Genomic selection in red clover (*Trifolium pratense*): A research project funded by SLU Grogrund - Centre for Breeding Food Crops

*Genomisk selektion i rödklöver (*Trifolium pratense*): ett forskningsprojekt finansierat av SLU Grogrund - Centrum för växtförädling av livsmedelsgrödor*

Mulatu Geleta, Cecilia Gustafsson, Elisabet Nadeau, Rodomiro Ortiz, David Parsons, Amanda Andersson and Linda Öhlund

Summary

Red clover is an important forage legume, which has a key role as feed for livestock in Sweden. Currently, Lantmännen uses crossbreeding methods to develop red clover cultivars for Nordic Europe in which new cultivars are synthetic populations. This process is time consuming and takes about 18 years from the initial cross to the approval of a new cultivar. Hence, introduction of modern methods is crucial to increase the efficiency of the breeding program. The project "Genomic selection in red clover (*Trifolium pratense*)" funded by SLU Grogrund aims to introduce genomic prediction of breeding values for selection (genomic selection; GS) in red clover for the improvement of forage yield, nutritional quality, host plant resistance and persistence. This project involves three departments at the Swedish University of Agricultural Sciences (SLU), Lantmännen Lantbruk, Lantbrukarnas Riksförbund (LRF) and Hushållningssällskapet Sjuhärad. The project began in July 2019 and its ongoing phase lasts until June 2024. In this project, 600 accessions of red clover comprising parts of the Scandinavian collection at NordGen and breeding populations and cultivars at Lantmännen and other relevant genotypes will be used for field trials at four sites across Sweden, starting in 2020. These accessions will be genotyped using genotyping-by-sequencing (GBS) method. Phenotyping in both the field and laboratory will be conducted for three consecutive years. GBS based genome-wide DNA markers and phenotypic data will be used for a genome-wide association study (GWAS) to identify genes and DNA markers associated with the target traits. A core set of such markers representing genetic diversity across the red clover genome (including those found through GWAS) will be used for the development and validation of genomic prediction models for use in a red clover GS-based breeding program.

Background

Sweden has over three million livestock and its production is among the best globally in terms of animal welfare. Maintaining such a high-quality livestock production requires continual improvements in forage production. Red clover (*Trifolium pratense*; Figure 1) is the most utilized forage legume in the country and plays a vital role in livestock agriculture. It is grown throughout the country, usually in association with grasses (Öhberg 2008). For example, 805 500 hectares of land were covered by ley in Sweden in 2016, of which 622 300 and 183 100 hectares were conventionally and organically grown, respectively (Statistics Sweden, 2017). During 2016, red clover was grown on 60% and 72% of conventionally and organically cultivated leys, respectively, indicating that red clover is a component of about two-third of the leys grown across Sweden.

Figure 1. Red clover at flowering stage. Original photos by Desirée Börjesdotter (left and middle) and Helena Holmkrantz (right).

In nature, red clover is a diploid species ($2n=2x=24$ chromosomes), but tetraploid red clover cultivars ($2n=4x=48$) have been bred through polyploidization and are under cultivation in Sweden for several decades. Both diploid and tetraploid red clover cultivars are currently grown in Sweden and both have late and medium-late flowering types. It exhibits a so-called homomorphic gametophytic self-incompatibility (GSI) and hence is outcrossing (Riday and Krohn 2010). As an efficient nitrogen-fixing legume, red clover contributes to the availability of nitrogen in the soil for subsequent crops (Tayler and Smith 1979). Hence, incorporating red clover in leys offers various advantages when compared with leys without forage legumes, including increased protein content of the forage and increased soil fertility as well as carbon capture. This is particularly important in organic farming where artificial fertilizers are not used.

Red clover is generally a short-lived perennial, and is harvested for only two or three years after sowing, most commonly with three harvests per year but both two and four cut systems are used (Linda Öhlund, Lantmännen, Svalöv, Sweden). Persistence is considered as a highly important trait in red clover; and it is highly desirable to develop cultivars that can persist several years, with stable yield and appropriate nutritional content. However, this is usually not the case as plants frequently die during the winter due to biotic and abiotic factors. Clover rot, caused by the fungus *Sclerotinia trifoliorum*, which attacks the plant during winter, is one of the major factors affecting the persistence of red clover. It not only leads to reduced persistence but also to decreased forage yield, as it kills some plants and weakens others (Öhberg et al. 2008).

