




# Trait characterization of genetic resources reveals useful variation for the improvement of cultivated Nordic red clover

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## Abstract

Red clover (*Trifolium pratense* L.) is the most important forage legume in the Nordic region, but its utilization is limited by poor persistency. The improvement of cultivated red clover can potentially take advantage of the numerous wild populations and landraces conserved in gene banks; however, there is often limited information available on the phenotypic and genetic characteristics of this material. We characterized 48 populations conserved at NordGen for a number of traits and compared them to commercial cultivars. The material was evaluated in field trials at four locations over two years and in an experiment under controlled conditions. Considerable variation was identified, with stem length, growth type and flowering date having the highest broad sense heritabilities. Traits related to plant size were strongly associated with late flowering and upright growth and differed between landraces/cultivars on the one hand and wild populations on the other. There was a large genotype by environment interaction on winter survival, which only partially correlated with freezing tolerance under controlled conditions. A majority of gene bank accessions exceeded the commercial cultivars in winter survival and freezing tolerance and can therefore be a genetic resource for future improvement of these traits. The phenotypic variation among accessions was associated with two main axes of climatic variation at the collection site. Petiole length of young plants under controlled conditions as well as plant size in the field increased with increasing summer temperature and decreasing summer precipitation, while number of leaves and an apparent vernalization requirement, recorded under controlled conditions, increased with decreasing annual and winter temperature. We discuss the implications these results have for collection, conservation and utilization of red clover genetic resources in the Nordic region.

## KEYWORDS

breeding, canonical correlation analysis, freezing tolerance, local adaptation, *Trifolium pratense*, winter survival

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## 1 | INTRODUCTION

Agriculture in large parts of Sweden, Finland, Norway and Iceland is grassland-based because the cold climate, short growing season, shallow soils and/or steep slopes make the land less suitable for other crops. Due to the warming effect of the North Atlantic Current this region has, however, a mild climate compared to other regions at the same latitudes (Helgadóttir et al., 2014). The unique combination of annual cycles of photoperiod and temperature necessitates the availability of cultivars of grassland species that are particularly adapted to this region. Temporal grasslands that are regularly re-sown cover 48%, 40% and 28% of the total agricultural area in Norway, Sweden and Finland, respectively (Helgadóttir et al., 2014; Statistics Sweden, 2013; Steinshamn et al., 2016). In Iceland, most of the agricultural area is covered by grasslands, but the majority of this is natural grasslands (Helgadóttir et al., 2014). The Nordic region is, like the rest of Europe, heavily dependent on imports of protein-rich biomass for feed and food (De Visser et al., 2014; Voisin et al., 2014). Greater cultivation of forage legumes can help reduce this protein deficit, and further increase sustainability, as their ability to make use of atmospheric  $N_2$  through symbiotic  $N_2$  fixation reduces the requirement for nitrogen fertilizer in the cropping system (Jensen et al., 2012; Lüscher et al., 2014; Reckling et al., 2016).

Red clover is the most important forage legume cultivated in northern Europe (Annicchiarico et al., 2015), including the Nordic region, where it is cultivated almost exclusively in mixtures with grasses over a wide range of climatic conditions (Helgadóttir et al. 2014; Steinshamn et al., 2016). Like most perennial forages, red clover is a cross-pollinated species with a high level of genetic variation within populations. It is thought to have originated near the Mediterranean basin (Boller et al., 2010). Domesticated red clover from Spain spread to other parts of Europe and reached the Nordic countries in the 18th century (Kjærgaard, 2003). As the use of red clover spread across Europe, a common practice was to re-sow seed harvested from a restricted area, often within a single farm. This led to the development of locally adapted landraces as a result of semi-conscious selection by the farmers, who exploited the natural selection by producing seeds from the surviving plants in the field (Boller et al., 2010). The use of landraces in Europe declined around 1970 with the increased use of highly productive cultivars developed by modern breeding (Boller et al., 2010). In the Nordic region, red clover breeding was first initiated in Sweden in the beginning of the last century, with several cultivars produced (Olsson, 1997). The first Finnish red clover cultivar 'Tammisto' was released in 1948, prior to which mainly Swedish cultivars were used in Finland (Valle, 1958). Although there was red clover breeding in Norway from the middle of the last century, the landrace 'Moldstad' dominated until the 1980s (Vestad, 1990). In Iceland, cultivars bred in other Nordic countries are cultivated. Current red clover breeding in Norway, Sweden and Finland is based mostly on progeny testing and phenotypic selection of full-sib families, sometimes combined with phenotypic mass selection, followed by creation of synthetic populations. Population improvement through recurrent phenotypic selection is also used.

Persistency is an important breeding goal, along with dry matter yield, disease resistance, seed yield and forage quality. Phenotypic evaluation and selection for persistency is complex, since many biotic and abiotic factors affect persistency (Annicchiarico et al., 2015; Ergon et al., 2019; Herrmann et al., 2008). Throughout the Nordic region, the main reason for limited persistency is the various stresses which affect the plants during the winter (Helgadóttir et al. 2014). Given the environmental variability and limited seed market in the Nordic region, the development of cultivars with wide adaptation is desired (Helgadóttir et al., 2000). Cultivars with wide adaptation will be even more important considering the high variability in the climatic conditions expected in the future (Ergon et al., 2018; Rapacz et al. 2014).

