Multi-environment Screening of Timothy (*Phleum pratense)* Breeding Material for Better Forage Yield in Sweden.

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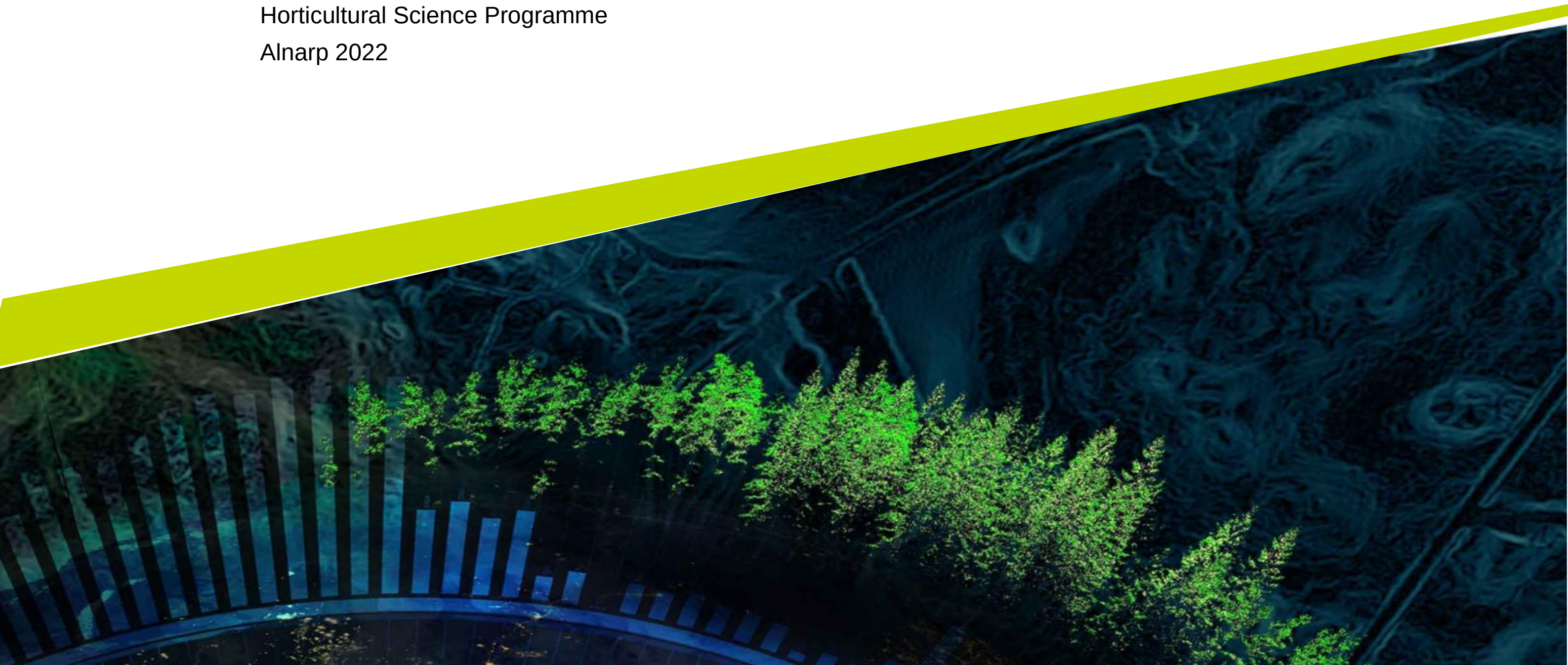

Multi-environment Screening of Timothy (*Phleum pratense*) Breeding Material for Better Forage Yield in Sweden.

Multi-miljöscreening av Timotej (Phleum pratense) Växtförädlingsmaterial för Bättre Foder Avkastning i Sverige.

Ibrahim Isam Abdi Ali Issa

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Abstract

Timothy (*Phleum pratense*) is one of the most widely used forage crops in hay and pasture grass mixtures in Nordic countries; it's also used in areas where it is one of the top forage crops harvested high yield, especially in boreal climates. There hasn't been any large-scale breeding of Timothy to develop varieties that are adapted for Swedish climate and standards, which is surprising given that Timothy is the main forage crop used in Sweden. This thesis is a part of a bigger project which aims for developing a "starter package" that offers a more efficient breeding method to commercial actors, which can be implemented together with traditional breeding for the development and improvement of new varieties of Timothy that enable sustainable farming.

This study is aimed to evaluate eco-agronomic traits such as fresh weight (cut), dry matter (DM), and botanical development stage (botdev) among advanced breeding material of timothy and estimate the impact of genotype-by-environment interactions (GEI) by testing the material at three geographic locations. A set of 264 modern accessions developed by Lantmännen were grown at three sites (Röbäcksdalen (RBD), Lövsta (LVT), and Svalöv) in Sweden together with two check varieties (Switch and Tryggve) that are well-known commercial varieties. The field trial was conducted according to a modified block design.

The results of an Anova indicated a significant difference in yield among the modern accessions. There was a significant difference between the length of growing degree days in RBD and LVT. The genetic variability analyses reveal the broad-sense heritability increase across the season from DM1 to DM3 and that the genetic gain was higher than environmental effects in LVT. However, the environmental effects were large for the traits DM1 and DM3 in RBD. The suggestion is that DM2 could be more efficiently bred among the modern accessions for the site RBD, while in LVT all three DM could be bred to increase the yield. A combination of PCA and GEI reveals a significant effect on accessions and the environment.

The conclusions are based on a combination of analyses such as PCA, GEI and genetic variability analyses and indicates suitable parental lines as we demonstrate here. Furthermore, these findings can speed up the breeding program and understanding of the GEI of Timothy in Sweden. There is an imperative need for a comprehensive understanding of the GEI of Timothy for today and future challenges and opportunities, to comply with climate changes. Additionally, more harvesting years and locations will provide a better understanding of the GEI of Timothy, an experiment of this kind is currently underway at the Swedish University of Agriculture.

Keywords: Phleum pratense, Multi-Environment, Forage yield, Sweden, Genotype-by-environment interaction

Sammanfattning

Timotej (*Phleum pratense*) är en av de mest använda fodergrödorna i hö- och betesgräsblandningar i de nordiska länderna. Timothy används också i områden där den är en av de främsta fodergrödorna som skördas med hög avkastning, särskilt i borealt klimat. Det har inte funnits någon "direkt" förädling av Timotej som är anpassad för svenskt klimat och svenska normer, vilket är förvånande när detta är den viktigaste fodergrödan som används i Sverige. Denna avhandling är en del av ett större projekt som avser till att utveckla ett "starterpaket" som erbjuder en effektivare förädlingsmetod till kommersiella aktörer, som kan implementeras tillsammans med traditionell förädling för utveckling och förbättring av nya sorter av Timotej som möjliggör ett hållbart jordbruk.

Syftet med denna studie var att utvärdera ekoagronomiska egenskaper som färskvikt (cut), torrsubstans (DM) och botaniskt utvecklingsstadium (botdev) bland avancerat förädlingsmaterial av Timotej och att uppskatta effekten av interaktioner mellan genotyp och miljö. En uppsättning av 264 förädlingslinjer som utvecklats av Lantmännen odlades på tre platser (Röbäcksdalen (RBD), Lövsta (LVT) och Svalöv) i Sverige och studerades tillsammans med två kontrollsorter (Switch och Tryggve). Fältförsöket genomfördes enligt en modifierad blockdesign.

Resultaten av Anova visade på en signifikant skillnad i avkastning mellan förädlingslinjerna. Det fanns en signifikant skillnad mellan längden på antal vegetationsdagar i RBD och LVT. Analyserna av den genetiska variabiliteten visar att arvbarheten i stort sett ökade under hela säsongen från DM1 till DM3 och att den genetiska vinsten var högre än miljöeffekterna i LVT. Miljöeffekterna var dock starka för egenskaperna DM1 och DM3 i RBD. Förslaget är att DM2 skulle kunna förädlas mer effektivt bland förädlingslinjerna för RBD, medan alla tre DM i LVT skulle kunna förädlas för att öka avkastningen. En kombination av PCA och GEI avslöjar en betydande effekt på accessioner och miljön.

Sammanfattningsvis är en kombination av PCA, GEI och analys av genetisk variabilitet visar på lämpliga föräldralinjer, vilket vi visar här. Dessutom kan dessa resultat påskynda förädlingsprogrammet och förståelsen av GEI för Timotej i Sverige. Det finns ett stort behov av en omfattande förståelse av Timotej GEI för dagens och framtidens utmaningar och möjligheter, för att kunna möta klimatförändringarna. Dessutom föreslås fler skördeår och platser för att ge en bättre förståelse av GEI hos Timotej, ett experiment av detta slag pågår för närvarande vid Sveriges lantbruksuniversitet.

