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Morphological and phenological consequences of *ex situ* conservation of natural populations of red clover (*Trifolium pratense* L.)

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Abstract

Ex situ seed banks provide an effective conservation and utilization system for crops and their wild relatives. Efforts are made to reduce genetic drift in conservation, where regeneration is a critical step. In the present study, we examined eight wild populations of red clover (*Trifolium pratense* L.) according to 13 morphological and phenological traits. Samples of original collected seed were grown and compared with plants from first and second *ex situ* generation, with commercial cultivars and landraces being included for purposes of comparison. Variance analysis and Tukey multiple comparisons of means showed that the commercial cultivars and landraces were clearly distinct from the wild populations and were excluded from the further analysis. Despite the fact that the wild accessions were collected from a geographically delimited region in Norway, they exhibited significant differences in several of the measured traits. The main phenotypic patterns remain after *ex situ* regenerations. However, the mean values for four of the examined traits (across accessions) did change significantly from one generation to the next. Two of the eight accessions had significantly changed from one generation to the next, a tendency was observed across all the studied traits. The results were discussed in terms of regeneration circumstances. Observed changes appeared to be directional, going from populations with predominantly wild morphological types towards plants more closely resembling the commercial cultivars. Such a directional change implies that selection or gene flow has been acting on the accessions during regeneration, rather than random changes owing to genetic drift.

Keywords: conservation; crop wild relative; diversity; *ex situ*; population; *Trifolium pratense*

Introduction

Ex situ seed banks (genebanks) provide an important conservation system for genetic diversity of cultivated plants (Abberton and Marshall, 2005; Herrmann *et al.*, 2008). In the seed banks, 1000 of individual plants can easily be stored and viability can be maintained for decades (Walters, 2004). For optimal longevity, seeds are stored under cold, ultra-dry conditions (Vertucci and Roos, 1990; Copeland and McDonald, 1995). *Ex situ* conservation also provide an efficient way of making genetic resources available (Wexelsen, 1965; Tucak *et al.*, 2013). A pivotal question is therefore: to what extent do seed banks efficiently conserve genetic diversity and how much genetic diversity is lost or changed during conservation?

Ex situ conservation systems usually follow these steps: (0) seeds are collected and stored as unique accessions with storage and viability monitoring. (1) When seeds get

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old, or if quantities become inadequate, regeneration is carried out – resulting in a new generation. This is done in fields at a research station. (2) This process is repeated whenever necessary, resulting in the new generations of the same accession. During these steps efforts are made to minimize forces causing genetic changes. However, regardless of whether conservation occurs in nature or in a seed bank, population change is to be expected, as forces continuously act on the genetic make-up of any living population (Ellstrand and Elam, 1993; Ouborg *et al.*, 2006). A new variation can be added through mutation or gene flow from other populations; the frequency of variation may change due to natural or artificial selection or genetic drift, and the variation may also be lost due to genetic drift (Van de Wouw *et al.*, 2010). In seed banks the goal is to minimize these changes. International standards (FAO, 2014) provide recommendations on collecting and regeneration practices such as isolation, pollinators and number of plants. We wanted to study how natural populations are influenced by an *ex situ* conservation system and examine the pattern of these changes, which allowing us to suggest potential improvements. In addition, we wished to describe the material in terms relevant for germplasm users. Morphological and phenological characterization has the advantage of offering a direct approach to traits of importance to agriculture and has therefore been extensively applied in breeding (Humphreys, 2005; Fjellheim *et al.*, 2007) as well as in the development of molecular methods to assist selection (Kölliker *et al.*, 2006, 2009). While the importance of diversity at species level is generally recognized, the role of diversity within populations and among individuals is frequently overlooked. Diversity is a prerequisite for selection, regardless of whether it is natural or targeted, as is the case in plant breeding (Reed and Frankham, 2003). Wild material can be used in breeding to broaden the gene pool of breeding populations and to facilitate adaptation to new environments (Allard, 1999) or new demands. The results of our study are of importance not only for practitioners and seed bank scientists, but also for researchers and breeders using genebank material in their work.

Material and methods

Plant material

Based on its importance as a forage crop and its out-crossing nature, red clover (*Trifolium pratense* L.) was selected as object of investigation for this study. The species' high protein content and nitrogen-fixing ability from air have led to its extensive use in grass–clover mixtures. Red clover became a key crop in Europe as part of the Viscount Townshend four-field crop rotation system, which was introduced in England in the seventeenth century (Ashton, 1948) and subsequently developed to what is known as the Norfolk four-course system (Martin *et al.*, 1976; Bruns, 2012). In this system, wheat was grown in the first year, turnips in the second, followed by barley with under-sown clover. The introduction of red clover has led to a rise in agricultural productivity and continues to play a major role, especially in organic agriculture in temperate regions. Red clover grows wild and there are 250,000 geo-referenced records globally (GBIF, 2014). The species has an incompatibility system that prevents self-pollination; insects are therefore the main pollinators (Taylor and Smith, 1979). Red clover is diploid ($2n = 14$) in the wild, but there are some tetraploid commercial cultivars.