The genetic resources found in landraces and wild populations are generally underutilized in plant breeding, both in the Nordic countries and in the rest of Europe (Brozynska et al., 2016; Rognli et al., 2013). Red clover is distributed all over the Nordic countries, but rarely in the alpine regions (Mossberg & Stenberg, 2012). Wild populations have probably either naturalized from cultivated populations or are the result of hybridization between wild and cultivated populations (Daugstad, 2016). In addition to over 100 red clover cultivars, NordGen (the Nordic Genetic Resource Centre) currently holds over 400 other red clover accessions in its collection. These accessions may be a source of variation for traits that are lacking or need improvement in cultivated material, such as superior winter survival or resistance to specific abiotic or biotic stresses. However, introducing such material in a breeding programme is challenging. It is likely to carry undesirable traits, most notably low biomass yield, which needs to be enhanced through subsequent breeding. The various accessions are poorly characterized, so identification of the most suitable ones is difficult. While molecular marker data may be useful for germplasm characterization (Greene et al., 2004; Kölliker et al., 2003; Pagnotta et al., 2011), characterizing the phenotypic variation in agronomically relevant traits is particularly valuable for plant breeding and germplasm conservation, since selectively neutral molecular genetic variation tend not to coincide with phenotypic variation (Riday, 2010; Rognli et al., 2013). Based on the premise that traits displayed by an accession reflect the selection pressures of the environment from which it originates, climatic variables at the collection sites can be used to predict the phenotype of gene bank accessions, helping breeders and gene bank managers to more effectively seek germplasm with specific phenotypic characteristics (e.g. Endresen et al., 2011; Hijmans et al., 2003; Khazaei et al., 2013). Adding high-density genomic data to the analyses can help identify valuable germplasm and be used in development of selection tools for breeding (Blanco-Pastor et al. 2020).

Large genetic variation for morphological and performance traits has been described in red clover material of various origin (Annicchiarico & Pagnotta, 2012; Dias et al., 2008; Greene et al., 2004; Kouamé & Quesenberry, 1993; Pagnotta et al., 2011; Rosso & Pagano, 2005; Tuck et al., 2013). However, only a few studies have evaluated red clover material of Nordic origin. Helgadóttir et al. (2000) evaluated yield and persistency of 13 cultivars of red

clover in pure stand and in mixture with timothy across five Nordic locations, while Solberg et al. (2015) characterized morphology and development of 16 Norwegian accessions from NordGen, grown as single plants in a greenhouse. The aim of the present study was to evaluate accessions of red clover in the Nordic collection at NordGen and compare them to commonly used cultivars in the region. We present results from spaced plant trials carried out at four locations across the Nordic countries, as well as from an experiment under controlled conditions, and describe i) variance components and broad sense heritability estimates, ii) correlations between traits and iii) relationships between phenotypic traits and climate variables at the collection sites.

