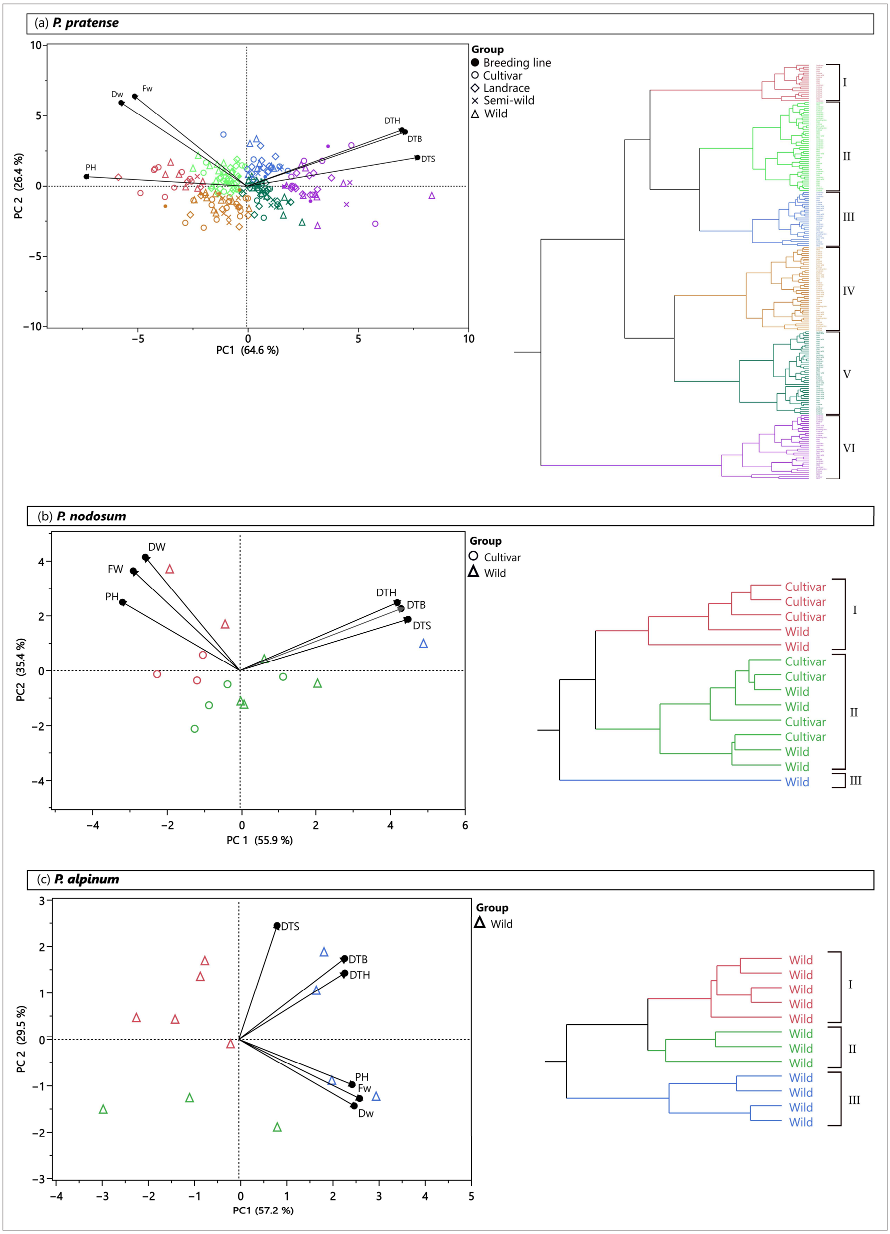


Figure 5. Principal component analysis (PCA) and cluster analysis based on all studied traits, days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the field for (**a**) *P. pratense*, (**b**) *P. nodosum* and (**c**) *P. alpinum*. Different colors in the PCA correspond to the clusters of accessions defined in the cluster analysis.

Timothy accessions falling within the same cluster in the PCA showed similarities in the development and growth traits rather than the type of accession. Accessions in cluster I (highlighted in red) had on average the highest PH, FW and DW, and reached the three developmental stages (DTS, DTB and DTH) earlier than the accessions in the other clusters (Figure 5a and Table 2). On the contrary, the accessions in cluster VI (highlighted in purple) showed on average the lowest yield and PH, and reached elongation, booting and heading later than any of the other clusters. The accessions in cluster II (highlighted in light green) and cluster III (highlighted in blue) had similar and relatively high FW and DW, but accessions in cluster II reached the three developmental stages earlier than those in cluster III. Both cluster IV (highlighted in brown) and cluster V (highlighted in dark green) consisted of relatively low-yielding accessions. The two clusters differed, however, in DTS, DTB and DTH. The total number of accessions of each group of accessions is shown in Table S5.

Table 2. Mean of each cluster for each studied trait in *P. pratense, P. nodosum* and *P. alpinum* in the field.

Species	**Cluster**	**Number of Accessions**	**Trait**					
			DTS (GDD)	**DTB (GDD)**	**DTH (GDD)**	**FW (g per Plant)**	**DW (g per Plant)**	**PH (cm)**
P. pratense	I	19	170.1	239.1	291.9	480.9	196.9	94.0
	II	46	182.1	252.0	302.1	448.2	176.7	85.6
	III	28	190.8	266.0	314.4	442.9	173.7	81.8
	IV	43	176.7	243.1	293.9	364.8	148.4	86.7
	V	43	187.5	255.5	305.7	364.0	142.4	78.7
	VI	33	201.4	270.6	318.5	339.3	133.2	71.1
P. nodosum	I	5	173.0	233.9	285.6	309.8	121.5	64.5
	II	8	177.1	239.0	288.2	221.9	87.2	58.7
	III	1	234.1	300.1	340.0	212.3	86.6	44.2
P. alpinum	I	5	188.9	232.4	279.5	69.0	25.4	27.0
	II	3	164.2	205.9	268.5	168.3	80.7	46.0
	III	4	187.0	252.7	307.0	386.5	136.9	59.6

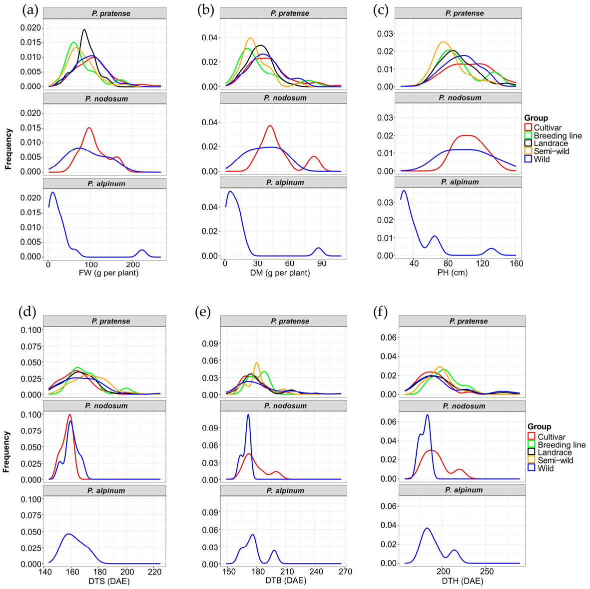
For *P. nodosum*, two clusters were formed with wild accessions and cultivars in both clusters (Figure 5b). In addition, a single wild accession did not cluster with any other accession. Cluster I (highlighted in red) consisted of accessions with, on average, the tallest plants as well as the highest FW and DW (Table 2). In contrast, the single accession (highlighted in blue) reached heading very late and had the lowest PH, FW and DW. The accessions in cluster II (highlighted in green) showed a phenotype in between the accessions in cluster I and the single accession.

The wild accessions of *P. alpinum* were divided into three clusters (Figure 5c). Cluster I (highlighted in red) was represented by accessions with very low PH, FW and DW, while cluster III (highlighted in blue) showed the highest yield (Table 2). Cluster II (highlighted in green) consisted of very early booting and heading accessions.

2.6.2. Greenhouse Trial

Differences in the distribution of the growth traits and the developmental stages were also found between species and between groups of accessions within species in the greenhouse trial (Figure 6a–f). However, the distribution pattern in the greenhouse differed partly from the pattern found in the field. For example, the variation in the distribution of PH was larger between groups of timothy accessions in the greenhouse than in the field (Figures 4c and 6c) and the variation in the developmental traits was larger between the wild accessions and the cultivars of *P. nodosum* in the field (Figures 4d–f and 6d–f).