For this study, Norwegian seed samples from the germplasm collection at NordGen were selected. This included eight populations classified as natural or wild in the geneband documentation system, two landraces and six commercial cultivars (Table 1). The wild populations (numbered 1–8) were selected randomly among accessions represented with seed samples from different generations: from (0.0) the originally collected material, (0.1) the first *ex situ* generation and (0.2) the second *ex situ* generation. Although keeping a reference sample of each generation has not been part of common procedure in seed banks, it proved possible to find eight accessions that were classified as wild material where the originally collected reference samples were still vigorous (with a germination ability above 70%) and where seeds from one or two new *ex situ* generations were available. The landraces and commercial cultivars (numbered 1C–8C) and in the following included in the term 'cultivars' were included for comparison. Of these Molstad, Pradi, Nordi, Lea and Bjursele were selected because they had been, or still are, commonly used in the region where the wild accessions were collected. Björn, Betty and Bredånger have been, or still are, cultivated in Northern Sweden and included as a reference. Details regarding collecting data and regenerations are given in Table 1. All natural populations were collected by the same team in the same year. Seeds were taken from at least 50 plants. All regenerations were done in Norway, either at the state-owned research station at Landvik or at Løken, and were done according to the standards set by the forage working group at the Nordic gene bank at that time; with the use of minimum 100 plants and a distance isolation of 50 m in a cereal field or the use of 50 plants isolated with cages and the use of pollinators.

Cultivation and characterization

A total of 22 plants of each accession and generation were cultivated in a greenhouse in Alnarp, Sweden (53°N, 13°E), each plant was grown in a 11 × 11 cm (1 L) pot filled with

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Table 1. Overview of the material included in this study. All wild accessions were collected and regenerated in Norway

Code	Type and generation	Accession number, collecting and regeneration information
1.0	Wild	NGB1571, collected 1980, Vingelen, 680 mamsl, 62°25′N, 010°52′E, from 50 m^2 natural population, field margin
1.1	1	First *ex situ* regeneration, 1987, Løken, 100 plants, distance isolation
1.2	2	Second *ex situ* regeneration, 2002, Landvik, 50–100 plants, cage isolation
2.0	Wild	NGB1572, collected 1980, Alvdal, 500 mamsl, 62°05′N, 010°39′E, from 100 plants, 1000 m^2, population from old meadow not used since 1953
2.1	1	First *ex situ* regeneration, 1985, Løken, 100 plants, distance isolation
2.2	2	Second *ex situ* regeneration, 2002, Landvik, 50–100 plants, cage isolation
3.0	Wild	NGB1574, collected 1980, Tufsingdalen, 780 mamsl, 62°20′N, 011°23′E, from natural population, natural mountain grassland
3.1	1	First *ex situ* regeneration,1985, Løken, 100 plants, distance isolation
4.0	Wild	NGB1575, collected 1980 at Atnabru, 725 mamsl, 61°51′N, 010°14′E, population from old meadow not used for many years
4.1	1	First *ex situ* regeneration, 1985, Løken, 100 plants, distance isolation
4.2	2	Second *ex situ* regeneration, 2002, Landvik, 50–100 plants, cage isolation
5.0	Wild	NGB1576, collected 1980 at Lom, 450 mamsl, 61°49′N, 008°30′E, from 100 plants, 200 m^2, natural population in a field margin close to farm
5.1	1	First *ex situ* regeneration, 1985, Løken, 100 plants, distance isolation
6.0	Wild	NGB1577, collected 1980 at Vågåmo, 400 mamsl, 61°53′N, 009°08′E, from 100 plants, 300 m^2, population from old meadow sawn in 1935
6.1	1	First *ex situ* regeneration, 1985, Løken, 100 plants, distance isolation
7.0	Wild	NGB1578, collected 1980 at Lesjaskog, 620 mamsl, 62°14′N, 008°17′E, from 100 plants, 200 m^2, population from old meadow in use
7.1	1	First *ex situ* regeneration, 1985, Løken, 100 plants, distance isolation
8.0	Wild	NGB13447, collected 1980 at Os, 725 mamsl, 62°33′N, 011°15′E, from 50 plants, 1000 m^2, natural population
8.1	1	First *ex situ* regeneration, 2002, Landvik, 50–100 plants, cage isolation
1C	Cultivar	NGB2183, Molstad, released 1953, donated 1986 from breeder
2C	Landrace	NGB2486, Bredånger, donated 1979 from maintainer
3C	Cultivar	NGB2745, Björn, released 1977, donated 1982 from breeder
4C	Cultivar	NGB7786, Pradi, released 1981, donated 1989 from breeder
5C	Cultivar	NGB11155, Nordi, released 1989, donated 1991 from breeder
6C	Landrace	NGB13203, Bjursele, donated 1997 from maintainer
7C	Cultivar	NGB13205, Betty, released 1992, donated 1997 from authority
8C	Cultivar	PL: 100236, Lea, released 2002, donated 2013 from breeder

mamsl, metres above mean sea level.