## 2 | MATERIAL AND METHODS

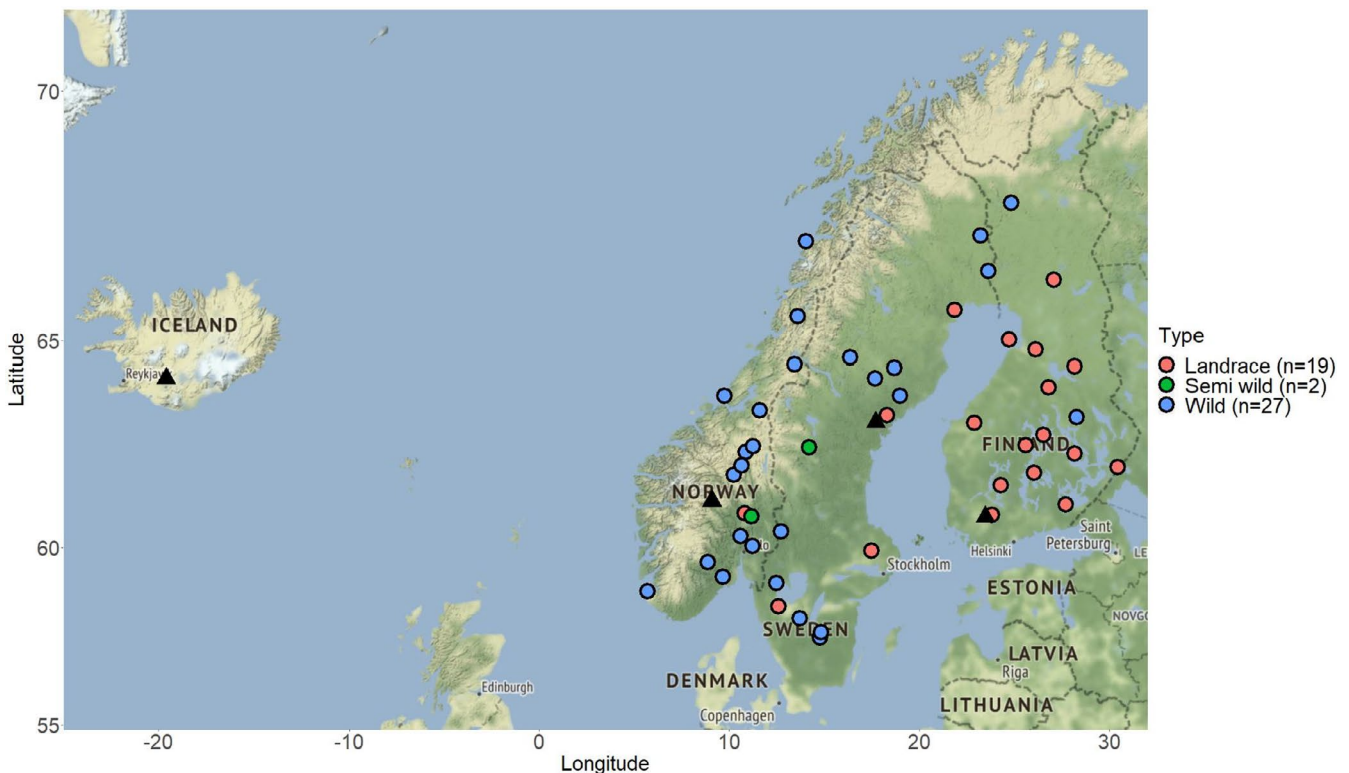
### 2.1 | Plant material

A total of 48 accessions of red clover were selected from the NordGen collection, representing the geographical distribution of the species in the Nordic region (Figure 1, Supplementary Table 1). This included 19 landraces, 2 semi-wild and 27 wild populations. The majority of Finnish populations in the collection are landraces, while the majority of Norwegian populations are wild, resulting in an uneven geographical distribution of population types. In addition, the commercial cultivars Lea, Linus and Gandalf (Graminor, Norway), SW Ares and SW Yngve (Lantmännen, Sweden) and Saija (Boreal,

Finland) were included in the experiment under controlled conditions, while Lea, SW Yngve and Saija were included in the field trials.

### 2.2 | Field trials

Spaced plant trials were established at Jokioinen, Finland (60°48'N, 23°29'E, 115 m a.s.l.), Korpa, Iceland (64°09'N, 21°84'W, 35 m a.s.l.), Løken, Norway (61°12'N, 9°06'E, 545 m a.s.l.) and Lännäs, Sweden (63°08'N, 17°43'E, 25 m a.s.l.) (Figure 1). Data describing the climate during the experiment (summer 2014 - spring 2016) at each location are presented in Supplementary Table 2. Plants were raised from seed in a greenhouse in spring 2014 and transplanted into bare ground during summer or early autumn at all locations except at Korpa, where plants were transplanted into a field which had been sown with a mixture of timothy (*Phleum pratense* L.), cv. Snorri, and smooth meadow grass (*Poa pratensis* L.), cv. Kupol. The different experimental layout at Korpa was implemented to avoid the very high winter mortality of red clover planted into bare ground seen in previous experiments at this location. Strong competition from the grass species was avoided by applying only a low level of N fertilizer in the sowing year. The experimental design was a complete randomized block with four replicates. Populations formed plots within each block, each made up of a group of five different genotypes. Each population was thus represented by 20 genotypes in total. The distance between individual plants within and between plots was 1 m. The plants were phenotyped for the following traits: autumn status



**FIGURE 1** Collection sites of the landraces (19), semi-wild (2) and wild (27) red clover populations (colored coded), and test locations where the field trials were conducted (black triangles)

in 2014; leaf area, stem length, flowering date, flowering score and yield score in 2015; winter survival and spring status in 2015 and 2016. A detailed description of all traits recorded is provided in Supplementary Table 3.

## 2.3 | Experiment under controlled conditions

Seeds were scarified with sandpaper, sown in a peat soil in early January and germinated in a greenhouse (59°40' N, 10°47' E) at 16°C with natural light supplied with additional metal halide lamps (approximately 100–125  $\mu\text{mol}/\text{m}^2\text{s}^{-1}$  PAR) to a photoperiod of 12 hr. After germination, individual young seedlings were transplanted into pots (28  $\text{cm}^3$ ) filled with peat soil and grown under the same conditions. When the plants were six weeks old, the number of leaves and the length of the longest petiole was recorded, and the plants were moved to cold acclimating conditions at 3–4°C, 12 hr photoperiod and 110  $\mu\text{mol}/\text{m}^2\text{s}^{-1}$  PAR, for two weeks. For determination of freezing tolerance (LT50; temperature required to kill 50% of the plants), cold acclimated plants were exposed to four different test temperatures. Six plants of each population were placed in a programmed freezing chamber initially set at 2°C. The temperature was first lowered from 2°C to –3°C at 1  $^\circ\text{C h}^{-1}$  and kept at this level for 12 hr to ensure even freezing, after which the temperature was lowered again by 1  $^\circ\text{C h}^{-1}$  down to the test temperature; –8°C, –11°C, –14°C or –17°C. When the temperature reached the test temperature, it was kept there for one hour before the temperature was raised, again by 1  $^\circ\text{C h}^{-1}$ , up to 2  $^\circ\text{C}$ . After thawing, the pots were returned to the greenhouse and kept there for three weeks, before survival of each plant was recorded. The experiment was repeated three times, resulting in approximately 18 plants per population and test temperature. The data from the three experiments were pooled before LT50 was calculated by probit analysis using PROC PROBIT in SAS 9.2 (SAS Institute, Inc., Car, NC, USA). For determination of timing of reproductive development, 9–10 non-acclimated seedlings of most populations were transplanted into 1-L pots with peat soil (one plant per pot) and kept in the greenhouse (conditions as above, but temperature occasionally exceeding 16  $^\circ\text{C}$ ) until they were approximately 3 months old. The photoperiod was then increased to 23 hr, and the number of days until the beginning of stem elongation was recorded. Stem elongation is the earliest sign of reproductive development in red clover, and we defined days to elongation as the number of days at 23 hr photoperiod until the longest internode measured at least 2 cm. Recording was done three times a week until 57 days had passed.