Greenhouse



(g)

Species	Group	CV		
		FW	DW	PH
P. pratense	Breeding line	48.3	62.0	26.5
	Cultivar	41.7	48.2	23.9
	Landrace	29.4	35.1	22.3
	Semi-wild	41.9	43.6	22.4
	Wild	36.7	40.7	20.4
P. nodosum	Cultivar	30.5	37.1	15.1
	Wild	44.9	41.6	25.7
P. alpinum	Wild	162.3	157.4	61.9

(h)

Species	Group	CV		
		DTS	DTB	DTH
P. pratense	Breeding line	7.3	4.7	7.6
	Cultivar	6.2	7.4	8.4
	Landrace	6.6	10.0	12.5
	Semi-wild	6.8	7.0	8.0
	Wild	8.5	12.7	15.6
P. nodosum	Cultivar	2.5	6.9	7.8
	Wild	3.3	2.5	2.9
P. alpinum	Wild	4.7	7.3	7.7

Figure 6. Distribution of studied traits in different groups of accessions of *P. pratense*, *P. nodosum* and *P. alpinum* in the greenhouse. (**a**) Fresh weight (FW), (**b**) dry weight (DW), (**c**) plant height (PH), (**d**) days to stem elongation (DTS), (**e**) days to booting (DTB), (**f**) days to heading (DTH), (**g**) coefficient of variation (CV) of growth traits, (**h**) CV of developmental stages.

Similar to what was found in the field trial, *P. alpinum* showed a much higher diversity for the growth traits than the other two species in the greenhouse (Figure 6g). Also, the wild accessions of *P. nodosum* had a higher CV than the *P. nodosum* cultivars for FW, DW and PH. While the wild accessions and cultivars had the highest diversity for FW and DW in the field, the breeding lines of timothy showed the highest CV for the growth traits in the greenhouse. The highest diversity for the developmental traits was found in the wild accessions of timothy, while the wild accessions of *P. nodosum* showed the lowest CV for DTB and DTH (Figure 6h).

A PCA based on all studied traits in the greenhouse also showed a large diversity among accessions in all the three *Phleum* species (Figure 7). Among the timothy accessions that reached heading, five clusters were formed. Both domesticated and wild accessions were found in all clusters. Accessions in cluster V (highlighted in dark green) had the highest yield and PH and reached the three developmental stages early (Figure 7a and Table 3). Cluster II (highlighted in light green) and III (highlighted in blue) included both low-yielding accessions, but the accessions in cluster III reached heading much later than the accessions in cluster II. Mid-early accessions were found in cluster IV (highlighted in brown). These accessions showed high FW and DW. The total number of accessions of each group of accessions is shown in Table S6.

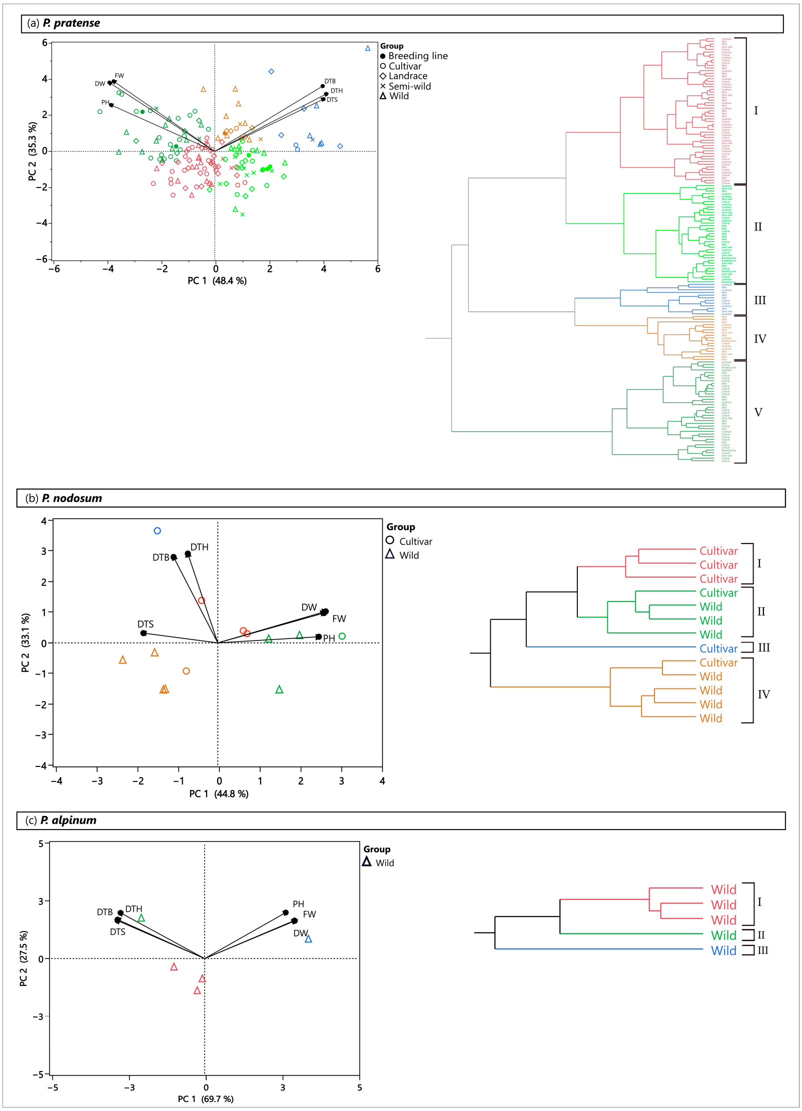


Figure 7. Principal component analysis (PCA) and cluster analysis based on all studied traits, days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the greenhouse for (**a**) *P. nodosum*, (**b**) *P. alpinum* and (**c**) *P. pratense*. Different colors in the PCA correspond to the clusters of accessions defined in the cluster analysis.

Table 3. Mean of each cluster for each studied trait in *P. pratense, P. nodosum* and *P. alpinum* in the greenhouse.

Species	Cluster	Number of Accessions	Trait					
			DTS (DAE)	DTB (DAE)	DTH (DAE)	FW (g per Plant)	DW (g per Plant)	PH (cm)
P. pratense	I	55	159.4	170.2	183.4	92.6	34.2	103.2
	II	37	169.3	180.9	195.6	70.0	25.1	80.2
	III	12	183.7	224.2	252.3	71.4	24.9	87.6
	IV	17	175.1	196.1	217.1	123.1	46.0	96.6
	V	38	160.2	174.5	184.9	143.9	56.9	125.7
P. nodosum	I	3	154.7	177.4	193.2	108.3	46.2	105.2
	II	4	156.7	167.5	179.6	141.5	60.1	128.0
	III	1	161.3	200.0	220.0	100.7	43.8	92.1
	IV	5	161.9	170.0	179.9	65.4	27.4	88.8
P. alpinum	I	3	161.2	174.7	187.2	3.1	1.6	44.1
	II	1	173.0	198.0	214.0	33.8	12.8	67.1
	III	1	154.0	163.5	176.5	223.6	87.5	132.0

The accessions of *P. nodosum* were grouped into three clusters (one more cluster than in the field) and a single cultivar that did not cluster with any other accession (Figure 7b). Cluster I (highlighted in red), which comprised only cultivars, cluster II (highlighted in green) and cluster III (highlighted in blue) had all high and similar mean FW and DW, while cluster IV (highlighted in brown) showed a low yield (Table 3). All clusters reached stem elongation early, but differed in DTB and DTH, where accessions in cluster III were very late.

P. alpinum formed one cluster with three accessions (highlighted in red, Figure 7c). The other two accessions did not cluster with each other or any other *P. alpinum* accession. The mean FW and DW in the accessions were very low in cluster I and one of the single accessions (highlighted in green) compared to *P. nodosum* and timothy (Table 3). Cluster I and this single accession (highlighted in green) also showed late booting and heading, while the other single accession (highlighted in blue) was early and high yielding.

2.7. Heritability

The broad-sense heritability (H^2) was lower in the field than in the greenhouse for all traits, except for PH which showed similar H^2 in both trials (Table 4). The largest difference was found for the developmental stages where H^2 was as high as 0.95 for both DTB and DTH in the greenhouse, while it was 0.37 for both traits in the field. In the greenhouse, the growth traits showed much lower H^2 compared to the developmental stages. However, in the field, the heritability for the growth traits and the developmental stages was more similar.

Table 4. Genotypic variance (V_g), environmental variance (V_e), phenotypic variance (V_p) and broad-sense heritability (H^2) of days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the field and in the greenhouse for *P. pratense*.