peat-based soil mixture (Hasselfors Spesialjord™, Hasselfors, Sweden, with long-term fertilizer) and inoculated with soil from a field close by. Seeding was performed in July 2013 and plants were overwintered in a temperate greenhouse (lower limit 5°C). They began to grow again in February 2014 as a result of natural sun heating. Irrigation was done automatically using a standard under-watering system. Additional fertilizers, 2 g/pot of 11-5-18 Micro™ (Yara, Oslo, Norway) were added twice, once in April and once in May. Characterization was initiated when plants began to grow and continued until the time of flowering, when each single plant was cut 2 cm above soil surface and measured. Our study was carried out in greenhouse conditions, with a higher average temperature and a lower light intensity than found in outdoors. Therefore, plants were taller than what is usually observed in fields (Vasiljevic *et al.*, 2000; Asci, 2011; Pagnotta *et al.*, 2011; Tucak *et al.*, 2013). Competition from neighbour plants may influence parameters such as stem length and plant weight. Therefore, the cultivars and wild accessions were cultivated on separate tables to avoid competition between larger cultivars and smaller wild accessions. The plants were harvested continuously and individually, as plants started to flower at different times. This harvesting method worked well and allowed us to capture the variation while also avoiding competition from the early or fast growing plants. However, a small percentage of very small plants were observed, and these were registered as missing plants. These came in addition to a few plants that were removed due to disease. Plants were characterized individually, and characterization was performed by the same single person throughout the entire period. The descriptors were based on UPOV (2014) with modifications and are given in Table 2.

Statistical analysis

Statistical work was done in R software (R Core Team, 2014). The R function *boxplot* was used to illustrate the distributions of the scores of the different descriptors. The following five descriptors were identified as categorical: Hair, White, LeafCol, GrowHab and EarlyG. Further eight descriptors provided numeric data and with a normal distribution: Weight, Flow, StemL, StemT, Nodes, LeafL, LeafW and DiamEa. In order to obtain an overview of the material, a principal component analysis was performed using the R function *princomp* (Everitt and Hothorn, 2011). This was done based on mean values of each accession and generation. The R function *heatmap* was used to demonstrate dendrograms for both accessions and variables in the same picture; dissimilarities are expressed as different colours. This is a two-way cluster analysis. Standard ANOVA tests using the R function *aov* were used to identify significant different in means. Significant descriptors were further analysed by using Tukey multiple comparisons of means (Crawley, 2013) with a 95% family-wise confidence level to identify the differences at the 5% level. These steps were carried out, first in a model including all data, then in a model including the three generations of wild material, then in a model including only the originally collected material and the first *ex situ* generation, and finally in a model including only the originally collected material. The accessions were also analysed according to their morphological types (morphs). Morphological types were identified using the 25th percentile of the boxplots of four descriptors which proved to be important in separating cultivars from wild material (Table 2). Each single plant in each generation of the wild accessions was scored by giving one point per criteria, which giving a maximum score of four points for cultivar morph and six points for wild morph (Table 2). Variance analysis and Tukey multiple comparisons of means were then applied to analyse the scores.

A Pearson correlation matrix (*scatterplot* in R) was set up to describe the relationship among descriptors. The following formula: $t = r\sqrt{\frac{n-2}{1-r^2}}$ was used to test the null hypothesis that the correlation coefficient of a population from which the sample has been taken is zero. The *t*-test for significance of the product-moment correlation coefficient r applies where n is the sample size.

The variations observed among individuals may be caused both by genetic, and environment and occasionally by genotype and environments interaction and error. In planned agriculture experiments, the coefficient of variation (CV) is often considered as a measure of precision. This is because it is a measure of the unexplained variation in the statistical model. However, CV is quite a useful measure of diversity in collected populations. It is also suitable for comparing different descriptors within each collection site. CV is defined as the standard deviation × 100 and divided by the mean (SD/mean) × 100. Since both the standard deviation (SD) and the mean have the same units, the division of one by the other cancels out the units and produces a numerical value which is independent of the scale used for the measurement. CV is an estimate of variability that is independent of sample size. Thus large means with large standard deviations may now be compared with small means with small standard deviations. CVs are useful for describing diversity in genetic resources, but this is not a method that has been much used in the past.