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Abbreviations

Anova	Analysis of variance
Botdev	Botanical development stage
Cut	Dry weight
DM	Dry matter
GAM	Genetic advance percent of the mean
GCV	Genotypic coefficient variance
GEI	Genotype-by-environment interactions
HSB	Broad-sense heritability
LVT	Lövsta
MBD	Modified augmented design
PCA	Principal component analysis
PCs	Principal components
PCV	Phenotypic coefficient variance
RBD	Röbäcksdalen
SLU	Swedish University of Agricultural Sciences
SP	Subjective picking

1. Introduction

1.1 Background

Timothy (*Phleum pratense L.*) forage crop belongs to the grass family *Poaceae* (*Gramineae*). Timothy is a cross-pollinating and hexaploid crop (2n=6x=42), though ploidy level varies within the genus Phleum from diploid to octoploid. Hexaploidy genotypes are the ones most commonly used for forage production in agriculture today (Tamaki et al. 2010; Stewart et al. 2011). Timothy is a long day plant, which requires a day length of 13 to 16.5 h for flowering, the hours vary between different varieties, and genotypes from higher latitudes are adapted to longer day lengths (Heide 1982; Junttila 1985).

Figure A: *Field of growing Timothy before first harvest.*

A small part of the arable lands (11 million km^2) throughout the world is allocated to produce crops for human consumption, while a greater part (40 million km^2) is for forage production and animal feed (Ritchie & Roser 2019). Also in Sweden is the largest part of agricultural lands used for growing forage crops which are mainly distributed as hay (Table 1, Figure B). Among forages, timothy is one of the most commonly used species both for hay and pasture grass mixtures.

Table 1*: Top 10 crops used in agriculture and the relevant cultivation area in Sweden 2021 (Jordbruksverket 2022)*

Agricultural land use of crops in Sweden 2021.	
Crops	**Area, hectare**
Forage crops	1050079
Winter wheat	436857
Spring barley	255696
Oat	174422
Canola	106127
Winter rapeseed	97001
Spring wheat	44741
Sugar beet	28702
Rye	25906
Winter barley	22860

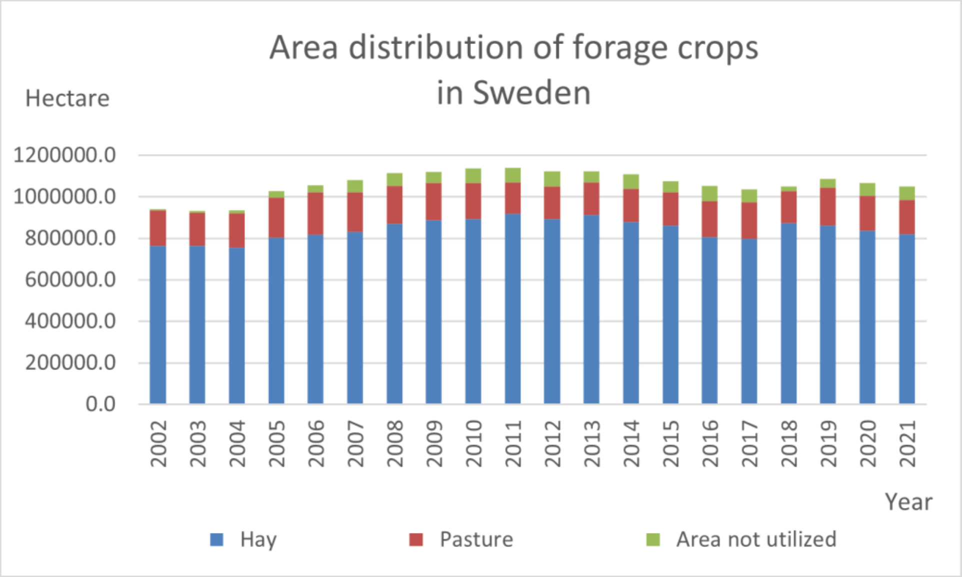

Figure B*: Description of the area distribution of forage crops in Sweden from 2002-2021 (Jordbruksverket 2022b).*

Timothy is also one of the most important pasture grasses grown in boreal climates such as Asia, North America, and the Nordic part of Europe. It’s an excellent crop for animal feed because of its’ high-quality nutritional value and physiological attributes, excellent winter hardiness, and wide range of photoperiod adaption. Timothy is also a hemicryptophyte and perennial, which means it can be grown and harvested across multiple seasons. Furthermore, Timothy has an extensive root system that helps limit soil erosion and nutritional leakage (Andrews & Gudleifsson 1983; Jönsson et al. 1992; Höglind et al. 2013). Forage grasses are harvested several times per season to optimize the nutritional value and yield. In Nordic countries it is usually harvested two to three times per year, but this depends heavily on the length of the growing season (Rinne & Nykänen 2000).

Plant architecture and size are important components which have major effects on desirable traits in forage crops, such as nutritional composition, lodging, a transition from vegetative to generative growth, photoperiod sensitivity, dry matter (DM), and will affect the forage yield production (Capstaff & Miller 2018). Many of these traits are complex quantitative traits that are affected by both genetic variations and influenced by environmental variations and genotype-by-environment interactions (GEI) (Bock et al. 2015).

Therefore, to produce high-yielding varieties with stable production and that produce forage with better nutritional compositions, genetic resources need to be tested using a multi-environmental field trial since research has shown that yield and nutritional value are strongly affected by environmental factors, such as temperature and length of the growing seasons (Gustavsson et al. 1995; Nordheim-Viken et al. 2009; Höglind et al. 2013; Solati et al. 2017). Furthermore, plant breeders are trying to understand GEI and how this impacts their breeding programs (Bocianowski et al. 2021; Egea-Gilabert et al. 2021; Acquaah, n.d.). One of the major focuses of plant breeders is to understand GEI in order to develop genotypes that are stable and well performing either in specific areas or varieties that are high-performing in general. However, this task is difficult due to the complexity of GEIs (Yau 1995; Ebdon & Gauch Jr. 2002).

Besides, analysis of variance (Anova) and multivariate analysis can be used to understand the variation among and within breeding material. Anova is an analysis tool that splits the dataset into two parts, random factors, and systematic factors. The systematic factors are assumed to influence the analysis while random factors don’t. Anova gives an overview if there is a significant difference between groups and/or treatments, however, it doesn’t describe the relationship between particular genotypes and environments (Bewick et al. 2004).

Principal component analysis (PCA) is one of the multivariate tests and a statical procedure for dimension reduction in large datasets aimed at explaining as much variation as possible with as few variables as possible. In a PCA, the original variables are translated to new and (often) fewer variables while minimizing the information loss. Importantly, these new variables, the principal components (PCs), are uncorrelated. The PCs are used for solving the eigenvector/eigenvalue problem. Researchers have been using this technique for several decades (Jolliffe & Cadima 2016), and its widely used by plant breeders to display relationships between specific genotypes and environments (Fjellheim et al. 2015; Das et al. 2017). Plant breeders and researchers have combined Anova and PCA to get a better understanding of GEI and it has been shown to also be useful for genotype evaluation.

1.2 Objectives

1.2.1 Aim

The aim of this project was initially to evaluate variation among advanced modern accessions developed by Lantmännen across different locations in Sweden in terms of forage yield and botanical development. In addition, we assessed the changes in relevant traits across the environmental gradient corresponding to the locations of the trials.

1.2.2 Research questions

- How much variation exists in essential agronomic traits within and between varieties and locations for the purpose of using Timothy as fodder crop?
- Which accessions are well-preforming in specific areas or overall, in Sweden?
- What needs to occur in the future to ensure a sustainable and high-yielding forage production for Timothy?

1.2.3 Purpose

If the questions are answered, they should provide valuable information about accessions which can be used for breeding Timothy for Swedish climates. This would provide a starter package that could be used for future research regarding Timothy accessions or for breeding of new varieties which are better adapted to the Swedish climate and standard.

2. Method

2.1 Plant material

A set of 264 modern accessions developed by Lantmännen, including two check varieties (Tryggve and Switch), were sown at three sites in Sweden; Röbäcksdalen Umeå, Lövsta Uppsala, and Svalöv in 2020. However, the data from Svalöv was not included in this study because of time constraints. Hereafter the modern accessions will be referred to as breeding material and accessions for simplicity.