Trial	Trait	V_g	V_e	V_p	H^2
Field	DTS	0.9	5.6	2.3	0.40
	DTB	0.6	3.8	1.5	0.37
	DTH	0.5	3.2	1.3	0.37
	FW	2449.5	24,289.1	8521.7	0.29
	DW	401.0	3708.0	1328.0	0.30
	PH	48.7	160.6	88.9	0.55

Table 4. *Cont.*

Trial	Trait	V_g	V_e	V_p	H^2
Greenhouse	DTS	99.2	111.6	127.1	0.78
	DTB	286.4	60.1	301.5	0.95
	DTH	505.0	98.9	529.7	0.95
	FW	745.9	3204.1	1546.9	0.48
	DW	125.2	559.9	265.1	0.47
	PH	315.3	1092.8	588.4	0.54

3. Discussion

Agriculture is challenged by an increased demand for food for a growing human population and thereby an increased demand for animal feed during a changing climate. To respond to this challenge, high-yielding cultivars adapted to grow in a changing environment need to be developed. In this context, wild populations and closely related species to the crop may serve as valuable genetic resources for the development of improved cultivars. The aim of our study was to provide knowledge about the phenotypic diversity in the forage crop timothy and two of its closely related species, *P. nodosum* and *P. alpinum*. We studied different growth and plant developmental traits in domesticated and wild accessions and identified potential genetic resources for timothy breeding.

The three *Phleum* species showed variation in growth. All groups of timothy accessions had a significantly higher FW, DW and PH than *P. nodosum* and *P. alpinum* in the field. Moreover, *P. nodosum* formed taller tillers than *P. alpinum*. The greater growth of timothy was expected as it is one of the most important forage crops in temperate regions and cultivated for its high biomass production. Cultivars of *P. nodosum* and *P. alpinum* are developed, but to the best of our knowledge not used in mixtures with other forage crops. However, in the greenhouse *P. nodosum* showed similar growth to timothy, while the plants of *P. alpinum* were very small and most of them did not produce elongated tillers. The cultivation of plants under different growing conditions in the greenhouse compared to in the field, such as differences in temperature, photoperiod and water and nutrient content in the soil, most likely contributed to the overall lower FW and DW in the greenhouse. For example, a longer photoperiod has been shown to stimulate growth and biomass production in *P. alpinum* [21]. The variation in the growing conditions seems to have also affected the heritability of the studied traits in timothy, where the heritability was higher in the greenhouse than in the field for almost all traits.

P. nodosum was, however, less affected by the different growing conditions in the field and the greenhouse than timothy and *P. alpinum*. The adaptation to different habitats may explain the difference in performance among the species in the two trials. *P. nodosum* is characterized as a lowland species with rapid growth and spread, while *P. alpinum* is primarily found at higher altitudes adapted to harsh mountainous environments [22]. The diploid *P. nodosum* and the tetraploid *P. alpinum* carry different genomes [13,14]. The genomic form of *P. nodosum* is described as B_NB_N and the genomic form of *P. alpinum* as R_ER_EXX. Our result suggests that the B_N genome carries genes for good and robust growth in different growing environments. In addition, cytological studies indicate that the hexaploid timothy carries two genomes of *P. nodosum* [15,16]. The polyploidization and duplication of the B_N genome in timothy is likely to have contributed to its large growth and biomass production. In fact, polyploidy has been shown to increase plant growth in wild plants and domesticated crops [23–25]. Polyploidization is an important tool in forage crop breeding, and the effect of genome duplication on biomass production and other traits would be of interest to explore.

A large phenotypic diversity was found within each *Phleum* species and the different groups of accessions. However, even though the diversity was high within the wild accessions and the cultivars of timothy, these two groups did not differ significantly in FW and DW in the field or in the greenhouse. Moreover, we did not find significant difference in spring growth between wild accessions and cultivars of *P. nodosum*. The similarity in the phenotype of wild accessions and cultivars as well as the large diversity among cultivars found in this study suggest that there has not been a strong directional selection towards increased yield during the domestication and breeding of timothy and *P. nodosum*. A strong directional selection towards a higher seed yield and other domestication traits such as resistance to seed shattering and apical dominance is found in annual grain crops [17–19,26], while in perennial forage crops, a high biomass production and forage quality are desirable [27–30]. The large changes in phenotypic traits in seed crops are a result of strong selection during many breeding cycles, while most of the forage crops have gone through relatively few cycles of selection. The human selection may also have been counteracted by cross-pollination in self-incompatible grasses such as timothy, increasing the diversity within the species.

In timothy, the undomesticated wild accessions showed a vigorous growth and high FW and DW, which are desirable traits for a forage crop. Our results suggest that the timothy cultivars have rather been selected and improved for other traits. We found a significant difference in plant development between the wild accessions and the cultivars of timothy both in the field and in the greenhouse. The group of timothy cultivars reached stem elongation, booting and heading earlier than the wild accessions. This shows that the breeding has favored a rapid plant development in spring growth, and thereby made repeated harvests of timothy possible during the same season. Also, in *P. nodosum*, the cultivars showed earlier stem elongation, booting and heading than the wild accessions in the field. The difference between the wild accessions and the cultivars in plant development was more pronounced in *P. nodosum* than in timothy, and in the field the *P. nodosum* cultivars reached heading much earlier than the timothy cultivars. The contrary was, however, found in the greenhouse, where the *P. nodosum* cultivars showed booting and heading later than the wild accessions, and reached heading at about the same time as the timothy cultivars. Differences in performance in the two growing environments were also found in timothy and *P. alpinum* accessions. Many domesticated and undomesticated accessions of timothy and a majority of the wild *P. alpinum* accessions did not flower in the greenhouse. The difference in the ability to flower in *P. alpinum* and timothy, and the opposite performance of the *P. nodosum* cultivars and wild accessions in the two growing environments, may be influenced by the difference in photoperiod. Also, the difference in length of vernalization between the six-week cold treatment in the greenhouse and the longer cold period in the field may have affected the development and ability to flower. Plants differ in their need for vernalization to flower, and winter-type plants of annual cereals and some perennial grasses such as ryegrass and tall fescue require vernalization [5,31,32].

The transition to the reproductive stage may also be affected by the photoperiod. In timothy, an increasing temperature and a longer photoperiod stimulate stem elongation during spring growth [5,8–10]. The need for vernalization for the transition to reproductive tillers differs between timothy accessions from different geographical origin [6–9]. While accessions in northern Scandinavia require vernalization to flower, more southern-growing accessions are not affected by the vernalization. This adaptation to the climate (e.g., photoperiod and temperature) at the cultivation and growing sites may explain the variation in heading time that we have found among accessions, and the different performance of some accessions in the field and the greenhouse. A weak correlation was found between the latitude of the geographical origin of the wild accessions of timothy and the heading time (Figure S1). A correlation between heading time and geographical origin in timothy has also been shown in other studies [7,10].

The large diversity that we have found in this study is of value for further pre-breeding studies. It also shows the potential to identify genetic resources for developing new improved timothy cultivars. The variation in days to stem elongation and flowering indicates the difference in response to photoperiod, where some accessions required a shorter photoperiod to reach flowering compared to other accessions. These accessions may therefore serve as important germplasm for development of improved cultivars for cultivation at lower latitudes. Accessions that respond to longer days for flowering could be used as germplasm for the development of new cultivars for northern latitudes. However, early cultivars having a vigorous growth and a high biomass production may have a lower nutritional value and forage quality than cultivars flowering later due to their faster production of elongated tillers [3,4]. This negative relationship between high yield and high forage quality is challenging in the breeding of forage grasses. Deviant accessions with high yield and delayed and suppressed flowering would therefore be of great interest for breeding. Interestingly, no strong correlation was found between the growth and plant developmental traits in our study, which indicates that these traits can be selected independently of each other. Timothy accessions with favorable traits could either be crossed with existing cultivars to transfer these traits into the crop or domesticated to make them adapted to grow on agricultural land.

Moreover, the relatively high yield of *P. nodosum* and its adaptation to low altitudes make this species an interesting candidate for the development of new forage crops. Selected accessions could be domesticated as a forage crop or crossed with already domesticated *P. nodosum* cultivars. On the other hand, *P. alpinum* may also be a potential source of genes for adaptation to colder and more stressful growing conditions. Genes from the wild relatives may also be transferred into the timothy forage crop through wide hybridization since Nordenskiöld [33] found that crosses between *P. nodosum* and *P. pratense*, and between *P. pratense* and *P. alpinum*, could give fertile hybrids.