Results

Overall patterns

The boxplots (Fig. 1) showed variation in all traits. Trait variation was found within the different generations of

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Table 2. Overview of the descriptors and morphological types as defined in the study. References to UPOV (2014) are given where relevant

Descriptor	Explanation and scale	Type/reference
Weight	Plant weight, measured in gram at flowering	Numerical
Flow	Time of flowering, days to start of flowering	Numerical
StemL	Stem length, measured in cm at flowering	Numerical
StemT	Stem thickness, measured in mm at flowering	Numerical
Nodes	Stem number of internodes at flowering	Numerical
LeafL	Leaf length, measured in mm at flowering	Numerical
LeafW	Leaf width, measured in mm at flowering	Numerical
DiamEa	Plant diameter in early spring (February), in cm	Numerical
Hair	Stem density of hairs, visual observation at flowering. Scale: 1 very low, 3 low, 5 medium, 7 high, 9 very high	Categorical/(UPOV, 2014)
White	Leaf intensity of white marks visual observation. Scale: 1 very low, 3 low, 5 medium, 7 high, 9 very high	Categorical/(UPOV, 2014)
LeafCol	Leaf colour, visual observation in the year of sawing. Scale: 3 = light green, 5 = medium green, 7 = dark green	Categorical/(UPOV, 2014)
GrowHab	Plant growth habit year of sawing. Visual observation. Scale: 1 erect, 3 semi-erect, 5 intermediate, 7 semi-prostrate, 9 prostrate	Categorical/(UPOV, 2014)
EarlyG	Early growth in spring, visual observation in April. Scale: 3 weak, 5 medium, 7 strong	Categorical
Cultivar morph	1. Plant weight > 110 g 2. Stem thickness >4.2 mm 3. Number of nodes >8 4. Leaf length >50 mm	
Wild morph	1. Plant weight ≤110 g 2. Stem thickness ≤4.1 mm 3. Number of nodes ≤8 4. Leaf length ≤50 mm 5. Stem hair ≥5 6. Growth habit ≥7	

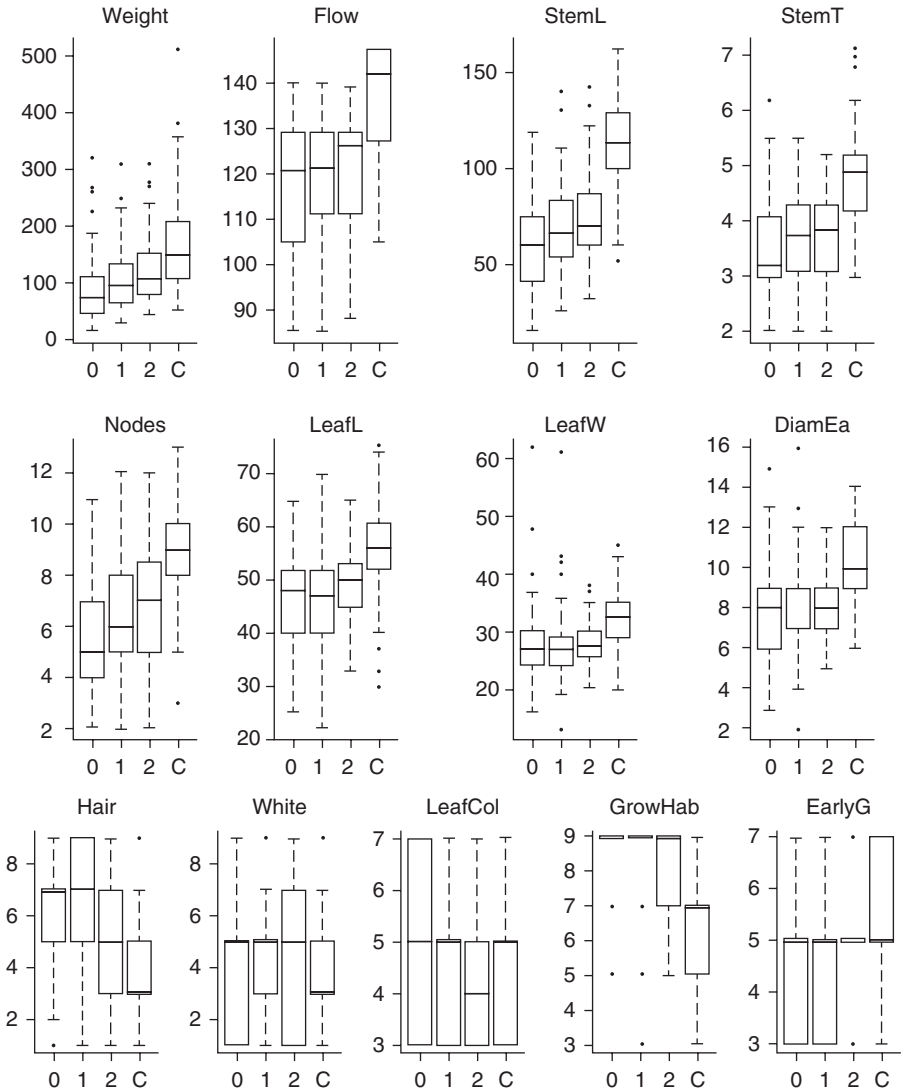

Fig. 1. Boxplots of characters where (0) = originally collected material, (1) = the first *ex situ* generation, (2) = the second *ex situ* generation and (C) = cultivars.