## 2.4 | Data analysis

Variance component analyses of the field data were conducted using the residual maximum likelihood (REML) procedure in DeltaGen as described by Jahufer and Luo (2018). The linear mixed model used for analysis was:

$$Y_{ijm} = M + g_i + l_j + (gl)_{ij} + b_{jm} + \varepsilon_{ijm}$$

where  $Y_{ijm}$  is the value of a trait measured in population  $i$  in replicate  $m$  at location  $j$ ;  $M$  is the overall mean;  $g_i$  is the random effect of population  $i$ ,  $N(0, \sigma_g^2)$ ;  $l_j$  is the fixed effect of location  $j$ ;  $(gl)_{ij}$  is the random effect of the interaction between population  $i$  and location  $j$ ,  $N(0, \sigma_{gl}^2)$ ;  $b_{jm}$  is the random effect of replicate  $m$  within location  $j$ ,  $N(0, \sigma_b^2)$ ; and  $\varepsilon_{ijm}$  is the residual effect for population  $i$  in replicate  $m$  at location  $j$ ,  $N(0, \sigma_\varepsilon^2)$ . The statistical significance of the variance components was further assessed using the likelihood ratio test. An estimate of entry-mean broad sense heritability ( $h_b^2$ ) (Falconer, 1989, hereafter referred to as just heritability), of the considered traits, was calculated by selecting the heritability option provided in DeltaGen (Jahufer & Luo, 2018), using the following formula:

$$h_b^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{gl}^2}{n_l} + \frac{\sigma_\varepsilon^2}{n_l n_b}}$$

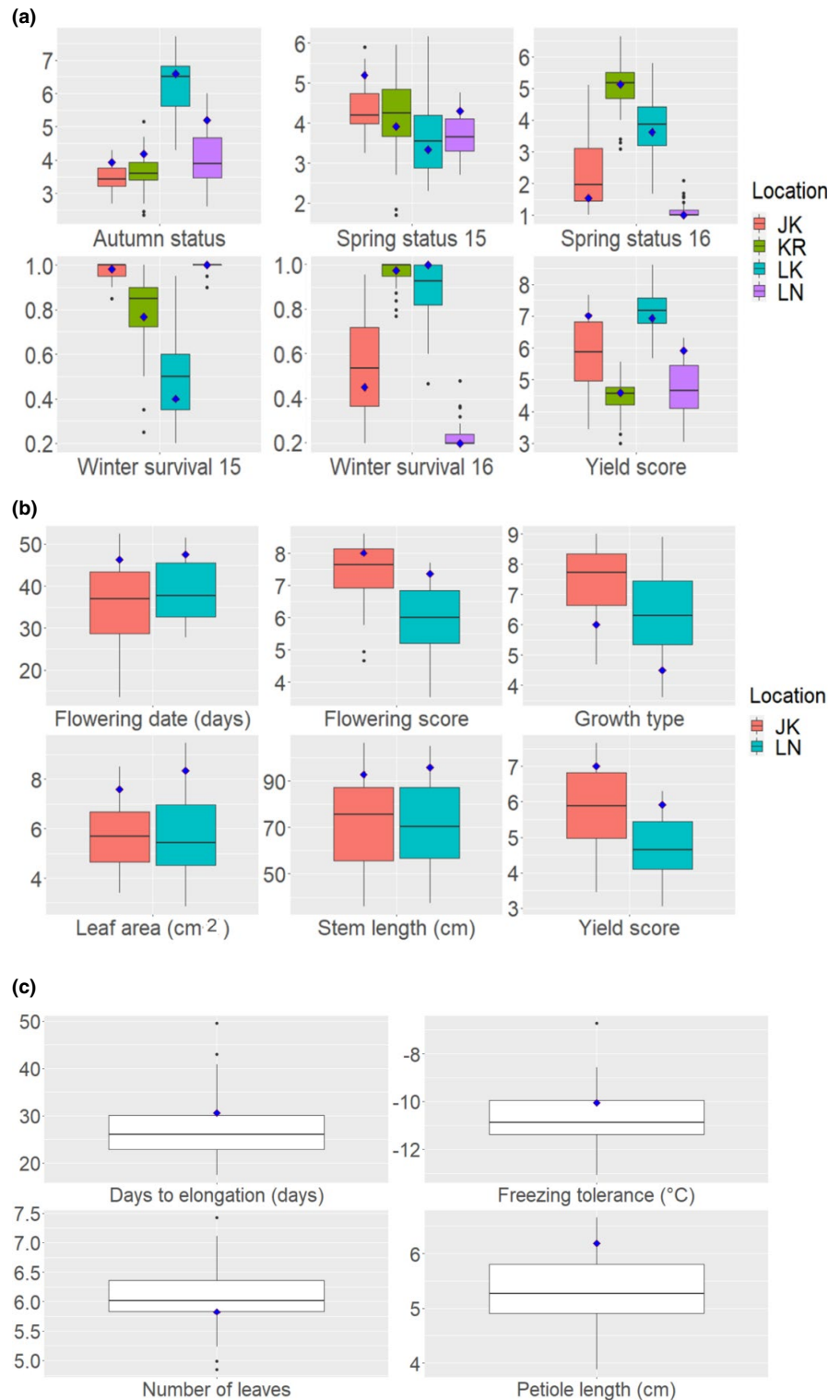
where  $\sigma_g^2$ ,  $\sigma_{gl}^2$  and  $\sigma_\varepsilon^2$  are the population, population by location and pooled error variance components and  $n_l$  and  $n_b$  are the number of locations and blocks per location, respectively. Genotypic correlations were estimated using the MANOVA option in DeltaGen (Jahufer & Luo, 2018) using a model which included the random effects of population, location and block within location for the correlation among different traits, and the random effects of population and block within location for the correlation of single traits among locations. These are genotypic and not genetic correlations (according to Falconer, 1989) because our experimental design did not allow the estimation of the additive genetic component of variance. Phenotypic correlations were calculated on averaged data from each population and location using the rcorr function (Harrell, 2020) in RStudio version 1.1.463 (RStudio: Integrated Development for R. RStudio, Inc., Boston).