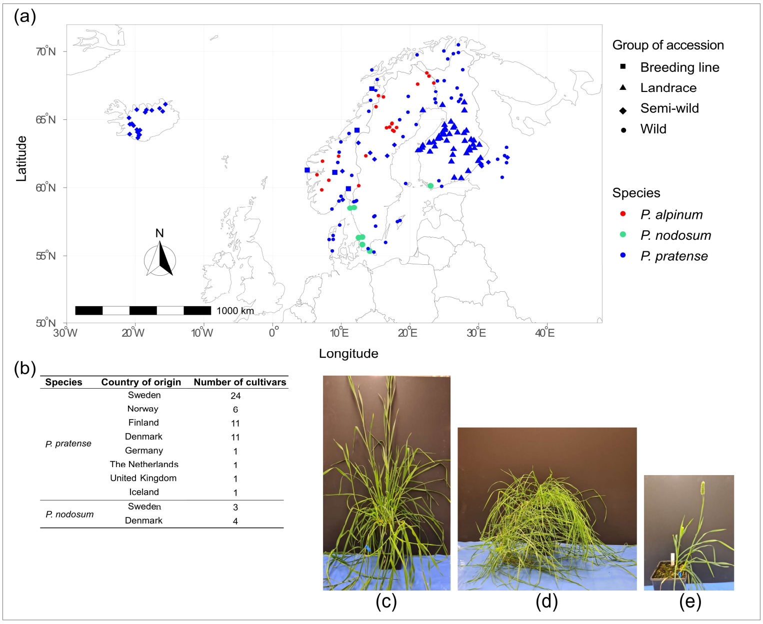
4. Materials and Methods

4.1. Plant Material

Clonal plants from 212 accessions of timothy (*P. pratense*), 14 accessions of *P. nodosum* and 18 accessions of *P. alpinum* originating from the Nordic countries, UK, Germany, the Netherlands and Russia were planted in the field (Table S7 and Figure 8). The same clonal plants, except four accessions (two timothy, one *P. nodosum* and one *P. alpinum*), were also studied in a greenhouse at the Plant Cultivation Facility, Uppsala BioCenter, Swedish University of Agricultural Sciences, Uppsala, Sweden. The seeds were provided by the genebank NordGen, Alnarp, Sweden. In the Nordic Baltic Genebanks Information System, *Phleum* accessions are described as wild, semi-wild (from populations nearby cultivated fields with potential gene flow between populations), landrace (from a cultivated variety adapted to a certain ecogeographical area), breeding line (group of related genotypes under evaluation in a breeding program) or cultivar (Table S7).

4.2. Pre-Cultivation and Cloning

Eight seeds of each accession were sown in low nutrient commercial potting soil (S-jord, Hasselfors Garden, Örebro, Sweden, Figure S2) in a climate chamber at the Plant Cultivation Facility, BioCenter, Swedish University of Agricultural Sciences, Uppsala, Sweden, with 16/8 h day/night photoperiod (350 µmol m^{-2} s^{-1} light), 22/17 °C day/night temperature and 65 percent humidity. After two months of growth, four randomly selected plants (genotypes) of each accession were cloned. Since the *Phleum* species are outcrossing, we considered each of the four plants within an accession genetically different. Vegetative tillers were gently separated from each other, and six of the tillers from each plant were individually transplanted into pots (15 cm × 15 cm × 20 cm) with commercial potting soil (P-jord, Hasselfors Garden, Örebro, Sweden). The cloned plants (totally six plants per genotype) were then cultivated in the greenhouse with a 16/8 h day/night photoperiod.



Species	Country of origin	Number of cultivars
P. pratense	Sweden	24
	Norway	6
	Finland	11
	Denmark	11
	Germany	1
	The Netherlands	1
	United Kingdom	1
	Iceland	1
P. nodosum	Sweden	3
	Denmark	4

Figure 8. (**a**) Collection sites of wild and semi-wild accessions, landraces and breeding lines of *P. pratense*, *P. nodosum* and *P. alpinum*, and (**b**) country of origin of developed cultivars based on passport data from the genebank NordGen, Alnarp, Sweden. Two months old plants of (**c**) *P. pratense*, (**d**) *P. nodosum* and (**e**) *P. alpinum* grown in a greenhouse.

4.3. Field Trial

In July 2020, four plants of each genotype were transplanted in clay soil in a farmer's field north of Uppsala, Central Sweden (60°00′ N, 17°42′ E). The field was located at an organic farm, and a low level of animal manure fertilizer was added. Manual weeding was carried out, and no herbicides were applied. In total, 244 *Phleum* accessions with 4 genotypes of each accession and 4 plants of each genotype were planted in a randomized complete block design with one plant of each genotype in each of the 4 blocks. The distance between the plants was 1 m within and between rows. The development and growth were studied during the season 2021, one year after planting, until the harvest in June the same year.

4.4. Greenhouse Trial

The other two plants of each genotype were grown in the greenhouse for two weeks and then given a vernalization treatment for six weeks at 4 °C under an 8 h photoperiod at 100 µmol m^{-2} s^{-1}, to stimulate flowering. After the vernalization, the plants were transferred back to the greenhouse (16/8 h; 22/17 °C) and placed in two blocks with one replicate of each genotype randomized within each block. In total, 240 *Phleum* accessions with 4 genotypes of each accession and 2 plants of each genotype were evaluated from September 2020 until the last plant was harvested in March 2021. During the experiment, the plants were given nutrient solution (N: 102 mg/L (NH_4: 40 mg/L, NO_3: 62 mg/L), P: 20 mg/L, K: 86 mg/L, S: 8 mg/L, Ca: 6 mg/L, Mg: 8 mg/L, Fe: 0.34 mg/L, Mn: 0.4 mg/L, B: 0.2 mg/L, Zn: 0.06 mg/L, Cu: 0.03 mg/L, Mo: 0.08 mg/L; Wallco Miljöcenter AB, Arlöv, Sweden) twice a month.

4.5. Evaluated Traits

The clonal plants grown in the greenhouse and in the field were individually studied for the same growth traits and developmental stages (Table 5). Development was evaluated by recording the number of days to stem elongation (DTS), days to booting (DTB) and days to heading (DTH). We considered a plant to have reached stem elongation when the first tiller internode started to elongate, and the inflorescence was palpable at least 1 cm above the stem base [34] in about one-fourth of the total number of tillers (vegetative and elongated tillers). A plant was considered to have reached booting when the tip of the inflorescence was palpable in the flag leaf sheath and heading when the tip of the head was visible above the flag leaf in about one-fourth of the total number of tillers (vegetative, elongated and heading tillers). In the greenhouse, DTS, DTB and DTH were measured by recording the number of days after emergence of the coleoptile, including the time of vernalization.

Table 5. Studied traits in individual plants of *P. pratense*, *P. nodosum* and *P. alpinum* in the field and in the greenhouse.

Trait	Abbreviation	Description
Fresh weight	FW	Fresh weight (g) of tillers cut at 3 cm above the soil surface
Dry weight	DW	Dry weight (g) of tillers cut at 3 cm above the soil surface
Plant height	PH	The average length of 5 tillers (cm) in the field and the average length of 9 tillers (cm) in the greenhouse
Days to stem elongation	DTS	Number of days from emergence of coleoptile until the first tiller internode started to elongate, and the inflorescence was palpable at least 1 cm above the stem base in about one-fourth of the total number of tillers
Days to booting	DTB	Number of days from emergence of coleoptile until the tip of the inflorescence was palpable in the flag leaf sheath below the flag leaf base in about one fourth of the total number of tillers
Days to heading	DTH	Number of days from emergence of coleoptile until the head was visible above the flag leaf base in about one-fourth of the total number of tillers

In the field, the number of days to reach a specific developmental stage was recorded according to the day-of-year calendar. Based on that, DTS, DTB and DTH were calculated as accumulated growing degree days (*GDD*)

$$GDD = \frac{T_{max} + T_{min}}{2} - T_{base} \quad (1)$$

where T_{max} is the daily maximum temperature, T_{min} is the daily minimum temperature and T_{base} (the base temperature) is the minimum temperature at which growth can occur for a plant species. The base temperature of 5 °C is used for timothy. Daily temperatures were recorded from January 2021 to December 2021 at the Swedish Metrological and Hydrological Institute's weather station 458, Uppsala, 59°90′ N, 17°59′ E. The accumulated growing degree days was calculated from the start of the meteorological growth that is defined as the first five consecutive days with a daily mean temperature above 5 °C in spring 2021 (Figure S3).

The plants in the greenhouse were harvested when the peduncle turned yellow below at least one head of the plant. However, 14 accessions of timothy and 12 accessions of *P. alpinum* remained in the vegetative stage in the greenhouse and did not form elongated or heading tillers. In addition, 36 accessions of timothy reached only the stem elongation stage. The 26 accessions with only vegetative tillers and the 36 accessions with elongated tillers were harvested at the end of the experiment, about 6 months after the vernalization treatment. All tillers were cut 3 cm above the soil surface. The length of 3 randomly selected vegetative tillers, 3 elongated tillers and 3 tillers with heads were then evaluated and the plant height (PH) was estimated as the average of the 9 tillers. For the plants that did not form elongated and heading tillers, the length of 9 vegetative tillers were measured. In addition, the fresh weight (FW) of all tillers was weighed. The plant material was then dried at 60 °C for 48 h and the dry weight (DW) was measured.