wild material and within the cultivars, also suggesting that there is differentiation between the wild accessions and the cultivars for several traits. A biplot PCA (Fig. 2) showed that, based on the mean values, the two first variance components explain 80% of the variation. The commercial cultivars and landraces were clearly distinct from the wild accessions but not from each other. The different generations of each of the wild accessions were to some extent clustering; however, the picture was not clear. Both first and second variance components were used to explain the variation among them. The descriptors of importance for explaining the variation were given as arrows in the biplot. The length of an arrow is a measure of the descriptors' variance. The angle between the arrows is a measure of the correlation between the descriptors, with a small angle expressing high correlation. All the eight continuous descriptors were positively correlated, pointing to the same side in the figure together with one of the categorical descriptors; early growth in spring. In general, their contribution to component 1 was larger than to component 2. The main accessions responsible for variation in the categorical descriptor leaf colour were 3.0, 3.1, 1.0 and 8.0. The accessions 1.2 and 2.0 also contributed to that descriptor, albeit to a lesser extent.

Based on mean values, the Pearson correlation matrix indicated a negative correlation (r significantly different from zero) between stem hair and plant weight as well as between stem hair and the other characters describing plant size (for all $P < 0.001$, $n = 19$). Similarly, a negative correlation coefficient was found between growth habit and the characteristics describing plant size.

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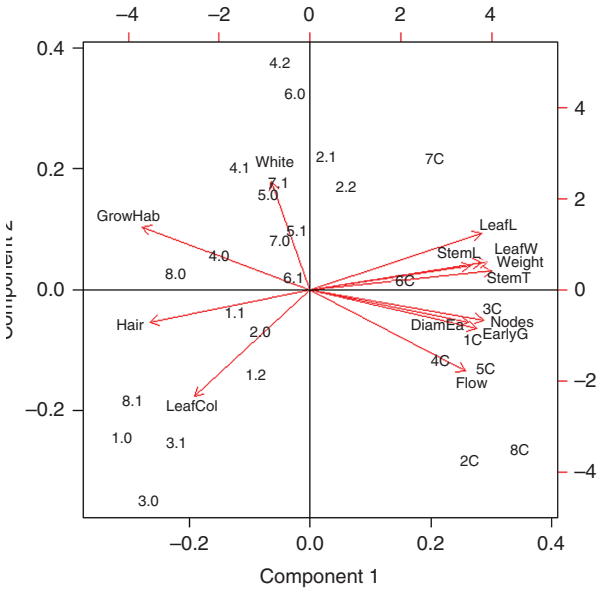

Fig. 2. Bi-plot PCA plot based on mean values of all traits for the different cultivars and the different generations of wild accessions (for decoding: Tables 1 and 2). The two plotted components explain about 80% of the variation (70.2 and 9.4 respectively).

Strong positive correlations were found between plant weight and stem thickness, stem length, number of nodes, leaf length, leaf width and flowering time (for all $P < 0.001$, $n = 19$). The variance analysis (ANOVA) showed significant differences among accessions in mean values for all numeric descriptors (all highly significant, $P < 0.01$) when the full data set was analysed. The result from Tukey multiple comparisons of means showed that the commercial cultivars and landraces differ for a few of the descriptors, but they both differ significantly from the wild material, independently of generation and for all descriptors.

Differences among and within wild accessions

The Tukey multiple comparisons of means showed significant differences in plant weight, stem length, stem thickness, leaf length, leaf width and early plant growth varied among the originally collected wild accessions (generation 0). The differences is illustrated in Fig. 3, where accessions 1 and 3 consist of a very high score for the wild morphological type in generation 0, while the other five accessions have a mixture of wild and cultivar morphs. Accession 1 was clearly different from accessions 2 ($P < 0.01$), 5 ($P < 0.01$) and 6 ($P < 0.05$), and accession 3 was different from accessions 2 ($P < 0.05$), 5 ($P < 0.01$) and 6 ($P < 0.05$). The variation, expressed as SD, did not differ significantly among the accessions. The CV was also quite similar (averages ranging from 25.5 to 31.0 in the accessions in generation 0, see Supplementary Table S1, available online). Looking at the averages for the descriptors, the highest CV value (57.7) was found for intensity of white leaf marks and the lowest CV value (5.8) was found for growth habit in spring.