Improvements in forage yield and quality are major targets in red clover breeding programs, and rapid biomass production without a significant reduction in nutritional quality is highly desirable. Significant variation exists in these traits among red clover cultivars and breeding populations. For example, Tucak et al. (2013) reported a dry matter yield (DMY) that varied from 14 to 26 t ha^{-1} within their study material originating from several countries in Europe including Sweden. Research has also shown significant genetic variation for protein degradability in red clover forage. There were highly significant variations in protein degradation rates and rumen undegradable protein among red clover germplasm (Broderick et al. 2004), thereby indicating the potential to develop red clover cultivars with improved protein utilization in ruminants through the application of modern plant breeding methods. Like in other crops, modern plant breeding approaches, such as genomic prediction of breeding values (GEBV) selection (or GS for short), and gene editing provide great opportunities for improvements of forage crops (Capstaff and Miller 2018). Hence, GS should be applied in red clover breeding programs to improve various desirable traits. However, the current forage breeding program at Lantmännen is conventional, in which new cultivars are developed through crossbreeding methods with subsequent release of synthetic populations. This process is time consuming, as it takes about 18 years from the initial cross to the approval of a new cultivar (Linda Öhlund; Lantmännen, Svalöv, Sweden). Hence, introducing modern plant breeding methods is vitally important to increase the efficiency of forage breeding programs.

Vision and goal

The project envisions the improvement of red clover through the use of modern plant breeding methods that involve dense-DNA markers and improved phenotyping methods. This will be realized through establishing a new and effective breeding method (GS) for the improvement of various desirable traits in red clover, and thereby contributing to efficient and sustainable meat and milk production that in turn contribute to the growth of Sweden's bio-based economy.

By introducing genomic selection (GS) in red clover breeding, we aim to improve not only forage yield but also nutritional quality, host plant resistance and persistence.

Materials and methods

This project targets medium-late and late flowering diploid as well as medium-late tetraploid red clover types. After evaluating available data on the Scandinavian red clover collection at NordGen (Table 1), breeding populations and cultivars at Lantmännen, 600 accessions comprising late and medium-late flowering diploids and medium-late flowering tetraploids have been selected for field trials at four sites in Sweden starting from 2020. These accessions will be genotyped by LGC Genomics using the genotyping-by-sequencing (GBS) method with Illumina NovaSeq 6000 and NextSeq 550. GBS is a highly efficient next-generation DNA sequencing method for simultaneous discovery of new SNP markers and genotyping of genetic material under study (Baird et al. 2008; Elshire et al. 2011). Each accession will be represented by 200 individual plants that will be pooled for DNA extraction, followed by GBS-based genotyping. Hence, data analysis will be based on genome-wide allele frequency profiles within each accession. GBS data analysis will be conducted using an already published red clover draft genome sequence (De Vega et al 2015) as a reference. Genotypic data will be used for genetic diversity analyses to estimate the overall genetic variation within the

Table 1. Country of origin, and germplasm type of 512 Scandinavian and eight Russian red clover accessions at NordGen.

Origin	No. acc.	I	B	CV	L	P	W	SW	ACC	TEM
Sweden	227	0	2	43	59	2	115	6	204	23
Norway	141	0	6	5	21	3	98	8	137	4
Finland	107	0	0	6	84	0	12	5	103	4
Denmark	37	6	4	25	0	1	1	0	34	3
Russia	8	0	0	0	0	0	8	2	8	0
Total	**520**	**6**	**12**	**79**	**164**	**6**	**234**	**21**	**486**	**34**

No. acc. = number of accessions; I = not defined; B = breeding and research material; CV = advanced cultivar; L = traditional cultivar/landrace/locally cultivated material; P = new and unverified material; W = wild; SW = semi-wild; ACC = accepted for long-term storage; TEM = accepted for short-term storage. Source: https://www.nordgen.org

Scandinavian red clover gene pool. Population structure and kinship among the accessions will also be analyzed, as they may have significant effects in marker-trait associations.

Phenotyping of the accessions at the four sites will be conducted for three consecutive years. Traits that will be studied include forage yield (both green mass and dry matter), clover rot resistance, persistence and forage quality. Traits that will be studied as part of nutritional quality will include the concentrations of crude protein, water-soluble carbohydrates, neutral detergent fiber (NDF), indigestible NDF (iNDF), and metabolizable energy. The phenotypic and genotypic data will be used for a genome-wide association study (GWAS) through the application of statistical models that incorporate kinship and population structure (Sul et al. 2016) to identify genes and DNA markers associated with the target traits.

A core set of genetic markers representing genetic diversity across the red clover genome including those that are known to be associated with various desirable traits will be used for the development and validation of genomic prediction models. Different models of Genomic Best Linear Unbiased Prediction (GBLUP) and other methods will be used for estimating GEBVs for each target trait. The accuracy of different prediction models will be assessed through various cross-validation schemes.

Discussion

This project introduces GS for red clover breeding in Sweden. GS is a relatively new plant breeding method where large number of dense-DNA markers are used to predict the genetic merit of individuals or breeding populations. GS is particularly suitable for the improvement of desirable traits governed by many genes, where marker-aided selection based on significant linkage between marker and trait is inefficient. GS is generally implemented through the development of genomic prediction models based on genotyped and phenotyped individuals or populations (training set), estimation of GEBVs of these individuals or populations, which are only genotyped in a validation set, and further used for selection of desirable genotypes in the breeding population.