A canonical correlation analysis (CCA) was performed to test the association of each of the phenotypic traits (population means) recorded, with variables describing the climate at the collection sites of the populations. Annual mean temperature, temperature seasonality, maximum temperature of the warmest month, minimum temperature of the coldest month, mean temperature in the warmest quarter, mean temperature in the coldest quarter, annual precipitation, precipitation seasonality, precipitation in the warmest quarter and precipitation in the coldest quarter at the place of origin of each of the 48 populations from the NordGen collection were retrieved from the Worldclim database (Worldclim.org) using the diva-gis software (diva-gis.org). In addition, we used latitude and altitude as descriptors of climate variation. Altitude and historical climate data for the time period 1950–2000 were obtained with 2.5 arcosec resolution (the highest resolution available with this software), corresponding to an area of 4.5 x 4.5 km. More information on the climatic variables used is provided in Supplementary Table 4. The CCA was performed using the function cca of the R package vegan (Oksanen et al. 2019) in RStudio

version 1.1.463. The squared canonical correlation coefficients were used to assess the proportion of the total variation explained by each canonical variable. The canonical variable loadings express the magnitude of each of the original trait's correlation with the derived canonical trait variables, and the original climate variable's correlation with the derived canonical climate variables (Vicario et al., 1989).

### 3 | RESULTS

Almost all plants established successfully at all locations, and there was both a significant variation among populations, and a relatively strong population by location interaction (GxE) on autumn status in the establishment year (Supplementary Table 5). Winter survival varied markedly between locations and years (Figure 2a). In



**FIGURE 2** Boxplots showing the variation in traits among Nordic red clover populations included in this study. (A), Autumn status, spring status, yield score (all on a scale from 1 to 9) and winter survival (as a proportion from 0 to 1) recorded at four locations; (B), traits recorded during the growing season at the two locations with good winter survival, and thus good representation of all populations (flowering score, growth type and yield score on a scale from 1 to 9; for growth type higher values mean more prostrate growth); (C), traits recorded in an experiment under controlled conditions. Data were averaged for each population prior to plotting. The blue dots correspond to averages of the cultivars included. Trait descriptions are given in Supplementary Table 3. JK, Jokioinen; KR, Korpa; LK, Løken; LN, Lännäs

the spring of 2015, a very good survival was observed at Jokioinen and Lännäs (an average of all populations of 98%–99%), while it was lower at Korpa (80%) and Løken (49%). During the next winter, the survival rate among the remaining plants was higher at Løken (86%) and Korpa (96%), while it was low at Jokioinen (44%) and almost zero at Lännäs. There was significant GxE for spring status and winter survival in 2015, and no significant variance between populations for these traits (Supplementary Table 5). For the yield score at the first cut in 2015, there was significant variation among populations and significant GxE. The heritability for yield score and autumn status was 0.45 and 0.52, respectively. Genotypic correlations between trait values at the different locations showed that the GxE was largely due to different responses of the populations to the growing conditions at Korpa compared to the other locations, as well as different responses to the winters at Korpa, Løken and Jokioinen/Lännäs (Table 1). The different behaviour of populations at Korpa was also evident from the fact that the proportion of plants that flowered during the 2015 growing season was considerably lower (35%) than at the other locations, where all plants flowered.

**TABLE 1** Genotypic correlation coefficients ( $p < .05$ ) for autumn status, spring status and yield score in 2014–2015 recorded in single plant trials with 51 Nordic red clover populations at four test locations. See Supplementary Table 3 for a description of the traits

Autumn status 2014	Jokioinen	Lännäs	Løken
Lännäs	0.70	-	
Løken	0.60	0.67	-
Korpa	0.22	0.01	0.18
Spring status 2015	Jokioinen	Lännäs	Løken
Lännäs	=0.64	-	
Løken	-0.13	-0.44	-
Korpa	0.20	0.39	0.14
Yield Score 2015	Jokioinen	Lännäs	Løken
Lännäs	0.80	-	
Løken	0.11	-0.25	-
Korpa	-0.04	-0.01	0.43

**TABLE 2** Genotypic correlation coefficients ( $p < .05$ ) and entry-mean broad sense heritability estimates ( $p < .05$ ) for the traits expressed during the growing season in single plant trials with 51 Nordic red clover populations at the two test locations that were not affected by winter mortality during the first winter (Jokioinen and Lännäs). See Supplementary Table 3 for a description of the traits

	Autumn status	Spring status	Flowering date	Flowering score	Growth type	Leaf area	Stem length	Yield score
Spring status	0.92	-						
Flowering date	0.84	0.76	-					
Flowering score	0.83	0.86	0.89	-				
Growth type	-0.96	-0.88	-0.88	-0.83	-			
Leaf area	0.96	0.89	0.88	0.79	-0.96	-		
Stem length	0.91	0.87	0.96	0.93	-0.95	0.92	-	
Yield score	0.90	0.90	0.90	0.95	-0.92	0.90	0.97	-
$h_b^2$	0.45 ± 0.10	0.47 ± 0.08	0.89 ± 0.02	0.69 ± 0.06	0.89 ± 0.02	0.65 ± 0.06	0.94 ± 0.01	0.75 ± 0.05

### 3.1 | Traits expressed during the growing season

Due to winter mortality and subsequent missing data, we based further analysis of traits expressed during the growing season on data collected after the non-selective winters at Jokioinen and Lännäs in 2015, in addition to autumn status at those locations in 2014. There was significant GxE for all traits except growth type, but the variance between populations was considerably larger than GxE for all traits except autumn and spring status (Supplementary Table 6, see also Figure 2b). The highest entry-mean broad sense heritabilities were estimated for stem length, growth type and flowering date (Table 2). The genotypic correlations between the different traits were all moderately high to high, with absolute correlation coefficients ranging from 0.76 to 0.97 (Table 2). Erect growth habit and late flowering were associated with high values for all growth and size-related traits. These traits were also positively correlated with petiole length (e.g.  $r = 0.80$  for yield score), and somewhat negatively correlated with number of leaves, recorded in the experiment under controlled conditions (Supplementary Table 7). Notably, the number of days to elongation recorded in the experiment under controlled conditions was not significantly correlated with flowering date in the field.