Changes over generations

From the initial boxplots and PCA analysis presented in Figs 1 and 2, indication of changes over generations of *ex situ* conserved wild red clover material could be detected.

The heat map (provided as Supplementary Fig. S1, available online) showed one cluster that includes the commercial cultivars and landraces and two clusters including the wild accessions, the first of which included both generations of accession 8 (8.0 and 8.1) and both generation of accession 3 (3.0 and 3.1) – but also the originally collected material from accession 1 (1.0). The other generations of accession 1 (1.1 and 1.2) were in the second cluster of the wild accessions. Both generations of accession 5 proved to be closely related, while the other accessions and generations all lay in the second cluster. However, they were found to be in various sub-clusters. The change in distribution of morphological types over generations is also illustrated in Fig. 3. In five of the eight accessions, the change was in the direction of higher scores for the cultivar morphs. However, the changes were only significant for accessions 2 and 4 ($P < 0.05$ for both).

The variance analysis showed significant differences in mean values among the generations. For four out of the eight numeric descriptors: plant weight ($P < 0.01$), stem length ($P < 0.01$), stem thickness ($P < 0.05$) and number of nodes ($P < 0.01$), respectively. For the categorical descriptors no clear differences among the generations were observed although the median value for stem hair was lower in generation 2 than in generations 0 and 1 (Fig. 1).

Table 3 shows the mean values and standard deviations across accessions of the originally collected material and the first *ex situ* generation (each with eight accessions). The second *ex situ* generation was excluded from this analysis since it was lacking for five out of the eight accessions. The analysis showed a significant difference between generations 0 and 1 for plant weight, stem length, stem thickness and number of nodes (the same descriptors as in the analysis of differences among generations 0, 1 and 2). By applying the Tukey multiple comparison analysis, two accessions (accession 1 and accession 2) were identified as having significantly changed in one or more of the traits when moving from the

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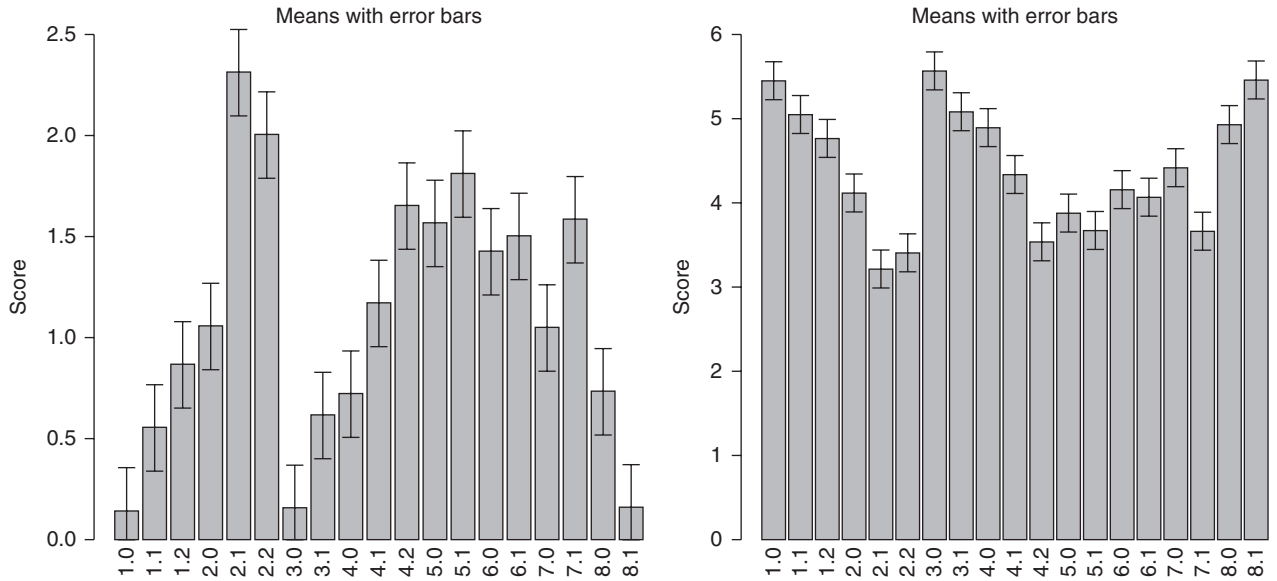

Fig. 3. Mean values with error bars for scores of cultivar morphs and wild morphs in wild accessions.

originally collected material to the first *ex situ* generation. Expression of the differences in relative values (relative to the average values) confirmed that accessions 1 and 2 had changed the most.