2.2 Location

Sweden has many different climatic zones but is usually broadly differentiated into three parts Norrland, Svealand, and Götaland. The field trials analyzed in this thesis were carried out in two locations in Sweden, Röbäcksdalen (RBD) (63.80688, 20.23675) and Lövsta (LVT) (59.83270, 17.70899), mainly grown during the main growing season of 2021. The two trial locations can be thought to represent north Norrland (RBD) and Svealand (LVT) of Sweden. The main difference between the two sites is the length of the growing season (Table 2). Norrland and Svealand are the two regions which have more forage production then crops for human consumption.

Table 2*: Length of the growing season during 2021 in the two locations. The vegetations period starts if the average temperature is +5°C over a minimum of 6 days and it ends when it's under +5°C over 6 days (SMHI 2022).*

Site	Days
North Norrland	143
Svealand	219

The growing degree days (GDD) were calculated based on the difference between mean daily temperature and tbase (+5°C), when the value is larger than tmax (+23°C) then it's replaced with tmax (Baskerville & Emin 1969). The GDD data was measured at Uppsala aut weather station for LVT and Umeå airport for RBD (SMHI 2022).

2.3 Field experiment

The field trials were set up based on a modified augmented design (MBD), which means there is no replication of accessions except for the controls. MBD is a useful method when there are too many accessions included in the design to reliably replicate them, for example, due to area restrictions or if the seed supply is limited. Each accessions gets measured and adjust for field variability and to estimate error variance, it is accessed because there are controls in each block that can be compared with each other and then adjust the mean of the accessions(Lin & Poushinsky 1983).

MBD was designed with 6 blocks for each of the two locations. Each block contains 52 plots, with 44 non-replicated accessions and two control varieties, Tryggve and Switch which have 4 replicates each per block (Table 3).

Table 3: *Details on how the MBD was designed for each location.*

Item	Details
Number of blocks	6
Number of treatments	266
Number of check treatments	2
Number of test treatments	264
Check treatments	SWITCH, TRYGGVE
Number of Traits	6
Traits	cut1, cut2, cut3, dm1, dm2, dm3

Each plot was randomly sown for the two sites and had an area of 13.5 m^2 at LVT and 10.5 at RBD, one accession per plot was sown. Between each plot, there is a space of 0.4-0.5m, and between every row is 0.11-0.22m.

All the typical agronomic practices in Sweden for Timothy such as fertilizers and weed control were applied if necessary, according to the regulation and recommended by jobrdbruksverket. The experimental design was provided by employees of Lantmännen and local field station personnel handled the field trials and collected the data. Phenotypical data such as fresh weight(cut), DM, and botanical stage of development (botdev) was collected three times per season from all plots. The sowing for respective location was conducted 2020-04-20 ((LVT) and 2020-06-08 (RBD). The different sow date was depended on the length of the season for each location. Before each harvest a botdev evaluation was conducted, from a scale of 1 to 7, 1 meant there was only leaves, 3 means some of the spike is visible, 5 means the spike is very visible and 7 meant its overflowing. The first and second cut was harvest 2021-06-22 and 2021-08-05 (+- 3 days). However, the third cut was harvested 2021-09-04 (RBD) and 2021-09-28 (LVT). Botdev was not collected for each stage because of limited time and resources.

2.4 Statical analysis

The experimental data analyses were based on six traits, DM 1, 2, and 3, and cut 1, 2, and 3. The data was used to calculate the adjusted mean in accordance with the MBD. The genotypic, phenotypic, and environmental variance was obtained by the expected value of the mean square according to the protocol by Federer & Searle (1976). Genotypic and Phenotypic coefficient variance (GCV, PCV) were calculated corresponding to Burton (1951, 1952). Using the method by Lush (1949) broad-sense heritability (hBS) was determined while genetic advance as a percentage of mean and category was calculated according to Johnson et al. (1955). The broad-sense heritability was calculated according to the method by Lush (1940). Genetic advance as per cent of mean and category is according to Johnson et al. (1955). Genetic variability analysis was retrieved based on the

Anova results. Genetic variability, hBS, and MBD was conducted by the package augmented RCBD in R (Aravind et al. 2021). To determent, the optimal number of clusters and, the GEI, the Anova mixed linear model, PCA, correlation, all statical analyses and graphs were generated in R (Charrad et al. 2014; R Core Team (2020). n.d.).

3. Results

3.1 Anova

The blocks showed a significant effect on the accessions in both sites which needed to be adjusted, except for cut 1 in LVT (*P*<=0.01, Table 4, Table 5), A block-adjusted Anova table was generated. The Anova for RBD data showed significant variation in cut-1 and cut-3 within the modern accessions (*P*<=0.05), and between the modern accessions and check varieties (*P*<=0.01, Table 6), while for LVT only cut-1 was significantly different within the modern accessions, and between the modern accessions and check varieties (*P*<=0.01, Table 4). In RBD, we observed a significant difference in DM1 and DM2 between modern accessions and check varieties, while there is a significant variation within the lines for only DM1 (Table 6). In LVT it showed significant differences in all three DMs within the modern accessions, however, there is only a significant difference between the modern accessions and check for DM3 (Table 7). There is high variability between the 264 modern accessions of Timothy for cut and DM.

Table 4: *Treatment-adjusted Anova for 266 modern accessions of Timothy in Röbäcksdalen. Df = Degree of Freedom, cut= Fresh weight, dm= Dry matter, Treatment = 266 accessions, ** <= Significant at or less than 0.01 Significance level, * <= Significant at or less than 0.05 Significance level, ns > No Significantly level higher than 0.05.*

Source	**Df**	**cut1**	**cut2**	**cut3**	**dm1**	**dm2**	**dm3**
Block (ignoring Treatments)	5	46.68 **	9.2 **	20.3 **	23.21 **	33.25 **	9.04 **
Treatment (eliminating Blocks)	265	6.73 *	2.21 ns	2.64 ns	1.04 ns	1.94 *	1.38 ns
Treatment: Check	1	0.04 ns	2.49 ns	68.48 **	6.96 *	35.48 **	1.45 ns
Treatment: Test and Test vs. Check	264	6.75 *	2.21 ns	2.39 ns	1.02 ns	1.81 *	1.38 ns
Residuals	41	4.33	2.3	1.76	1.23	1.14	1.64
Block (ignoring Treatments)	5	46.68 **	9.2 **	20.3 **	23.21 **	33.25 **	9.04 **

Table 5: *Treatment-adjusted Anova for 266 modern accessions of Timothy in Lövsta. Df = Degree of Freedom, cut= Fresh weight, dm= Dry matter, Treatment = 266 accessions, ** <= Significant*

*at or less than 0.01 Significance level, * <= Significant at or less than 0.05 Significance level, ns > No Significantly level higher than 0.05.*

Source	**Df**	**cut1**	**cut2**	**cut3**	**dm1**	**dm2**	**dm3**
Block (ignoring Treatments)	5	15.58 ns	32.17 **	169.09 **	29.32 **	125.98 **	141.3 **
Treatment (eliminating Blocks)	265	16.02 **	3.64 ns	5.36 ns	8.39 ns	2.17 ns	1.75 ns
Treatment: Check	1	52.05 *	81.25 **	129.15 **	2.77 ns	42.77 **	42.19 **
Treatment: Test and Test vs. Check	264	15.89 **	3.35 ns	4.89 ns	8.41 ns	2.02 ns	1.59 ns
Residuals	41	8.42	5.4	6.55	5.7	1.89	1.17
Block (ignoring Treatments)	5	15.58 ns	32.17 **	169.09 **	29.32 **	125.98 **	141.3 **

Table 6: *Block-adjusted Anova for 266 modern accessions of Timothy in Röbäcksdalen. Df = Degree of Freedom, cut= Fresh weight, dm= Dry matter, Treatment = 266 accessions, ** <= Significant at or less than 0.01 Significance level, * <= Significant at or less than 0.05 Significance level, ns > No Significantly level higher than 0.05.*

Source	**Df**	**cut1**	**cut2**	**cut3**	**dm1**	**dm2**	**dm3**
Treatment (ignoring Blocks)	265	7.29 *	2.36 ns	2.99 *	1.36 ns	2.25 **	1.42 ns
Treatment: Check	1	0.04 ns	2.49 ns	68.48 **	6.96 *	35.48 **	1.45 ns
Treatment: Test vs. Check	1	159.25 **	0.12 ns	33.23 **	14.18 **	22.64 **	2.49 ns
Treatment: Test	263	6.74 *	2.37 ns	2.63 ns	1.29 ns	2.04 *	1.42 ns
Block (eliminating Treatments)	5	17.14 **	1.39 ns	1.56 ns	6.47 **	16.84 **	7.06 **
Residuals	41	4.33	2.3	1.76	1.23	1.14	1.64