Through the application of GS, candidate cultivars with superior traits such as forage yield, nutritional quality as well as host plant resistance and improved persistence can be identified early in the breeding process. With GS, the need for time-consuming field trials will decrease, both the breeding program and the breeding cycle will be shortened, and selection intensity may increase.

The main project outputs will be a customized genotyping array and newly developed and validated prediction models for GS, which will be further utilized to design a new modern red clover breeding program specifically adjusted to the Swedish germplasm and conditions. Overall, this will lead to new cultivars with superior traits, contributing to a profitable forage production across Sweden.

Project participants and collaborations

This project is coordinated by SLU Associate Professor Mulatu Geleta and is being implemented through collaboration of researchers at three Departments of SLU [Department of Plant Breeding (MG, Professor Rodomiro Ortiz and Dr. Cecilia Gustafsson), Department of Agricultural Research for Northern Sweden (Professor David Parsons) and Department of Animal Environment and Health (Associate Professor Elisabet Nadeau)], Lantmännen Lantbruk (Linda Öhlund; Plant Breeder), Hushållningssällskapet Sjuhärad (EN) and Lantbrukarnas Riksförbund (Amanda Andersson). A new licentiate student is being recruited and will be actively engaged in different aspects of the project including field trials, phenotyping and genotyping and data analysis.

There will be a close collaboration between this project and another SLU-Grogrund funded project on new breeding methods for timothy grass, as red clover and timothy are the two major outcrossing forage crops that share a perennial growth habit. We also collaborate with an SLU Grogrund project working on host plant resistance to various pathogens as well as other on-going projects on red clover. Genomic resources and tools generated in these projects will be utilized in our project.

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Sammanfattning

Rödklöver, en av våra viktigaste foderväxter, spelar en avgörande roll svensk animalieproduktion. För närvarande tillämpar förädlingsprogrammen i rödklöver på Lantmännen endast konventionella metoder, där nya sorter utvecklas genom korsningsförädling för att senare marknadsföras som syntetiska populationer. Denna process är tidskrävande och det

tar cirka 18 år från den första korsningen till godkännandet av en ny sort. Därför skulle införandet av moderna förädlingsmetoder vara avgörande för att öka effektiviteten. Projektet ”Genomiskt urval i rödklöver (*Trifolium pratense*)”, finansierat av SLU Grogrund, syftar till att införa genomiskt urval (GS) i rödklöver för att förbättra foderutbytet, protein- och fiberkvaliteten, samt sjukdomsresistens och uthållighet. Projektet omfattar tre avdelningar vid Sveriges lantbruksuniversitet (SLU), Lantmännen Lantbruk, Lantbrukarnas riksförbund (LRF) och Hushållningssällskapet Sjuhärad. Projektperioden sträcker sig från juli 2019 till juni 2024. Från och med 2020 kommer 600 olika accessioner (genotyper) av rödklöver, både ur samlingen på NordGen och avelspopulationer vid Lantmännen, att användas för fältförsök på fyra platser i hela Sverige. Accessionerna kommer att vara genetiskt beskrivna (genotypade) med hjälp av en sekvenseringsmetod (GBS). Fenotypning på både fält- och laboratorienivå kommer att genomföras under tre år i rad. Särskilda GBS-baserade DNA-markörer och fenotypiska data kommer att användas för en s.k. *genome-wide association study* (GWAS) för att identifiera gener och genetiska markörer som är associerade med de studerade egenskaperna. En utvald uppsättning av genetiska markörer som representerar den genetiska mångfalden över hela rödklövergenomet, inklusive dem som identifierats genom GWAS, kommer att användas för att utveckla och bekräfta genomiska prediktionsmodeller för senare användning av ett GS-baserat förädlingsprogram i rödklöver.

Mulatu Geleta Dida är docent vid SLU, Inst. för växtförädling (Alnarp)
mulatu.geleta.dida@slu.se

Cecilia Gustafsson vid SLU, Inst. för växtförädling (Alnarp)
cecilia.gustafsson@slu.se

Elisabet Nadeau är forskningsledare vid SLU, Institutionen för husdjurens miljö och hälsa (Skara)
elisabet.nadeau@slu.se

Rodomiro Ortiz är professor i växtförädling vid SLU, Inst. för växtförädling (Alnarp)
rodomiro.ortiz@slu.se

David Parsons är professor vid SLU, Inst. för norrländsk jordbruksvetenskap (Umeå)
david.parsons@slu.se

Amanda Andersson är verksamhetsansvarig vid LRF Växtodling
amanda.andersson@lrf.se

Linda Öhlund är foderväxtförädlare vid Lantmännen Lantbruk
linda.ohlund@lantmannen.com