Discussion

Diversity and trait relationships

The study established a close correlation with several traits, and that commercial cultivars and landraces differed significantly from wild red clover. The wild plants had more prostrate growth habits, were generally much lower and had more stem hairs than the cultivars and landraces. Hairs are known to protect plants from adverse UV radiation (Karabourniotis *et al.*, 1993; Roy *et al.*, 1999) and insects. Low plant growth generally protects plants from cold (Pecetti and Piano, 2002; Singh *et al.*, 1995). In red clover breeding, relatively tall plants with many nodes and large leaves are often used as a criterion in the selection with a view to increasing yields (Tucak *et al.*, 2013). However, intense selection on one trait may affect other traits. Less stem hair could be one such effect and this may be the result of a lack of selection for hairs. Pecetti *et al.* (2008) reported plastic response in growth habit, but our study does not confirm this as the wild material has retained its prostrate growth habit also after *ex situ* regeneration. Flowering times did not differ much among the wild accessions in our study. Flowering time is acknowledged as one of the main traits affecting germplasm adaptation to natural environments (Izawa, 2007). Our wild collections were from the same region and thus adapted to similar latitude and local climate and similar flowering time was therefore expected. However, a clear difference was found between the cultivars and landraces on one side and the wild material on the other side. If earlier flowering time is sought for cultivars at any point, the wild material can be used as a source of genetic variation. However, our analysis also showed that late flowering was correlated to high yield.

Unique accessions

Even though the wild accessions in this study were collected from different locations within a relatively limited geographical scope in Norway, they display significant differences in several of the measured traits (weight, stem length, stem thickness, node number, leaf length, leaf width and early growth diameter) as well as significant differences in morphs, but with coefficient of variance at a similar level. This suggests that there is morphological differentiation at this geographic scale for red clover and that this should be considered in the development of conservation strategies. All accessions were collected from the same year, following the same collecting protocols, but the details on collecting sites vary (Table 1). The two accessions with the highest percentage of wild morphs were both collected from true natural populations, while the accessions with more cultivar like morphs were from old meadows or natural habitats close to cultivation, such as field margins. What has been regarded as a natural population of red clover could have been a relic from a previous cultivation or a mixture of true natural and previously cultivated red clover. Such a population can be termed

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Table 3. Mean values and standard deviation across all wild accessions for the different traits of the originally collected material (generation 0) and the first *ex situ* generation (generation 1) ($n = 8$ for both). The lower line shows the *P*-values from Tukey multiple comparisons of the differences of means

Generation	Plant weight (g)	Flowering time (days)	Stem length (cm)	Stem thickness (mm)	Number of nodes	Leaf length (mm)	Leaf width (mm)	Early growth (cm)
0	85.2 ± 51.5	118 ± 13.9	59.7 ± 21.3	3.5 ± 0.8	5.5 ± 2.0	46.9 ± 9.0	27.1 ± 5.3	7.8 ± 1.9
1	102.7 ± 50.1	119 ± 13.1	68.5 ± 19.4	3.7 ± 0.8	6.4 ± 2.1	46.9 ± 8.3	27.2 ± 5.4	7.7 ± 1.9
P-value	0.008	NS	0.001	0.027	0.001	NS	NS	NS

Ns, not significant.

as a 'semi-wild' population; however, this has not been the practice so far.

Interaction between germplasm and environment has previously been reported in red clover (Maki *et al.*, 1974) and is also known from related species (Song and Walton, 1975; Berger *et al.*, 2002). A recent study of snow clover (*Trifolium pratense* subsp. *nivale*) has revealed differences among wild populations in different Italian valleys (Pecetti *et al.*, 2008). In their study, differences were observed both in flowering time, flowering colour, growth habit and type and susceptibility to mildew; however, the overall pattern of phenotypic diversity was similar among the valleys.

Our results can be discussed in a broader context of *ex situ* and *in situ* conservation of crop wild relatives (Zizumbo-Villarreal *et al.*, 2005; Andrianasolo *et al.*, 2013; Christie *et al.*, 2014; Greene *et al.*, 2014; Hoban and Schlarbaum, 2014). There may be numerous factors important for a population's structure. An *ex situ* collection can be seen as a genetic snapshot of a population. Therefore, a solid documentation is essential for the conservation of crop wild relatives.