Table 7: *Block-adjusted Anova for 266 modern accessions of Timothy in Lövsta.Df = Degree of Freedom, cut= Fresh weight, dm= Dry matter, Treatment = 266 accessions, ** <= Significant at or less than 0.01 Significance level, * <= Significant at or less than 0.05 Significance level, ns > No Significantly level higher than 0.05.*

Source	**Df**	**cut1**	**cut2**	**cut3**	**dm1**	**dm2**	**dm3**
Treatment (ignoring Blocks)	265	15.82 **	4.1 ns	8.23 ns	8.74 *	4.12 **	3.98 **
Treatment: Check	1	52.05 *	81.25 **	129.15 **	2.77 ns	42.77 **	42.19 **
Treatment: Test vs. Check	1	426.38 **	0.57 ns	1.55 ns	14.29 ns	2.9 ns	18.37 **
Treatment: Test	263	14.12 *	3.82 ns	7.79 ns	8.74 *	3.98 **	3.78 **
Block (eliminating Treatments)	5	26.52 *	7.75 ns	16.77 *	10.66 ns	22.71 **	22.86 **
Residuals	41	8.42	5.4	6.55	5.7	1.89	1.17

3.2 Cluster analysis

To get a better understanding of the grouping within breeding populations, a cluster analysis was done in R. The optimal cluster scheme and groups were produced using the k-means algorithm in the R package NbClust, which choose randomly centroids and then assigns the closest ones to the center by using the Euclidean distance between points (Figure C).

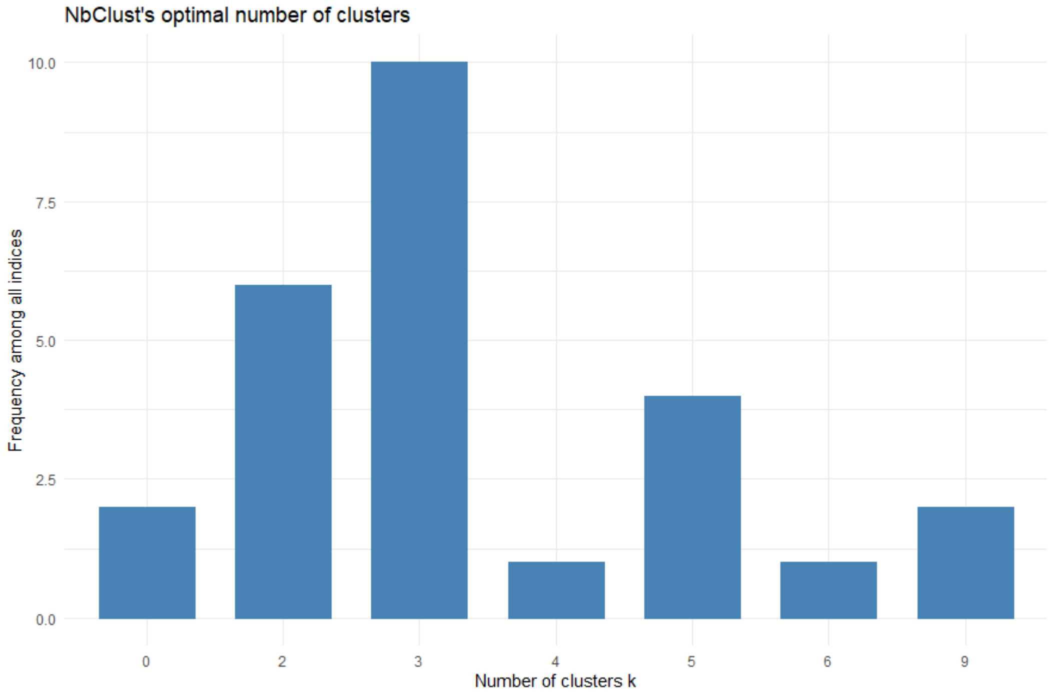

Figure C*: Optimal cluster group determined by K-means on the package by NbClust. Two clusters proposed at 0 as the best result, 6 clusters proposed at 2 as the best result, 10 clusters proposed at 3 as the best result, 1 cluster proposed at 4 as the best result, 4 clusters proposed at 5 as the best result, 1 cluster proposed at 6 as the best result, 2 clusters proposed at 9 as the best result. In total 26 indicate were proposed.*

A biplot was generated from the cluster analysis results based on the 266 Timothy accessions that are tested in two different environments. In each environment, the accessions are scored for three different traits dm1, dm2, and dm3. A Neighbourhood-Based clustering with constraints, grouped samples in three clusters which is the most optimal clustering scheme (Figure C). Cluster-III is the largest cluster, containing 221 accessions from the RBD site and 9 accessions from the LVT site. Of these 230 accessions there are 9 duplicates, which could mean that they can be good candidates for the specific site. Cluster-I contains a total of 160 accessions, with 159 from the LVT site and 1 from the RBD site, and in, this cluster one accession is duplicated. The last cluster-II contains 142 accessions with 98 accessions from the LVT site and 44 accessions from the RBD site. For this cluster, 22 duplicate accessions are present (Figure D).

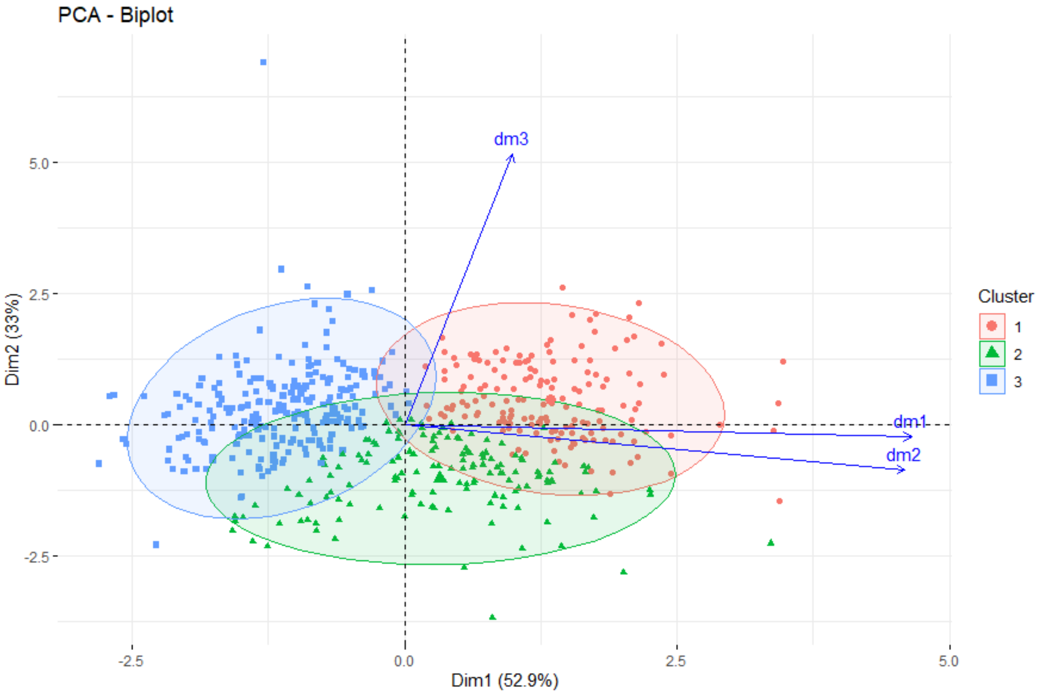

***Figure D**: PCA biplot over 266 Timothy accessions over two locations and the three cluster groups are dived in different colors. Accessions closer to a specific DM had the best performance in that DM.*

3.3 Genetic variability and heritability

The genetic variance analysis is only performed if there is a significant difference between the sum of the square of Treatment: Test in Anova. The PCV, GCV, and hBS are categorized as low, medium, and high according to their value see Tables 8 and 9.