Wild/semi-wild populations had a more prostrate growth type, earlier flowering and generally smaller plant size on average than landraces and cultivars, and cultivars did not outperform the best landraces in yield score (Table 3, Figure 2b).

### 3.2 | Winter survival and spring status after selective winters

To focus on informative data sets, and avoid many missing values, analysis of variation in winter survival was based on the first selective winter at Korpa, Løken and Jokioinen (2015, 2015 and 2016, respectively). Winter survival and spring status were strongly correlated within all three data sets ( $r = 0.95$ – $0.98$ ). Heritability for winter survival and spring status was very low (Supplementary Table 8). While there was a moderate genotypic correlation between winter survival at Løken and Jokioinen ( $r = 0.55$ ), winter survival at Korpa

**TABLE 3** Analysis of variance to assess the difference between population types of the Nordic red clover populations used in the field trials (A) and in the controlled experiment (B). Group means followed by different letters are significantly different following the Tukey's test for multiple groups comparisons

A				
Trait	Cultivars (n = 3)	Landraces (n = 19)	Wild (n = 29)	P-value
Autumn status	4.57 <sup>a</sup>	4.10 <sup>a</sup>	3.49 <sup>b</sup>	***
Spring status 15	4.75 <sup>a</sup>	4.20 <sup>a</sup>	3.81 <sup>b</sup>	**
Flowering date	46.92 <sup>a</sup>	43.19 <sup>a</sup>	32.47 <sup>b</sup>	***
Flowering score	7.69 <sup>a</sup>	7.13 <sup>a</sup>	6.33 <sup>b</sup>	**
Growth type	5.25 <sup>a</sup>	6.16 <sup>a</sup>	7.56 <sup>b</sup>	***
Leaf area	7.98 <sup>a</sup>	6.62 <sup>a</sup>	5.00 <sup>b</sup>	***
Stem length	94.40 <sup>a</sup>	84.10 <sup>a</sup>	62.10 <sup>b</sup>	**
Yield score	6.50 <sup>a</sup>	5.90 <sup>a</sup>	4.81 <sup>b</sup>	***
Winter survival Korpa 2015	0.77	0.84	0.78	NS
Winter survival Løken 2015	0.40	0.45	0.52	NS
Winter survival Jokioinen 2016	0.32 <sup>ab</sup>	0.25 <sup>a</sup>	0.57 <sup>b</sup>	***
B				
Trait	Cultivars (n = 6)	Landraces (n = 19)	Wild (n = 29)	P-value
Days to elongation	30.58	26.51	27.08	NS
Number of leaves	5.83	6.01	6.14	NS
Petiole length	6.19 <sup>a</sup>	5.67 <sup>a</sup>	4.92 <sup>b</sup>	***
Freezing tolerance	-10.06	-10.48	-10.98	NS

\*,  $p < .05$ ; \*\*,  $p < .01$ ; \*\*\*,  $p < .001$ ; NS, not significant

had a weak negative genotypic correlation with winter survival at the two other locations ( $r = -0.38$  and  $-0.23$ , Supplementary Table 8). Similarly, freezing tolerance (i.e. low LT50 value) was only partly correlated with winter survival at Jokioinen in 2016 (phenotypic,  $r = -0.49$ ), and not with winter survival at Løken or Korpa in 2015 (Supplementary Table 7).

Wild/semi-wild populations had significantly better winter survival at Jokioinen than landraces, while cultivars were intermediate. There were no significant differences between population types in winter survival at Løken or Korpa or in freezing tolerance (Table 3). However, the range in population averages of these traits was large, and cultivars were among the poorest 50% (Figure 2b, 2c).

### 3.3 | Relationships between winter survival and traits expressed during the growing season and between traits and the environment at the collection sites

The CCA of the phenotypic traits and the climatic variables resulted in two significant canonical correlations with  $R^2$ -values of 0.83 ( $p < .003$ ) and 0.79 ( $p < .05$ ), respectively. The first canonical variable for the phenotypic data set had the strongest correlation with petiole length recorded under controlled conditions (Table 4).

Flowering time, field traits related to plant size (except autumn status at Korpa) and LT50 were moderately correlated to both canonical variables, but more so to the first than to the second. The number of leaves and days to elongation under controlled conditions, as well as winter survival at all locations, were more correlated with the second canonical variable than with the first. For the climate data set, the first canonical variable had the highest correlations with temperature and precipitation during summer. This variable may partly be explained by the difference between wild populations (largely from Norway) and landraces (largely from Finland) (Figure 1, Supplementary Table 1). Thus, considering the sign of the coefficients, populations from locations with warm summers and low precipitation tended to have long petioles, and to some extent other traits related to large plant size (and vice versa). The second canonical variable had the strongest correlations with mean annual temperature and temperature during winter. Thus, populations originating from locations with low annual temperatures and in particular cold winter temperatures tended to have many leaves, late stem elongation (probably due to a facultative vernalization requirement; see Discussion), good winter survival at Løken and Jokioinen, but low winter survival at Korpa. Interestingly, the correlations of autumn status and winter survival at Korpa had the opposite sign compared to those of the same traits at the other two locations, for both canonical variables. It is