In the field, the plants were harvested when 30–50 percent of the tillers on a plant had reached the booting to heading stage. At harvest, the height of five elongated tillers (2 tall, 1 medium height and 2 short tillers) of each plant was measured from soil surface and PH was estimated as the average of these tillers. The tillers were cut 3 cm above the soil surface, and the FW and DW of the plants were recorded. Plants within the same block were harvested within 3 days and the whole experiment was harvested within 12 days.

4.6. Data Analysis

Datasets were analyzed using the software RStudio [35] and JMP ver. 15 SAS Institute Inc., Cary, NC, USA. All traits showed normally distributed residuals and were therefore analyzed with parametric Analysis of Variance (ANOVA) tests. Since the number of accessions differed among the three *Phleum* species, and the number of accessions differed within each group of timothy accessions (wild, semi-wild, landraces, breeding lines and cultivars), an unbalanced nested design was used in the ANOVA to compare species initially. In this model, genotype was nested within accession, and accession was nested within species. Genotypes within accession and species were considered as random effect in the nested model. A normal ANOVA was then used for comparisons of different types of accessions within each species. In addition, the LSMeans Differences Tukey HSD test was performed to study the relationship among groups of accessions within species. Student's *t*-test was used for pairwise comparisons. Phenotypic diversity within each group of accessions was evaluated by calculating the coefficient of variation (CV)

$$CV = \frac{\delta}{\mu} \times 100 \tag{2}$$

where δ is the standard deviation and μ is the mean of the trait within the group.

To analyze and display relations among accessions based on all studied growth and developmental traits, we used the multivariate analyses principal component analysis (PCA) and cluster analysis. Moreover, Pearson correlation was used to evaluate and estimate the pattern and level of association between traits. The broad-sense heritability (H^2) was estimated for each trait using the R packages "inti" and "variability" in RStudio

$$H^2 = \frac{\delta_g^2}{\delta_p^2} \tag{3}$$

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.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants12193494/s1. Figure S1: Correlation between days to heading (DTH) in the field and latitude coordinate of the collection site of wild *P. pratense* accessions. Figure S2: Cloning of *Phleum* genotypes planted in the field and in the greenhouse. Figure S3: Accumulated growing degree days (GDD) calculated from meteorological growth start (defined as the first of five consecutive days with daily mean temperature above 5 °C) for 2021 in Uppsala, Sweden based on the climate data from the Swedish Meteorological and Hydrological Institute, Norrköping, Sweden. Table S1: Unbalanced-nested analysis of variance (ANOVA) for the components block, species, accession and genotype for the studied traits days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the field. DF: degrees of freedom, ns: non-significant, *: $p < 0.05$, **: $p < 0.01$. Table S2: Unbalanced-nested analysis of variance (ANOVA) for the components block, species, accession and genotype for the studied traits days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the greenhouse. DF: degrees of freedom, ns: non-significant, *: $p < 0.05$, **: $p < 0.01$. Table S3: Mean and SD of days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in *P. pratense*, *P. nodosum* and *P. alpinum* in the field. GDD: Growing degree days. Mean values that do not share the same letter are significantly different among species according to Tukey HSD, $p < 0.05$. Table S4: Mean and SD of days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in *P. pratense*, *P. nodosum* and *P. alpinum* in the greenhouse. DAE: days after coleoptile emergence. Mean values that do not share the same letter are significantly different among species accessions according to Tukey HSD, $p < 0.05$. Table S5: Number of accessions of each group in each cluster in *P. pratense*, *P. nodosum* and *P. alpinum* in the field. Table S6: Number of accessions of each group in each cluster in *P. pratense*, *P. nodosum* and *P. alpinum* in the greenhouse. Table S7: Information of the studied accessions of *P. pratense*, *P. nodosum* and *P. alpinum* from the genebank NordGen, Alnarp, Sweden.

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Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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Supplementary materials to:

Phenotypic Diversity in Domesticated and Wild Timothy Grass, and Closely Related Species for Forage Breeding

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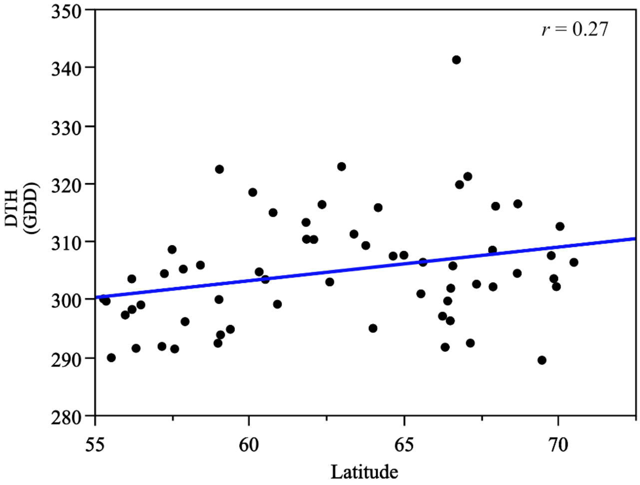

Figure S1. Correlation between days to heading (DTH) in the field and latitude coordinate of the collection site of wild *P. pratense* accessions.

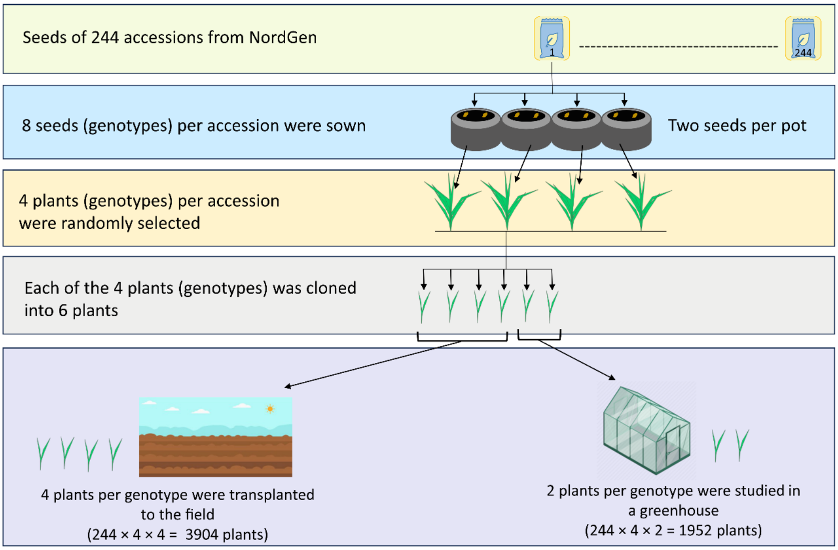

Figure S2. Cloning of *Phleum* genotypes planted in the field and in the greenhouse.

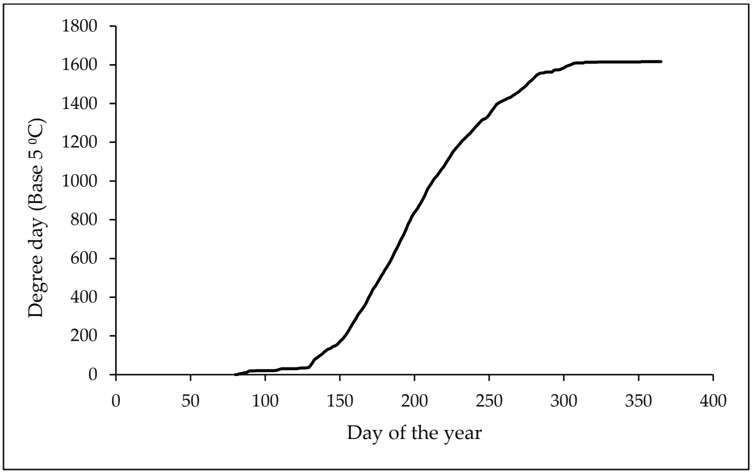

Figure S3. Accumulated growing degree days (GDD) calculated from meteorological growth start (defined as the first of five consecutive days with daily mean temperature above 5 °C) for 2021 in Uppsala, Sweden based on the climate data from the Swedish Meteorological and Hydrological Institute, Norrköping, Sweden.

Table S1. Unbalanced-nested analysis of variance (ANOVA) for the components block, species, accession and genotype for the studied traits days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the field. DF: degrees of freedom, ns: non-significant, *: $p < 0.05$, **: $p < 0.01$.