***Table 8:** Broad-sense heritability (H^2) categorized according to (Robinson 1966).*

H^2	**Category**
$x \leq 30$	Low
$30 \leq x \leq 60$	Medium
$x \geq 60$	High

Table 9: *Genotypic- and phenotypic- coefficient variance categorized according to (Sivasubramaniam & Madhavamenon 1978).*

***CV* (%)**	**Category**
x ≤ 10	Low
10 ≤ x ≤ 20	Medium
x ≥ 20	High

In RBD for dm1 and dm3 there is no significant difference between Treatment: Test (Table 6), which led to no reason for running the genetic analysis for RBD (Table 10). In RBD there is a low PCV for all three traits. However, trait dm2 has a medium hBS, but hBS is subjected to trial error. To retrieve more reliable information for desirable traits for selection, the estimation of genetic advance as a percentage of the mean (GAM) should be the base for selection (Burton 1952). The PCV and GCV for dm2 show there is a low variance within the population, and GAM was estimated to have a low probability for the trait to be passed on.

Table 10: *Genetic variability analysis for Röbäcksdalen. PV = Phenotypic variation, GV = Genotypic variation, EV = Environmental variation, GCV = Genotypic coefficient variance, PCV = phenotypic coefficient variance, ECV = environmental coefficient of variation, hBS = Broad-sense heritability (H^2), GA = Genetic advance and 5 % selection in a large population which is normal disturbed, GAM = Genetic advance as per cent of mean.*

Trait	**dm1**	**dm2**	**dm3**
Mean	20.17	24.43	20.44
PV	1.14	2.04	1.42
GV		0.9	
EV	1.23	1.14	1.64
GCV		3.89	
GCV.category		Low	
PCV	5.3	5.85	5.82
PCV.category	Low	Low	Low
ECV	5.5	4.38	6.26
hBS		44.08	
hBS.category		Medium	
GA		1.3	
GAM		5.32	
GAM.category		Low	

The GCV and PCV for all three traits in LVT show a low estimation, except for PCV dm1 which indicates a medium category. The difference between PCV and GCV is slightly high for dm1, while for dm2 and dm3 there is just a little variation. HBS for dm1 and dm2 imply a medium category, while dm3 indicates high. This effect the GAM results which suggest a low category for dm1 and dm2, while dm3 is medium, however, dm2 is close to a high value in hBS (Table 11).

Table 11: *Genetic variability analysis for Lövsta. PV = Phenotypic variation, GV = Genotypic variation, EV = Environmental variation, GCV = Genotypic coefficient variance, PCV = phenotypic coefficient variance, ECV = environmental coefficient of variation, hBS = Broad-sense heritability (H^2), GA = Genetic advance and 5 % selection in a large population which is normal disturbed, GAM = Genetic advance as per cent of mean.*

Trait	**dm1**	**dm2**	**dm3**
Mean	25.52	27.76	20.68
PV	8.74	3.98	3.78
GV	3.04	2.09	2.61
EV	5.7	1.89	1.17
GCV	6.84	5.2	7.81
GCV.category	Low	Low	Low
PCV	11.59	7.19	9.4
PCV.category	Medium	Low	Low
ECV	9.35	4.96	5.24
hBS	34.81	52.4	68.96
hBS.category	Medium	Medium	High
GA (5%)	2.12	2.16	2.77
GAM	8.32	7.77	13.38
GAM.category	Low	Low	Medium

3.4 Correlation

There is a strong correlation between biomass produced by accessions at DM-1 and -2. However, there is a clear distinction between the first two DMs and DM3 that are presented in Figure D. Cluster-I is strongly correlated with DM-1 and -2, while Cluster-III seems to perform better at the third cut compared to Cluster-I. Cluster-II have a non-significant correlation with DM3.

3.5 Growing degree days

LVT has a longer period of the season because of its location compared to RBD (Tables -2 and -12). The first harvest was done on 2021-06-22, the GDD for dm1 was 2411.90°C for LVT, while RBD had a value of 1396.85°C. The second harvest was done on 2021-08-05, Dm2 had a value of 3001.80°C for LVT and 1901.25°C for RBD. The third harvest was done on 2021-09-04 for RBD and the GDD value for dm3 was 2167.50°C. For LVT the third harvest was done 2021-09-28 and the GDD value for dm3 was 3449.00°C (Figure E).

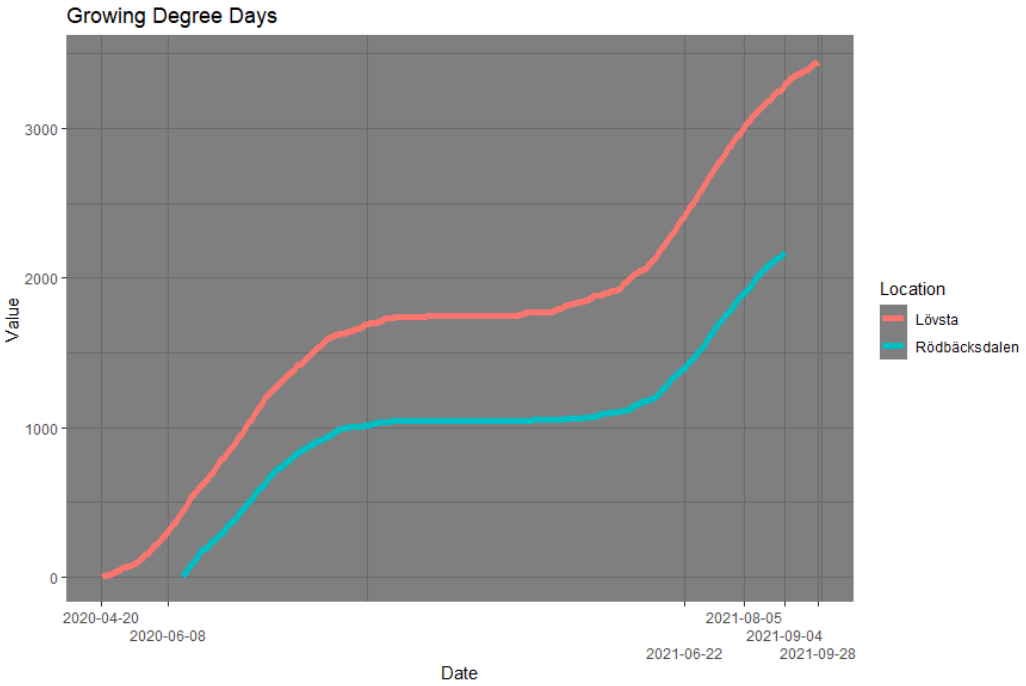

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Figure E: *Growing degree days. 2020-04-24 and 2020-06-08 are referring to the sowing dates for each location. 2021-06-22 = dm1, 2021-08-05 = dm2, 2021-09-02 = dm3 for Rödbäcksdalen, 2021-10-01 = dm3 for Lövsta.*

Table 12: *GDD and mean of DM. Mean of all accessions in the specific site. %/Plot = dry matter % per plot.*

	RBD		LVT	
	GDD	Mean (%/Plot)	GDD	Mean (%/Plot)
DM1	1396.85	20.13	2411.9	25.52
DM2	504.4	24.43	589.9	27.76
DM3	266.25	20.44	447.2	20.68

3.6 Genotype by environment interaction

GEI was determined using Finlay-Wilkinson regression (FW) (Lian & de los Campos 2015). The location has a high estimate, indicating that the site of the trials has a strong and significant effect on the productivity of the accessions. There is no overall significant difference among the accessions across the two trials and three DMs (Table 13). However, there is a significant accession by location effect, indicating that the trial location has a strong effect on both the actual yield of the different accessions but also the ranking of accessions within sites (Figure F).