**TABLE 4** Canonical correlation analysis of phenotypic traits and climatic conditions at the collection site of 48 Nordic wild, semi-wild or landrace red clover populations. The correlations between each phenotypic trait and climate variable and their respective first two canonical variables (Can 1 and Can 2). Phenotypic traits recorded in an experiment under controlled conditions were included, together with traits recorded in field trials at four locations. See Supplementary Table 3 for a description of the traits and Supplementary Table 4 for a description of climate variables. When location is not indicated, field traits are averages of data recorded before and after the non-selective winter at Jokioinen and Lännäs in 2014–2015. Correlations in bold are at least twice as strong for the indicated canonical variable than for the other

Phenotype			Climate		
Original variable	Can 1	Can 2	Original variable	Can 1	Can 2
Autumn status	-0.64	0.34	Altitude	0.40	-0.50
Autumn status Korpa	0.18	-0.18	Latitude	0.22	-0.34
Autumn status Løken	-0.61	0.32	Mean annual temp.	-0.19	<b>0.62</b>
Flowering date	-0.68	0.46	Temp. seasonality	-0.35	-0.30
Flowering score	-0.52	0.35	Max. temp. warmest month	<b>-0.73</b>	0.28
Growth type	0.59	-0.50	Min. temp. coldest month	0.06	<b>0.47</b>
Leaf area	-0.64	0.34	Mean temp. warmest quarter	-0.65	0.49
Stem length	-0.66	0.48	Mean temp. coldest quarter	0.06	<b>0.51</b>
Yield score	-0.53	0.42	Annual precipitation	0.57	0.32
Spring status 2015	-0.50	0.31	Precip. seasonality	0.01	<b>-0.15</b>
Winter survival Jokioinen 2016	0.49	-0.61	Precip. warmest quarter	<b>0.58</b>	0.21
Winter survival Korpa 2015	-0.16	<b>0.42</b>	Precip. coldest quarter	0.55	0.31
Winter survival Løken 2015	0.02	<b>-0.40</b>			
Days to elongation	0.11	<b>-0.49</b>			
Number of leaves	0.13	<b>-0.61</b>			
Petiole length	<b>-0.79</b>	0.34			
Freezing tolerance	-0.31	0.21			

also notable that while winter survival at Jokioinen was to some extent correlated with both canonical variables, winter survival at Løken was only correlated to the second variable. These results are in agreement with the strong G×E observed for winter survival and are supported by the variation in correlations between winter survival in the different locations and other traits (Supplementary Table 7 and Supplementary Table 8).

## 4 | DISCUSSION

### 4.1 | Traits expressed during the growing season

Positive correlations between erect growth, plant size, stem length and leaf size have also been found in very diverse material of red clover from different parts of the world (Annicchiarico & Pagnotta, 2012; Greene et al., 2004; Herrmann et al., 2008; Pagnotta et al., 2011; Rosso & Pagano, 2005; Solberg et al., 2015; Tucak et al., 2013). In these studies, erect growth habit and large plants are sometimes associated with early flowering, sometimes with late flowering, probably depending on both the origin of the material, and the conditions at the test site.

Interestingly, the number of days to stem elongation in the controlled experiment was not correlated with flowering time in

the field. Red clover flowers in response to long photoperiods and increasing temperatures; the first visible sign of reproductive development is the initiation of stem elongation. The photoperiod requirement for Nordic red clover is around 16–18 hr (Lunnan, 1989; Van Dobben, 1964). There is little requirement for vernalization in red clover, but in some populations flowering is stimulated by a cold treatment (Fejer, 1960; Van Dobben, 1964). In a previous study of vernalized plants of three Norwegian cultivars, it was found that the temperature requirement for onset of stem elongation was saturated between 10 and 14°C, while the temperature requirement of flower bud initiation was higher (Ergon et al., 2016). Days to elongation in our controlled experiment was measured at 16°C and 23 hr photoperiod. These conditions are likely to have been saturating for the stimulation of elongation. It therefore appears that the variation in timing of stem elongation reflects either variation in a facultative vernalization requirement (as the plants were not vernalized), or variation in loss of juvenility. Variation in flowering date in the field trials in 2015 (older and vernalized plants) probably reflects variation in photoperiod and temperature responses, controlled by other genes. Given the weak requirement for vernalization in red clover, all plants were probably fully vernalized at all locations. Despite this, a large proportion of plants did not flower at Korpa in 2015. Korpa is the most Northern location with the longest photoperiod in summer,



so photoperiod cannot be the reason for this. Thus, the low summer temperature (mean of 9°C) remains as the most likely cause of limited flowering at Korpa. Autumn status was another trait that was expressed differently at Korpa compared to the other three locations. Korpa belongs to a different agroclimatic zone than Jokioinen (Björnsson, 1993; Helgadóttir & Björnsson, 1994). In addition to being the northernmost location, Korpa is characterized by an oceanic climate with cooler and wetter summers than the other locations; this was also the case in 2014 (Supplementary Table 2). Analysis of GxE on yield in perennial ryegrass also showed that populations behaved differently at Korpa compared to other Nordic locations with more continental climates (Helgadóttir et al., 2018). Both the oceanic climate and the rapid shortening of daylength in the fall may have affected growth as well as growth cessation of the various populations differently at Korpa relative to the other locations. Also, at Korpa the red clover single plants were planted in a field previously sown with a mixture of timothy and smooth meadow grass. Although grass growth was limited by low N fertilization, we cannot rule out that clover-grass interactions may have affected the growth of red clover plants belonging to different populations differently.