Source of Variation	**DF**	**Mean Square**					
		DTS	**DTB**	**DTH**	**FW**	**DW**	**PH**
Block	3	9566.1**	54169.1**	82409.2**	1033059.0**	399403.0**	25279.9**
Species	2	2429.6*	16319.5**	16604.1**	1764388.0**	277685.0**	44013.9**
Accession (Species)	236	1698.5**	2200.5**	1701.2**	54460.4**	8847.4**	830.7**
Genotype (Accession(Species))&Random	671	930.2	1240.5	957.3	38146.5	5405.4	359.6
RSquare Adj	-	0.4	0.5	0.5	0.3	0.4	0.7

Table S2. Unbalanced-nested analysis of variance (ANOVA) for the components block, species, accession and genotype for the studied traits days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the greenhouse. DF: degrees of freedom, ns: non-significant, *: $p < 0.05$, **: $p < 0.01$.

Source of Variation	**DF**	**Mean Square**					
		DTS	**DTB**	**DTH**	**FW**	**DM**	**PH**
Block	1	19.2 ^{ns}	17.7 ^{ns}	23.3^{ns}	420.4^{ns}	44.6 ^{ns}	206.9 ^{ns}
Species	2	2297.8**	872.8**	1531.2**	82984.3**	13030.8**	53878.2**
Accession (Species)	210	445.1**	673.0**	1085.0**	7157.8**	1248.3**	2513.2**
Genotype (Accession(Species))&Random	359	153.0	104.8	144.6	4164.1	782.6	1438.4
RSquare Adj	-	0.8	0.9	0.9	0.5	0.5	0.6

Table S3. Mean and SD of days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in *P. pratense*, *P. nodosum* and *P. alpinum* in the field. GDD: Growing degree days. Mean values that do not share the same letter are significantly different among species according to Tukey HSD, $p < 0.05$.

Species	**DTS (GDD)**	**DTB (GDD)**	**DTH (GDD)**	**FW (g per plant)**	**DW (g per plant)**	**PH (cm)**
P. pratense	185.1±0.5^{ab}	254.4±0.6^{a}	304.4±0.5^{a}	400.2±3.4^{a}	158.9±1.3^{a}	82.4±0.3^{a}
P. nodosum	178.9±2.2^{b}	240.9±2.5^{b}	290.6±2.1^{b}	254.5±14.5^{b}	100.6±5.5^{b}	60.4±1.3^{b}
P. alpinum	187.2±6.5^{a}	237.1±7.1^{b}	291.2±6.4^{b}	186.7±43.6^{b}	72.2±16.4^{b}	44.1±3.9^{c}

Table S4. Mean and SD of days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in *P. pratense*, *P. nodosum* and *P. alpinum* in the greenhouse. DAE: days after coleoptile emergence. Mean values that do not share the same letter are significantly different among species accessions according to Tukey HSD, $p < 0.05$.

Species	**DTS (DAE)**	**DTB (DAE)**	**DTH (DAE)**	**FW (g per plant)**	**DW (g per plant)**	**PH (cm)**
P. pratense	165.9±0.5^{a}	178.0±0.6^{a}	192.7±0.7^{a}	97.2±2.0^{a}	36.1±0.9^{b}	96.1±1.6^{a}
P. nodosum	158.2±1.4^{b}	170.4±1.6^{b}	182.4±1.9^{b}	103.4±6.8^{a}	43.8±2.9^{a}	105.1±4.0^{a}
P. alpinum	162.9 ±4.8^{ab}	178.0±4.0^{a}	191.0±4.7^{a}	18.8±11.6^{b}	7.5±5.0^{c}	36.2±6.8^{b}

Table S5. Number of accessions of each group in each cluster in *P. pratense, P. nodosum* and *P. alpinum* in the field.

Species	**Cluster**	**Total number of accessions**	**Number of accessions in each group**				
			Cultivar	**Breeding line**	**Landrace**	**Semi-wild**	**Wild**
P. pratense	I	19	9	-	3	1	6
	II	46	9	2	15	4	16
	III	28	7	1	9	4	7
	IV	43	17	4	6	7	9
	V	43	7	-	11	11	14
	VI	33	7	3	11	3	9
P. nodosum	I	5	3	-	-	-	2
	II	8	4	-	-	-	4
	III	1	-	-	-	-	1
P. alpinum	I	5	-	-	-	-	5
	II	3	-	-	-	-	3
	III	4	-	-	-	-	4

Table S6. Number of accessions of each group in each cluster in *P. pratense*, *P. nodosum* and *P. alpinum* in the greenhouse.

Species	Cluster	Total number of accessions	Number of accessions in each group				
			Cultivar	Breeding line	Landrace	Semi-wild	Wild
P. pratense	I	55	18	-	18	3	16
	II	37	8	4	9	8	8
	III	12	2	4	-	1	5
	IV	17	1	1	5	2	8
	V	38	20	2	4	2	10
P. nodosum	I	3	3	-	-	-	-
	II	4	1	-	-	-	3
	III	1	1	-	-	-	-
	IV	5	1	-	-	-	4
P. alpinum	I	3	-	-	-	-	3
	II	1	-	-	-	-	1
	III	1	-	-	-	-	1

Table S7. Information of the studied accessions of *P. pratense*, *P. nodosum* and *P. alpinum* from the genebank NordGen, Alnarp, Sweden.

Row	**Species**	**Accession number**	**Country of origin**	**Type**	**Latitude**	**Longitude**
1	*Phleum pratense*	NGB10779	Norway	Wild	59.03	9.66
2	*Phleum pratense*	NGB10784	Norway	Wild	60.31	10.61
3	*Phleum pratense*	NGB1096	Finland	Landrace	64.61	25.04
4	*Phleum pratense*	NGB1097	Finland	Landrace	63.94	25.76
5	*Phleum pratense*	NGB1098	Finland	Landrace	63.11	21.88
6	*Phleum pratense*	NGB1102	Finland	Landrace	64.51	26.46
7	*Phleum pratense*	NGB1104	Finland	Landrace	63.96	24.79
8	*Phleum pratense*	NGB1111	Finland	Landrace	64.41	26.33
9	*Phleum pratense*	NGB1114	Finland	Landrace	64.24	25.06
10	*Phleum pratense*	NGB1115	Finland	Landrace	64.34	25.13
11	*Phleum pratense*	NGB11156	Norway	Cultivar		
12	*Phleum pratense*	NGB1117	Finland	Landrace	66.06	24.74
13	*Phleum pratense*	NGB1120	Finland	Landrace	63.44	27.99
14	*Phleum pratense*	NGB1121	Finland	Landrace	62.84	28.63
15	*Phleum pratense*	NGB1122	Finland	Landrace	62.51	28.24
16	*Phleum pratense*	NGB1127	Finland	Landrace	62.83	26.36
17	*Phleum pratense*	NGB11427	Sweden	Cultivar		
18	*Phleum pratense*	NGB11428	Sweden	Cultivar		
19	*Phleum pratense*	NGB11429	Sweden	Cultivar		
20	*Phleum pratense*	NGB11430	Sweden	Cultivar		
21	*Phleum pratense*	NGB11431	Sweden	Cultivar		
22	*Phleum pratense*	NGB11677	Denmark	Cultivar		
23	*Phleum pratense*	NGB132	Finland	Landrace	63.48	29.31
24	*Phleum pratense*	NGB13218	Sweden	Cultivar		
25	*Phleum pratense*	NGB13219	Sweden	Cultivar		
26	*Phleum pratense*	NGB13220	Sweden	Cultivar		
27	*Phleum pratense*	NGB13221	Sweden	Cultivar		
28	*Phleum pratense*	NGB13222	Sweden	Cultivar		
29	*Phleum pratense*	NGB13223	Sweden	Cultivar		
30	*Phleum pratense*	NGB13224	Sweden	Cultivar		
31	*Phleum pratense*	NGB13226	Sweden	Cultivar		
32	*Phleum pratense*	NGB13227	Finland	Cultivar		
33	*Phleum pratense*	NGB13228	Sweden	Cultivar		
34	*Phleum pratense*	NGB1324	Sweden	Wild	64.16	17.68
35	*Phleum pratense*	NGB1331	Sweden	Wild	63.76	18.99
36	*Phleum pratense*	NGB135	Finland	Landrace	61.21	28.49
37	*Phleum pratense*	NGB13524	Sweden	Cultivar		
38	*Phleum pratense*	NGB138	Finland	Landrace		
39	*Phleum pratense*	NGB13884	Sweden	Breeding line		
40	*Phleum pratense*	NGB13894	Finland	Cultivar		
41	*Phleum pratense*	NGB13976	Norway	Landrace		
42	*Phleum pratense*	NGB14008	Norway	Landrace		
43	*Phleum pratense*	NGB14009	Norway	Landrace		

Table S7. Continued.