Table 13: *Accession and environment interaction. Name = Accession, Pvalue is significant if it is 0.05 or less. FW was estimated on the total yield of each accession.*

	Estimate	SE	PValue	X2.5.LL	X97.5.UL
V(location)	39.87998	1.138887	2.50E-10	35.85712	43.90283
V(name)	0.74699	0.397687	3.17E-01	-0.65775	2.151726
V(location:name)	4.262341	0.140167	8.80E-10	3.767235	4.757447
V(e)	4.262341	0.140167	8.80E-10	3.767235	4.757447

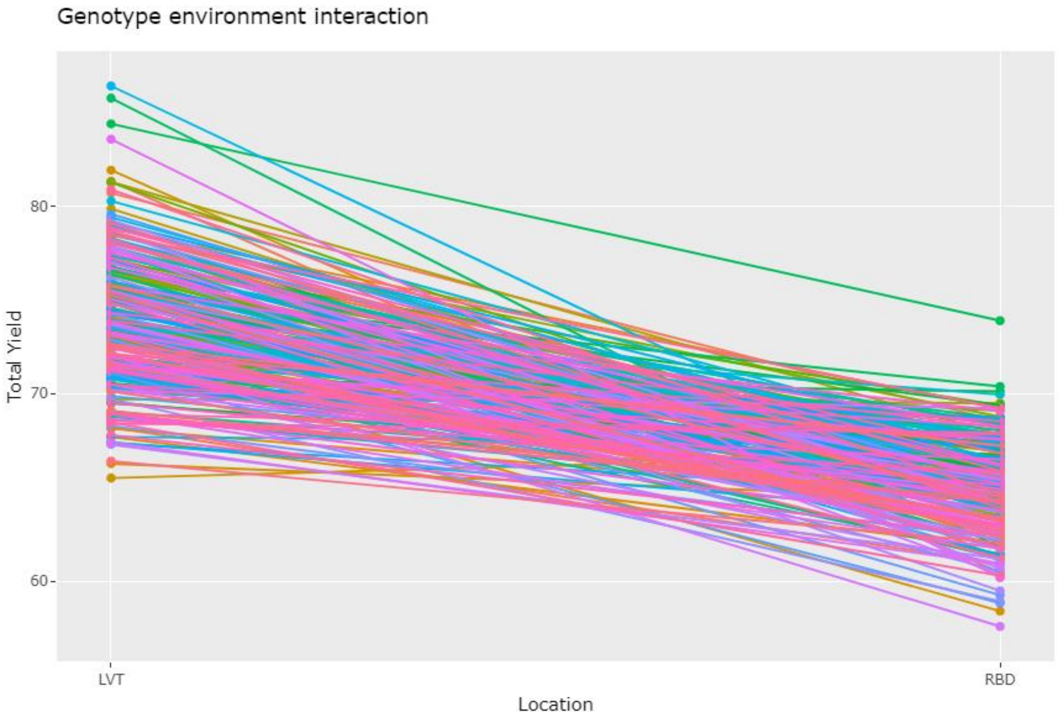

Figure F: *Accession and environment interaction. Total Yield = addition of dm1, dm2 and dm3, RBD = Röbäcksdalen, LVT = Lövsta. Display how rankings change between two sites for each accession.*

3.7 Principal component analysis (PCA)

Eigenvalues and the variance explained from the PCA of the three DMs are presented in Table 14. PC1 explained 52.9 %, PC2 33 % and PC3 14.1 % of the total variation. Everything above 1 eigenvalue can be accepted as a descriptor of the variance in the collected data, according to Kaiser rules (Kaiser 1960). The eigenvalues of PC1 are 1.58, which is higher than 1 and accepted as a descriptor, PC2 has a value of 0.99 which could be accepted as a descriptor, even if it doesn't fit the requirement Table 14. PCA was made which assigned PC1 to stand for DM1 and DM2, while PC2 stands for the third DM which are provided in Figure D.

Table 14*: PCA: The value of Eigenvalue, Variability (%), and Cumulative (%) variance for each component derived from PCA.*

	Eigenvalue	Variability	Cumulative
PC1	1.586	52.856	52.856
PC2	0.991	33.023	85.879
PC3	0.424	14.121	100.000

If the goal is to obtain accessions which are performing similarly at both sites, then it becomes more difficult to choose depending on the method. Three methods were used, one which is based on the score difference between LVT and RBD for the PCs, and it can't be higher than 0.1, that will be called script for simplicity. One which is based on subjective picking from the graph, it will be called SP for simplicity. The selection of accession is nearly the same between the script and SP method for PC1. The only difference was that SP choice G126, and script choice G174, the rest were the same (Appendix 1). For PC2 there was a big difference between the script and SP methods, the only accession both methods selected was G144 (Appendix 2).

4. Discussion

There is a large literature on ryegrass which is one of the dominating forage grasses across Europe and North America (Wilkins 1991; Humphreys et al. 2010). However, for Timothy there are comparably few studies available and the available ones are focused on genotypes which are adapted for Poland, Canada, Finland, or Norway (Heide 1982; Junttila 1985; Rinne & Nykänen 2000; Jing et al. 2013; Bocianowski et al. 2021). Notable there are few projects that are focused on Scandinavia, which is surprising given that Timothy is the most important forage grass in the region and serves as the backbone in agriculture from an economic perspective (Höglind et al. 2001, 2013). Considering future climate change, with predicted higher temperatures and more common droughts (Lin 2011; Juhola et al. 2017), there is a clear need to evaluate current Timothy modern accessions in Sweden with the aim of developing new varieties with high adaptability (Uleberg et al. 2013).

By generating GEI models, we gain a direct biological interpretation of how the biological process works, such as the effect of drought, temperature, photoperiod on root system, gene expression, biochemical process, etc (Chapman 2008; Teressa et al. 2021). In the present study we evaluated 266 modern accessions of Timothy by applying methods aimed at detecting GEI. These accessions will be used in future breeding programs but also for introducing biological processes in agriculture which has been conducted by other authors in Ryegrass and Timothy (Radkowski et al. 2020; Bocianowski et al. 2021; Fois et al. 2021). However, the biological processes are limited because generally in many experiments the expression of the genotype are recommended on specific soil type and climate conditions. Furthermore, not to comprised the yield of Timothy and ensure high nutritive value it becomes more urgent to understand the GEI under several different environmental condition (Bertrand et al. 2008; Jing et al. 2013; Altieri et al. 2015; Palit et al. 2020).

The Anova results reveal that there are significant differences both among the modern accessions and between check varieties and modern accessions. At RBD differences were found for cut-1 and cut-3, while at LVT differences were found only for cut-1. It shows the same pattern in both sites, which could be the result that the fresh weight is weighted directly in the tractor after they are harvested and not under control circumstances. Improper use of equipment may have occurred. Furthermore, it can also be affected by a difference of about 1 to 3 days between the harvest within accessions on the same sites. If more harvesting year was conducted, then it would show more precise result and the Anova result would probably change.

The result for the Anova for DM at RBD reveals there are significant differences among modern accessions for only DM2, while for DM1 and DM3 there were no significant differences among the modern accessions. The genetic variability analysis suggests that the EV is higher than GV for these time points, which could be attributed to a large environmental effect on the traits DM1 and DM3. The conclusion of the combination of Anova and genetic variability analysis suggests that DM2 could be more efficiently bred within the modern accessions for the site RBD, and the accessions with the highest value of DM2 have potential to be selected as parental lines, to gain higher yield. However, there could be variation within the modern accessions for traits DM1 and DM3, but the environmental effect was on the other hand too strong, and no significant difference was observed in Anova analysis. To gain more precise results regarding the variation within the modern accessions, the suggestion is to include more harvesting years in the trials.

DM at LVT shows significant differences among the modern accessions for all three traits. The genetic variability analyses reveal that the hSB values increase across the season from DM1 to DM3, and genetic effects in DM2 and DM3 were higher than environmental effects. These results suggest that there is a higher probability to increase the yield of trait DM3 and DM2 than DM1 for the next generations due to the higher heritability at that stage. Furthermore, there are candidate accessions that could be used as parental lines for the LVT site. As with RBD, the accessions with the highest value of either DM1, DM2 or DM3 could be chosen as parental for a breeding program, to gain the highest yield for the site LVT and specific trait.

The LVT site gained more GDD compared to RBD which resulted in greater yield for every trait of DM (Table 12). The mean and GDD revel that the season at LVT is longer than at RBD and it’s in a warmer temperature zone. Furthermore, postponing the first harvest results in more yield in DM1, however, it has an opposite effect on regrowth and decrease for DM2 and DM3, similar results were found by Rinne & Nykänen (2000). Additionally higher GDD than 288°C or postponed harvest results in less nutritional value, but higher fiber content in DM1 (Rinne & Nykänen 2000; Nissinen 2001). If the goal is to produce more yield, then it is favourable to have more GDD, however, if the goal is to produce feed for intensive animal feed, then its more favourable to harvest earlier and less GDD then 288°C which results in less fiber content and higher crude protein

From the PCA candidate parental lines can be selected, depending on which traits and site are the breeding goal. The PC1 is highly correlated with the traits DM1 and DM2, while PC2 is correlated with DM3. Here are some scenarios which can be drawn from the PCA, but other conclusion can also be draw from the data of the PCA. Figure G visualizes PC1 vs PC1. The best candidate from this can be picked out either from the positive side of the x-axis (LVT) or y-axis (RBD). Furthermore, the candidates that works for both sites are close to the line through the origin.

PCA is a well-used method to eliminate redundancy and to reveal patterns in large data sets. It can also be a tool that can speed up and improve breeding programmes. It has been widely used by plant breeders for many varieties of crops and fodder species to find parental candidates for breeding programmes or further studies (Das et al. 2017; Kujur et al. 2017; Hamidou et al. 2018; Girgel 2021).