#### 4.2 | Winter survival and its relationship to other traits

Winter survival at Jokioinen and to some extent at Løken was negatively correlated with plant size. This is in agreement with earlier studies in Italian red clover (Annicchiarico & Pagnotta, 2012) as well as in Nordic white clover populations (Aasmo Finne et al., 2000). It is possible that the populations with more above-ground growth (particularly if they grow late in the autumn) tend to allocate less resources to storage tissues in the tap root and crown. Lack of organic reserves can reduce the ability to survive the winter and regrow the following spring. Thus, the variation we observed may represent the differences between 'competitors', allocating resources to growth and 'stress tolerators', allocating resources to storage and maintenance, as described in Grime's classical plant strategy theory (Grime, 1977). Winter survival is a very complex trait. Winter stresses include freezing, ice encasement, soil movements, waterlogging, drought, fungal pathogens and lack of light (Bélanger et al., 2006; Rapacz et al., 2014). Winter survival depends on various stress resistance mechanisms and is strongly influenced by cold acclimation and growth cessation in autumn, as well as deacclimation and growth resumption in spring (Rapacz et al., 2014). Freezing tolerance, measured under controlled conditions, can be used as a proxy for cold acclimation status and winter survival ability in some grass species (Gusta et al., 2001; Hulke et al., 2008; Waldron et al., 1998) and in white clover (Annicchiarico et al. 2001). However, freezing tolerance is not always correlated with winter survival, because other stresses than freezing may be predominant, and because resistance mechanisms towards the various stress factors are multiple and likely to be, to some extent, genetically

independent. This was also shown in our experiments; there was considerable GxE for winter survival, and freezing tolerance was correlated to some extent with winter survival at Jokioinen in 2016, but not at Løken or Korpa in 2015, suggesting that freezing was an important winter stress at Jokioinen but that other winter stresses were prevalent at the two other locations, in the respective years. The lack of correlations between LT50 and winter survival in the field could also be due to the method of testing freezing tolerance, such as cold acclimation conditions and duration of the freezing treatment. A prolonged freezing test, LD50 (lethal duration time for 50% kill), may be better correlated to winter survival in the field. Waalen et al. (2011) found that the LD50 test was more effective than the LT50 test in identifying freezing tolerance in canola cultivars. Surprisingly, winter survival at Korpa was negatively correlated with winter survival at Jokioinen and Løken, both according to genotypic and canonical correlations. The oceanic climate typical for Iceland is characterized by less severe winter temperatures, and plants are subjected to stresses such as frequent freeze-thawing cycles, waterlogging and ice encasement. The different winter survival at Korpa may also be related to the different expression of autumn status at Korpa, as discussed above. Moreover, soil moisture and excessive rainfall during the acclimation was demonstrated to negatively affect the capacity of plants to obtain a full acclimation (Bélanger et al., 2006).

#### 4.3 | Breeding prospects and management of gene bank material

Due to high GxE for winter survival, breeding of cultivars with improved persistency and broad adaptation to the Nordic region requires testing at several locations over several years. Alleles involved in various processes and mechanisms will have to be combined, either at individual or population level, to ensure adaptation to the variation in Nordic winters (e.g. as initiated by Helgadóttir et al., 2000). This may be even more important in the prospect of increasing variability in the winter climate expected in the future at Nordic latitudes (Rapacz et al. 2014).

Controlled experiments aiming at characterizing LT50 and other specific winter survival-related traits may speed up the breeding process and help ensure that several traits are combined. For example, successful enhancement of freezing tolerance and winter survival in the field was realized through the recurrent selection of alfalfa and red clover plants surviving controlled freezing treatments (Bertrand et al., 2016; Castonguay et al., 2009). However, it is clear from our results that characterizing only LT50 is not sufficient when breeding for Nordic conditions, as it explains only a portion of the variation in winter survival. We found very strong positive correlations between yield score and stem length in the field, and petiole length measured on young plants in the greenhouse. The latter trait could be used in rapid evaluation of a large number of individuals.

Several gene bank accessions outperformed the cultivars, particularly for winter survival, but also for yield and flowering scores,

suggesting that they can be utilized in further improvement of persistency, biomass and seed yield. Also, the large variation in morphological traits such as growth type, leaf area and stem length that we found can be exploited for breeding cultivars with various morphologies, adapted to different end uses and agronomic systems (e.g. for grazing).

A gene bank collection should be as broad as possible, representing most of the existing variation. At the same time, there are economical limitations, so the redundancy should be kept low. Our results suggest that the Nordic red clover collection should at least contain accessions which capture the variation along the two identified phenotype climate gradients in order to cover the variability present in the region. Additional gradients may be identified in future studies with more accessions, capturing larger variation in climatic conditions. Development of predictive models based on environmental information, phenotypic data as well as genomic data, would further increase the precision in conservation and utilization of Nordic red clover germplasm.

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#### AUTHOR CONTRIBUTIONS

Anna Palmé, Áslaug Helgadóttir, Merja Veteläinen and Petter Marum planned the field trials. Áslaug Helgadóttir, Linda Öhlund, Kristin Daugstad and Mika Isolanti conducted the field trials. Áshild Ergon and Maria Ahlin Moen planned and conducted the experiment under controlled conditions. Stefano Zanotto analysed the data. Áshild Ergon and Odd Arne Rognli supervised the data analysis. Stefano Zanotto and Áshild Ergon wrote the paper. All authors commented on the manuscript and approved the final version.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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