Row	**Species**	**Accession number**	**Country of origin**	**Type**	**Latitude**	**Longitude**
44	*Phleum pratense*	NGB14174	Sweden	Cultivar		
45	*Phleum pratense*	NGB14175	Sweden	Cultivar		
46	*Phleum pratense*	NGB143	Finland	Landrace	63.54	28.91
47	*Phleum pratense*	NGB14328	Finland	Landrace	63.77	24.23
48	*Phleum pratense*	NGB14336	Finland	Landrace	63.9	25,00
49	*Phleum pratense*	NGB14375	Finland	Cultivar	61.23	26.18
50	*Phleum pratense*	NGB14378	Finland	Cultivar	63.81	26.42
51	*Phleum pratense*	NGB14416	Finland	Wild	60.51	24.22
52	*Phleum pratense*	NGB14417	Finland	Wild	60.1	24.63
53	*Phleum pratense*	NGB14467	Finland	Landrace	63.65	24.43
54	*Phleum pratense*	NGB14469	Finland	Landrace	61.9	26.04
55	*Phleum pratense*	NGB14470	Finland	Landrace	64.1	24.78
56	*Phleum pratense*	NGB145	Finland	Landrace	62.89	29.39
57	*Phleum pratense*	NGB14618	Finland	Landrace	63.59	24.06
58	*Phleum pratense*	NGB147	Finland	Landrace	63.18	29.21
59	*Phleum pratense*	NGB15094	Denmark	Cultivar		
60	*Phleum pratense*	NGB151	Finland	Landrace	62.71	28.71
61	*Phleum pratense*	NGB1526	Sweden	Wild	57.58	18.56
62	*Phleum pratense*	NGB153	Finland	Landrace		
63	*Phleum pratense*	NGB1537	Sweden	Wild	57.49	18.18
64	*Phleum pratense*	NGB15461	Denmark	Wild	56.2	8.83
65	*Phleum pratense*	NGB15477	Denmark	Wild	56.48	8.82
66	*Phleum pratense*	NGB15487	Denmark	Wild	56.19	8.23
67	*Phleum pratense*	NGB15502	Norway	Cultivar	60.29	12.08
68	*Phleum pratense*	NGB15556	Norway	Semi-wild	61.21	10.16
69	*Phleum pratense*	NGB156	Finland	Landrace	60.69	27.48
70	*Phleum pratense*	NGB157	Finland	Landrace	63.16	30.11
71	*Phleum pratense*	NGB158	Finland	Landrace	62.16	30.29
72	*Phleum pratense*	NGB159	Finland	Landrace	61.94	29.96
73	*Phleum pratense*	NGB16264	Sweden	Wild	57.91	14.89
74	*Phleum pratense*	NGB16268	Sweden	Wild	57.85	14.75
75	*Phleum pratense*	NGB16271	Sweden	Wild	57.16	15,00
76	*Phleum pratense*	NGB1671	Denmark	Cultivar		
77	*Phleum pratense*	NGB1672	Denmark	Cultivar		
78	*Phleum pratense*	NGB1673	Denmark	Cultivar		
79	*Phleum pratense*	NGB1674	Denmark	Cultivar		
80	*Phleum pratense*	NGB1675	Denmark	Cultivar		
81	*Phleum pratense*	NGB1676	Denmark	Cultivar		
82	*Phleum pratense*	NGB1677	Denmark	Cultivar		
83	*Phleum pratense*	NGB16790	Finland	Landrace		
84	*Phleum pratense*	NGB17194	Norway	Wild	70.49	27.06
85	*Phleum pratense*	NGB17200	Norway	Wild	69.46	25.51
86	*Phleum pratense*	NGB17202	Norway	Wild	70.04	24.94
87	*Phleum pratense*	NGB17203	Norway	Wild	69.76	21.04

Table S7. Continued.

Row	Species	Accession number	Country of origin	Type	Latitude	Longitude
88	*Phleum pratense*	NGB18354	Finland	Landrace	65.66	27.94
89	*Phleum pratense*	NGB18357	Finland	Landrace	66.25	27.92
90	*Phleum pratense*	NGB18367	Finland	Landrace	64.19	28.23
91	*Phleum pratense*	NGB18462	Sweden	Semi-wild	62.33	16.69
92	*Phleum pratense*	NGB18489	Sweden	Semi-wild	62.08	14.84
93	*Phleum pratense*	NGB18498	Sweden	Semi-wild	62.54	14.21
94	*Phleum pratense*	NGB18511	Sweden	Semi-wild	63.31	12.49
95	*Phleum pratense*	NGB18566	Sweden	Semi-wild	63.16	17.74
96	*Phleum pratense*	NGB20365	Finland	Landrace	64.06	26.92
97	*Phleum pratense*	NGB20369	Finland	Landrace	64.02	27.89
98	*Phleum pratense*	NGB20791	Iceland	Semi-wild	64.24	-20.68
99	*Phleum pratense*	NGB20792	Iceland	Semi-wild	64.27	-20.23
100	*Phleum pratense*	NGB20793	Iceland	Semi-wild	64.09	-20.31
101	*Phleum pratense*	NGB20794	Iceland	Semi-wild	63.9	-20.62
102	*Phleum pratense*	NGB20795	Iceland	Semi-wild	63.86	-20.53
103	*Phleum pratense*	NGB20796	Iceland	Semi-wild	63.67	-20.37
104	*Phleum pratense*	NGB20798	Iceland	Semi-wild	63.95	-21.18
105	*Phleum pratense*	NGB20799	Iceland	Semi-wild	64.57	-21.74
106	*Phleum pratense*	NGB20801	Iceland	Semi-wild	64.66	-21.21
107	*Phleum pratense*	NGB20802	Iceland	Semi-wild	64.7	-21.51
108	*Phleum pratense*	NGB20803	Iceland	Semi-wild	65.13	-21.08
109	*Phleum pratense*	NGB20804	Iceland	Semi-wild	65.73	-20.21
110	*Phleum pratense*	NGB20805	Iceland	Semi-wild	65.71	-19.62
111	*Phleum pratense*	NGB20806	Iceland	Semi-wild	65.54	-19.44
112	*Phleum pratense*	NGB20807	Iceland	Semi-wild	66.12	-16.39
113	*Phleum pratense*	NGB20808	Iceland	Semi-wild	65.82	-17.54
114	*Phleum pratense*	NGB20809	Iceland	Semi-wild	65.6	-17.99
115	*Phleum pratense*	NGB20810	Iceland	Semi-wild	65.68	-18.4
116	*Phleum pratense*	NGB21082	Norway	Semi-wild	59.13	10.21
117	*Phleum pratense*	NGB2166	Norway	Landrace		
118	*Phleum pratense*	NGB2167	Norway	Breeding line		
119	*Phleum pratense*	NGB2169	Norway	Cultivar		
120	*Phleum pratense*	NGB2180	Norway	Cultivar		
121	*Phleum pratense*	NGB2181	Norway	Cultivar		
122	*Phleum pratense*	NGB22615	Denmark	Wild	55.27	14.76
123	*Phleum pratense*	NGB2355	Sweden	Cultivar		
124	*Phleum pratense*	NGB2356	Sweden	Cultivar		
125	*Phleum pratense*	NGB24047	Norway	Breeding line	61.12	9.06
126	*Phleum pratense*	NGB24050	Norway	Breeding line	61.12	9.06
127	*Phleum pratense*	NGB24053	Norway	Breeding line	59.93	11.04
128	*Phleum pratense*	NGB24056	Norway	Breeding line	67.28	14.45

Table S7. Continued.