The top 10 accessions for specific PC and site were compared to see if there is any similarity or difference. One criterion was that all the accessions that were picked performed better than both check varieties. The top accessions for PC1 (Figure G and H) for the specific site RBD or LVT showed a generally high total yield of the specific traits, and the total yield (Appendix 1). There was no similarity for the modern accessions that had the highest score for a specific site. So, the modern accessions that perform well in RBD was not performing well in LVT vice versa. Some interesting accessions for PC1 in RBD is genotype91(G91), G96, G98, and for LVT it is G46, G100, G151.

The selection of accessions, between the script and SP method had only difference on one accessions (Appendix 1 and 2). However, the SP method choose accessions which have a higher score of PC1 or PC2 in the site LVT because the SP method isn't only based on the PC score but takes also into account the results of the genetic variability analysis. This could explain that there were differences between the two methods for PC2.

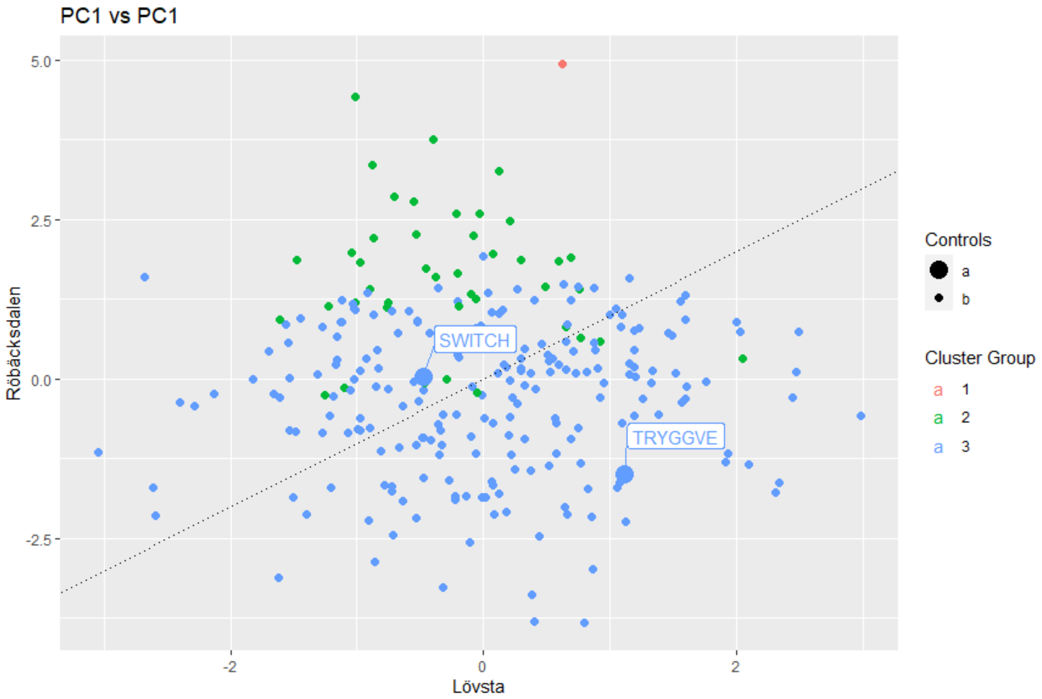

Figure G: *Plot of PC1 vs PC1, which stand for two first dm. The controls are described as the bigger circle and the accession as smaller circles. The best performing accession for specific location is at the end of their axis. The accession which are performing well on both locations are close to the line passing through the origin, so the top right corner is the top accessions for both locations.*

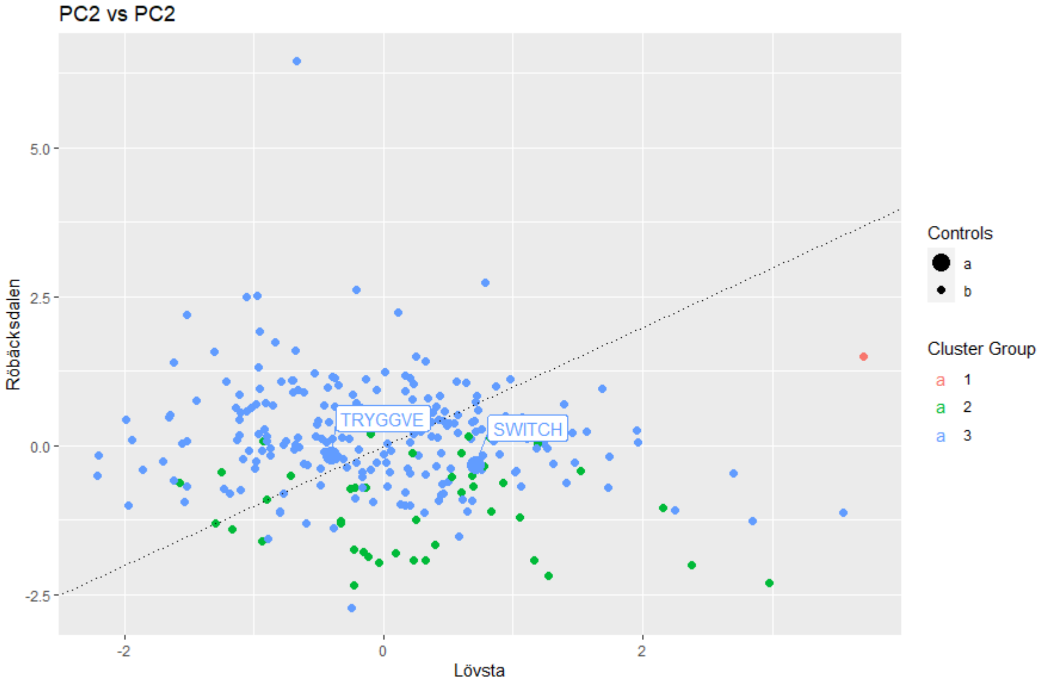

Figure H: *Plot of PC2 vs PC2, which stand for the third dm. The controls are described as the bigger circle and the accession as smaller circles. The best performing accession for specific location is at the end of their axis. The accession which are performing well on both locations are* *close to the line passing through the origin, so the top* right *corner is the top accessions for both locations.*

If the goal is to obtain resilient accessions which perform well at both sites, then yield would likely be lower compared to accession that are well-preforming at a single site. So, there are a trade-offs for adaptation between sites which are decreasing yield and which makes it difficult to develop varieties that perform well across two different climate zones. Nevertheless, such varieties could be preferred by the farmers as under current climate change assuring yield stability for across seasons might be preferable due to climate uncertainty (Beitnes et al. 2022). However, the issues are complex, and breeder need to take into a lot of considerations into account when developing new varieties for the farmers (Atlin et al. 2001; Teressa et al. 2021). The prediction of the current and future climate change in Scandinavia is estimated to increase temperature and $CO_{2.}$ Nevertheless, also other changes to the climate will likely occur, such as more extreme weather variation (Field et al. 2012; Ergon et al. 2018). With increasing temperature also comes the risk of new and more frequent weeds and pests (Eckersten et al. 2008). The production of Timothy is already now encountering some challenges such as drought, pests and weeds which are directly correlated to climate change. While it seems to increase yield and new opportunities, it comes with important challenges that require a comprehensive understanding of resistance and adaption for abiotic and biotic stress for specific climate zones (Tubiello et al. 2007; Hakala et al. 2011; Höglind et al. 2013). Furthermore, selection can't only be based on overcoming challenges, but also needs to be balanced with biodiversity (Erisman et al. 2016; Begna 2022; Ceccarelli & Grando 2022). Thus, a broad range of genetic material to select from is needed to counter future challenges and opportunities.

5. Conclusion

There is a need to evaluate GEI in current modern accessions of Timothy in Sweden as it is currently the most important forage grass. An Anova indicated that there were significant differences in yield among the modern accessions. There was a significant difference between the length of the season in RBD and LVT which impacts how the modern accessions perform and interact with local climate. The PCA indicated modern accessions that were high yielding for a specific site and trait. In this study we have performed a comprehensive evaluation of the phenotypical data provided by Lantmännen on 266 modern accessions of Timothy to investigate the GEI effects. A combination of genetic variability analyses, PCA and GEI indicate suitable parental lines furthermore these findings can speed up breeding and understanding of Timothy interaction with boreal and nemoral climate. There remains an urgent need of a comprehensive understanding of GEI of Timothy in specific Sweden, to comply with ongoing and future climate change. Furthermore, more harvesting years and locations will give more precise candidate for specific trait and demand, experiment of this kind is currently underway in Grogrund project.

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Popular science summary

From an economic perspective, livestock and dairy production are the backbone of agriculture in northern Europe, because arable land is limited for economic crops such as cereals. Timothy is the most grown forage crop in Sweden, and therefore has an important impact on the production of high yield and high nutritional quality feed for our livestock. It is able to produce high quality yield under a wide range of environmental conditions, compared to English ryegrass (*Lolium perenne*) which is the most common forage grass in Europe and is less persistent to cold climate conditions. Production of Timothy can contribute to many ecosystem services, reducing nutritional leakage, soil erosion, and increasing the organic matter in soil.

Our climate is changing, and temperatures are expected to increase in northern latitudes. This may increase the yield of forage crops but also introduce extreme weather changes and conditions in autumn/spring, such as severe temperature drops, snow cover and surface ice, reducing the ability of crops to cope with abiotic stress. This will affect the growing condition of Timothy thereby highlighting the importance of new varieties which can uphold the high quality, yield and growing security for Swedish agriculture and farmers.

There hasn’t been any “direct breeding” of Timothy in Sweden and the research is very limited for varieties which are adapted to Scandinavia. This thesis is part of a wider project that aims to develop a “starter package” to implement improved plant breeding methods and techniques for important agronomic traits, in turn improving cost efficiency and speeding up the breeding program of Timothy. Mapping of the genome and evaluation of modern varieties and landraces are essential. Thus, a breeding program can start with a wide genomic variation to make it sustainable in the long term.

This thesis project evaluated important agronomic traits in 266 varieties of Timothy in two locations in Sweden (Lövsta and Röbäcksdalen). Principal component analysis (PCA) was conducted to compress the traits to PC1 and PC2 that explained 85.68 % of the variation. Thereafter a genotype-environment interaction (GEI) was conducted to understand which accessions are good candidates for a specific area and to find accessions that are stable in both areas. Furthermore, accessions which didn’t perform well was also found. The results for GEI indicate there was significant accession by location affect, which has a strong impact on the performance. There is a difference between the length of the season and GDD between RBD and LVT.

Genetic variability analyses indicate whether the trait is passed on to the next generation, which indicates that all traits for LVT would be hereditary and that it is higher probability to increase the yield of DM3 then DM1. However, trait DM1 and DM3 for RBD would not be passed on because the environmental affect was too strong on the modern accessions. The PCA suggested well performing modern accessions which could be candidates for specific sites and traits. Furthermore, it indicated whether the choice is a accession which perform well in both locations, then it would generate less yield which is a trade-off. A combination of PCA, genetic variability and GEI gives a good estimation of candidates for specific sites and traits. This project provides estimation of the performance and stability in the modern accessions, which provided valuable information for breeding programs and GEI of Timothy.

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Appendix 1

Supplementary Table 1. Top 10 modern accessions with highest PC1 scores in Röbäcksdalen. DM are measured as %/Plot.

Name	DM1	DM2	TotalDM1+2	Total
90	20.15	29.01	49.16	69.38
91	20.37	30.32	50.69	70.12
96	20.98	30.94	51.92	73.89
98	21.79	28.18	49.97	70.38
103	20.14	27.83	47.97	68.39
115	19.42	29.83	49.25	68.39
117	21.37	27.36	48.73	68.04
119	21.71	27.22	48.93	68.34
123	20.00	28.23	48.23	67.52
146	19.29	28.66	47.95	67.37

Supplementary Table 2. Top 10 modern accessions with highest PC1 scores in Lövsta. DM are measured as %/Plot.

Name	DM1	DM2	TotalDM1+2	Total
5	25.81	21.94	47.75	68.21
9	28.94	25.56	54.50	77.25
17	29.01	26.50	55.51	78.20
46	31.98	27.22	59.20	81.28
71	30.99	26.48	57.47	81.26
100	35.97	28.25	64.22	85.75
144	29.77	27.47	57.24	80.27
151	35.58	28.26	63.84	86.40
160	29.01	26.62	55.63	79.14
183	28.22	26.44	54.66	78.10

Supplementary Table 3. Top 10 accessions which perform well in both sites. They are based on subjective picking and PC1. DM are measured as %/Plot.

	Röbäcksdalen				Lövsta			
Name	DM1	DM2	TotalDM1+2	Total	DM1	DM2	TotalDM1+2	Total

3	21.34	25.37	46.71	68.51	28.60	29.12	57.72	80.70
7	21.99	25.08	47.07	66.88	27.02	26.59	53.61	74.92
47	20.04	25.32	45.36	66.95	26.80	27.24	54.04	74.77
81	21.01	24.89	45.90	66.97	26.22	26.68	52.90	73.76
126	19.21	25.63	44.84	63.68	29.46	27.62	57.08	77.80
140	19.29	26.23	45.52	66.44	26.27	25.87	52.14	73.03
145	18.40	25.60	44.00	66.07	25.28	28.47	53.75	75.50
167	19.87	24.75	44.62	65.89	23.28	26.77	50.05	70.95
168	19.48	25.31	44.79	67.15	26.21	28.28	54.49	75.97
256	20.08	24.81	44.89	65.89	25.36	30.18	55.54	78.66

Supplementary Table 4. Top 10 accessions which perform well in both site and are based on difference between the PC1 scores for the sites is <=0.1. DM are measured as %/Plot.

	Röbäcksdalen				Lövsta			
Name	DM1	DM2	TotalDM1+2	Total	DM1	DM2	TotalDM1+2	Total
3	21.34	25.37	46.71	68.51	28.60	29.12	57.72	80.70
7	21.99	25.08	47.07	66.88	27.02	26.59	53.61	74.92
47	20.04	25.32	45.36	66.95	26.80	27.24	54.04	74.77
81	21.01	24.89	45.90	66.97	26.22	26.68	52.90	73.76
140	19.29	26.23	45.52	66.44	26.27	25.87	52.14	73.03
145	18.40	25.60	44.00	66.07	25.28	28.47	53.75	75.50
167	19.87	24.75	44.62	65.89	23.28	26.77	50.05	70.95
168	19.48	25.31	44.79	67.15	26.21	28.28	54.49	75.97
174	20.68	23.96	44.64	64.18	24.91	27.83	52.74	73.52
256	20.08	24.81	44.89	65.89	25.36	30.18	55.54	78.66

Appendix 2

Supplementary Table 5. Top 10 modern accessions with highest PC2 scores in Röbäcksdalen. DM are measured as %/Plot.

Name	DM3	Total
57	22.38	69.49
134	23.78	69.95
152	22.69	67.49
155	24.11	66.88
156	23.74	67.64
159	23.29	67.68
160	23.37	67.40
164	23.68	68.71
207	29.10	69.22
221	22.92	68.25

Supplementary Table 6. Top 10 modern accessions with highest PC2 scores in Lövsta. DM are measured as %/Plot.

Name	DM3	Total
4	18.43	81.32
31	21.10	81.91
36	19.29	78.07
41	19.72	79.86
96	19.15	84.38
100	21.53	85.75
102	17.22	76.79
151	22.56	86.40
212	21.09	83.56
253	20.59	80.88

Supplementary Table 7. Top 10 accessions which perform well in both sites. They are based on subjective picking and PC2. DM are measured as %/Plot.

	Röbäcksdalen		Lövsta	
Name	DM3	Total	DM3	Total
3	21.80	68.51	22.98	80.70

58	21.32	64.01	20.56	76.49
71	21.17	64.99	23.79	81.26
144	21.33	67.29	23.03	80.27
167	21.27	65.89	20.90	70.95
189	20.94	65.67	20.35	75.39
194	21.87	64.22	22.16	79.25
219	21.65	64.20	21.32	76.96
220	21.20	65.43	21.19	76.69
256	21.00	65.89	23.12	78.66

Supplementary Table 8. Top 10 accessions which perform well in both sites. They based on difference between the PC2 scores for the sites is <=0.1. DM are measured as %/Plot.

	Röbäcksdalen		Lövsta	
Name	DM3	Total	DM3	Total
62	20.89	67.29	17.12	70.16
69	20.82	67.74	21.69	76.63
71	21.17	64.99	23.79	81.26
120	18.64	65.41	20.20	69.04
127	19.17	65.88	20.21	70.30
144	21.33	67.29	23.03	80.27
171	19.39	64.27	22.62	74.40
216	20.83	62.80	19.43	73.18
256	21.00	65.89	23.12	78.66
257	20.62	63.20	19.77	72.55

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