Row	**Species**	**Accession number**	**Country of origin**	**Type**	**Latitude**	**Longitude**
129	*Phleum pratense*	NGB24061	Norway	Breeding line	61.29	5.04
130	*Phleum pratense*	NGB24064	Norway	Breeding line	64.23	12.29
131	*Phleum pratense*	NGB24163	Finland	Landrace	64.84	25.98
132	*Phleum pratense*	NGB24165	Finland	Landrace	62.94	28.69
133	*Phleum pratense*	NGB24169	Russian Federation	Wild	60.76	33.44
134	*Phleum pratense*	NGB24184	Russian Federation	Semi-wild	61.58	30.46
135	*Phleum pratense*	NGB24185	Russian Federation	Semi-wild	61.69	30.28
136	*Phleum pratense*	NGB24192	Russian Federation	Semi-wild	61.73	30.39
137	*Phleum pratense*	NGB24205	Russian Federation	Semi-wild	61.86	31.43
138	*Phleum pratense*	NGB24214	Russian Federation	Wild	62.08	32.78
139	*Phleum pratense*	NGB24215	Russian Federation	Wild	61.83	34.08
140	*Phleum pratense*	NGB24221	Russian Federation	Semi-wild	62.21	34.21
141	*Phleum pratense*	NGB24235	Russian Federation	Wild	62.34	33.79
142	*Phleum pratense*	NGB24247	Russian Federation	Wild	62.98	34.08
143	*Phleum pratense*	NGB2528	Sweden	Wild	58.98	11.83
144	*Phleum pratense*	NGB2530	Sweden	Wild	59.01	12.08
145	*Phleum pratense*	NGB2532	Sweden	Wild	59.06	12.23
146	*Phleum pratense*	NGB2597	Sweden	Breeding line		
147	*Phleum pratense*	NGB2755	Sweden	Cultivar		
148	*Phleum pratense*	NGB2756	Sweden	Cultivar		
149	*Phleum pratense*	NGB2757	Sweden	Cultivar		
150	*Phleum pratense*	NGB2776	Finland	Landrace	60.73	26.46
151	*Phleum pratense*	NGB2777	Finland	Landrace	63.04	23.63
152	*Phleum pratense*	NGB2795	Finland	Landrace	62.96	21.78
153	*Phleum pratense*	NGB2796	Finland	Landrace	62.76	21.48
154	*Phleum pratense*	NGB2798	Finland	Landrace	62.91	21.73
155	*Phleum pratense*	NGB2799	Finland	Landrace	62.73	21.19
156	*Phleum pratense*	NGB2816	Finland	Landrace	63.31	22.83
157	*Phleum pratense*	NGB2817	Finland	Landrace	63.36	23.46
158	*Phleum pratense*	NGB2823	Finland	Landrace	62.63	22.93
159	*Phleum pratense*	NGB2836	Finland	Landrace	62.39	26.24
160	*Phleum pratense*	NGB2850	Finland	Landrace	63.46	25.36
161	*Phleum pratense*	NGB2933	Norway	Wild	59.38	9.97
162	*Phleum pratense*	NGB4053	Denmark	Wild	57.24	9.78
163	*Phleum pratense*	NGB4066	Finland	Cultivar		
164	*Phleum pratense*	NGB4067	Finland	Cultivar		

Table S7. Continued.

Row	Species	Accession number	Country of origin	Type	Latitude	Longitude
165	*Phleum pratense*	NGB4070	Finland	Cultivar		
166	*Phleum pratense*	NGB4122	Denmark	Cultivar		
167	*Phleum pratense*	NGB4141	Iceland	Cultivar		
168	*Phleum pratense*	NGB4349	Sweden	Wild	55.53	13.91
169	*Phleum pratense*	NGB4351	Sweden	Wild	56.33	12.53
170	*Phleum pratense*	NGB4509	Norway	Wild	60.9	11.53
171	*Phleum pratense*	NGB4511	Norway	Wild	61.84	9.49
172	*Phleum pratense*	NGB4520	Norway	Wild	58.41	8.64
173	*Phleum pratense*	NGB4534	Norway	Breeding line		
174	*Phleum pratense*	NGB4548	Denmark	Wild	55.36	8.69
175	*Phleum pratense*	NGB6779	Sweden	Wild	55.98	13.39
176	*Phleum pratense*	NGB7154	Norway	Wild	68.66	14.46
177	*Phleum pratense*	NGB7159	Norway	Wild	66.41	14.28
178	*Phleum pratense*	NGB717	Finland	Wild	66.24	23.76
179	*Phleum pratense*	NGB719	Sweden	Wild	66.51	23.64
180	*Phleum pratense*	NGB722	Sweden	Wild	66.69	19.76
181	*Phleum pratense*	NGB728	Sweden	Wild	65.54	19.71
182	*Phleum pratense*	NGB729	Sweden	Wild	64.99	18.51
183	*Phleum pratense*	NGB732	Sweden	Wild	64.64	20.91
184	*Phleum pratense*	NGB733	Sweden	Landrace	65.68	21.88
185	*Phleum pratense*	NGB739	Finland	Wild	67.06	23.76
186	*Phleum pratense*	NGB742	Finland	Wild	67.88	24.99
187	*Phleum pratense*	NGB743	Finland	Wild	67.86	24.01
188	*Phleum pratense*	NGB744	Finland	Wild	68.68	27.69
189	*Phleum pratense*	NGB747	Finland	Wild	69.84	26.33
190	*Phleum pratense*	NGB748	Finland	Wild	69.93	27.06
191	*Phleum pratense*	NGB752	Finland	Wild	67.34	26.88
192	*Phleum pratense*	NGB756	Finland	Wild	66.49	27.36
193	*Phleum pratense*	NGB7566	Norway	Wild	62.59	9.69
194	*Phleum pratense*	NGB7572	Norway	Wild	63.99	11.13
195	*Phleum pratense*	NGB7577	Norway	Wild	63.38	9.94
196	*Phleum pratense*	NGB758	Finland	Wild	66.79	27.48
197	*Phleum pratense*	NGB759	Finland	Wild	66.33	27.09
198	*Phleum pratense*	NGB763	Finland	Wild	66.58	26.04
199	*Phleum pratense*	NGB7707	Norway	Wild	67.96	15.23
200	*Phleum pratense*	NGB7710	Norway	Wild	67.14	15.03
201	*Phleum pratense*	NGB7717	Norway	Wild	65.61	14.01
202	*Phleum pratense*	NGB7785	Norway	Cultivar		
203	*Phleum pratense*	NGB8363	Finland	Cultivar		
204	*Phleum pratense*	NGB8381	Denmark	Cultivar		
205	*Phleum pratense*	NGB8418	Finland	Cultivar		
206	*Phleum pratense*	NGB9285	Finland	Cultivar		
207	*Phleum pratense*	NGB9290	Finland	Cultivar		
208	*Phleum pratense*	NGB9301	United Kingdom	Cultivar		

Table S7. Continued.

Row	Species	Accession number	Country of origin	Type	Latitude	Longitude
209	*Phleum pratense*	NGB9302	Germany	Cultivar		
210	*Phleum pratense*	NGB9924	Netherlands	Cultivar		
211	*Phleum pratense*	Switch	Sweden	Cultivar		
212	*Phleum pratense*	Tryggve	Sweden	Cultivar		
213	*Phleum nodosum*	NGB13229	Sweden	Cultivar		
214	*Phleum nodosum*	NGB13230	Denmark	Cultivar		
215	*Phleum nodosum*	NGB13231	Sweden	Cultivar		
216	*Phleum nodosum*	NGB14477	Finland	Wild	60.15	23.09
217	*Phleum nodosum*	NGB1678	Denmark	Cultivar		
218	*Phleum nodosum*	NGB16972	Sweden	Wild	55.81	13.04
219	*Phleum nodosum*	NGB17008	Sweden	Wild	58.54	11.28
220	*Phleum nodosum*	NGB17010	Sweden	Wild	58.5	11.3
221	*Phleum nodosum*	NGB17012	Sweden	Wild	56.37	12.82
222	*Phleum nodosum*	NGB1724	Denmark	Cultivar		
223	*Phleum nodosum*	NGB1725	Denmark	Cultivar		
224	*Phleum nodosum*	NGB2378	Sweden	Cultivar		
225	*Phleum nodosum*	NGB4352	Sweden	Wild	55.39	14.16
226	*Phleum nodosum*	NGB4364	Sweden	Wild	56.33	12.53
227	*Phleum alpinum*	NGB1335	Sweden	Wild	64.74	17.39
228	*Phleum alpinum*	NGB1336	Sweden	Wild	64.69	17.36
229	*Phleum alpinum*	NGB1337	Sweden	Wild	64.41	16.59
230	*Phleum alpinum*	NGB1338	Sweden	Wild	64.46	17.04
231	*Phleum alpinum*	NGB1340	Sweden	Wild	64.43	18.06
232	*Phleum alpinum*	NGB1341	Sweden	Wild	64.23	17.49
233	*Phleum alpinum*	NGB18474	Sweden	Wild	62.33	13.58
234	*Phleum alpinum*	NGB31885	Norway	Wild	60.55	8.18
235	*Phleum alpinum*	NGB31886	Norway	Wild	59.84	7.16
236	*Phleum alpinum*	NGB31887	Norway	Wild	62.3	9.62
237	*Phleum alpinum*	NGB770	Sweden	Wild	67.63	21.13
238	*Phleum alpinum*	NGB772	Sweden	Wild	68.21	22.78

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Climate-resilient cultivars of timothy with high forage yield and quality are needed to maintain and enhance livestock production in the Nordic countries. This thesis evaluated wild and domesticated accessions of timothy and two related species. New insights into the phenotypic and genetic diversity and signatures of selection on the genomes of these species are provided. These findings serve as a basis for future research and development of genomic-based breeding strategies.

Yousef Rahimi received his graduate education at the University of Tehran, Iran, and acquired a M.Sc. in Plant breeding, he received his undergraduate degree in Agronomy and plant breeding with a specialization in Plant breeding at Bu-Ali Sina University, Iran.

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