Ex situ *conservation can change morphology*

Our study shows that the main phenotypic patterns persist also after *ex situ* regenerations. Despite the persistence of the dominant patterns, some changes were also identified. Across accessions, the mean values for four of the examined traits changed from one generation to the next. This indicates that changes have taken place during *ex situ* regeneration. Some of the examined accessions were affected more than others, suggesting that circumstances during regeneration play an important role. When change is observed it seems to be directional, going from populations with predominantly wild morphological types towards plants more closely resembling commercial cultivars. A directional change implies that either selection or gene flow has affected the accessions during regeneration, rather than random changes as a result of genetic drift. This directional change can be observed both in the boxplots (Fig. 1) and the illustration of morphological types (Fig. 3). Plants of the cultivar type are taller and larger than the wild type, potentially giving them a competitive advantage in terms of light and resulting in a higher seed production. In this way selection may increase the frequency of genes associated with the cultivar morph. An alternative or supplementary explanation may be gene flow from cultivars during regeneration. Isolation distances or isolation cages are used in connection with regeneration of red clover (Table 1) and should substantially limit gene flow from nearby cultivars; however, low-level gene flow may

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nevertheless occur. Regenerations were performed following the standard regeneration procedures at that time and seeds were harvested in bulk from at least 50 individual plants. The same generations were regenerated at the same location and most accessions also in the same year and with good harvest.

Another finding in our study is that the variation (expressed as standard deviation across accessions) did not change significantly from one generation to the next. In general, there is an expectation that genetic variation is lost through genetic drift during regeneration. However, it can be difficult to predict the effect on morphology and this may depend on the trait's genetic background. When the loci affecting a trait are completely additive population genetics theory predicts a decrease in genetic diversity (Falconer and Mackay, 1996), but if there is dominance or epistasis, a bottleneck in the population size may result in a higher level of variation. An increase in quantitative genetic variation after a bottleneck has been demonstrated in a number of experimental studies (Van Buskirk and Willi, 2006; Taft and Roff, 2012). Since we do not know the genetic background for the observed traits in red clover, it is difficult to interpret the results with any degree of certainty. However, we cannot exclude that variation has been lost on the molecular level but continues constant at the morphological and phenological level.

Ex situ *management recommendations*

There is evidence that *ex situ* conservation has maintained alleles that have been lost from *in situ* populations, for example in barley landraces resistant to strains of powdery mildew (Jensen *et al.*, 2012). However, there is evidence that *ex situ* conservation has caused genetic drift and leads to changes in the traits of the conserved material. This has, for example, been shown to be the case for the common bean (Gomez *et al.*, 2005; Negri and Tiranti, 2010) and maize landraces (Soleri and Smith, 1995). Good initial sampling and proper regeneration practice are essential to *ex situ* conservation. Small population sizes have a strong effect, predominantly on outcrossing and self-incompatible plant species (Leimu *et al.*, 2006; Honnay and Jacquemyn, 2007). Sampling technique is also important (Richards *et al.*, 2007). The negative impact of small population size on plant fitness has been established (Leimu *et al.*, 2006), as has the relationship between population size and genetic diversity (Van Treuren *et al.*, 1991; Ellstrand and Elam, 1993; Frankham, 1996; Dittbrenner *et al.*, 2005; Hensen and Oberpieler, 2005). Leino *et al.* (2013) showed that seed banks may only harbour a subset of the alleles originally found in meta-populations. They also link this fact to how populations are maintained. For domesticated cross-pollinated species, Marshall and Brown (1983 and 1995) recommend a population size of more than 50 plants to ensure a 95% likelihood of including alleles occurring in the population with a frequency of 5% or more. Crossa *et al.* (1993), on the other hand, suggested 160–210 individuals. Regeneration strategies should be made to minimize genetic drift, selection and external gene flow and recollection should in some cases be considered over regeneration (Brown *et al.*, 1997).

As the number of plants used for regeneration will often be limited, genetic drift will inevitably have an impact on the population. The expected effects are loss of genetic diversity and random changes in gene frequencies. Regeneration will therefore result in decreased heterogeneity in later generations compared with the initially collected material. If genetic drift is the dominant force, the frequency of traits will increase or decrease randomly. However, this is not what we observed in our study; instead, a directional change was identified. The data presented here on the cause of such a change is not conclusive. However, it is evident that the change is most likely a result of either selection or gene flow. Measures to prevent these processes should therefore be further assessed with a view to minimizing the change in gene bank populations of this and similar species, while also reducing regeneration costs. Balanced harvesting (harvesting the same number of seeds from each plant) has been suggested as an approach to minimize the effect of genetic drift and selection. However, this approach is very costly and implementing it would seriously reduce the number of accessions that can be stored in gene banks, thus reducing the total amount of variation that can be conserved. Alternative approaches include increasing distances between plants to reduce competition and hence selection; to always use isolation cabins rather than isolation distance to decrease gene flow; and to use larger population sizes to reduce genetic drift, even though there is no strong evidence for the latter in this data set. The regeneration standards currently used at NordGen for forage crops specify that 100 individuals should be used for regeneration, harvesting should be done in bulk and that isolation cabins are the preferred isolation approach for red clover; however, isolation distance of 100 m or more are also allowed. This is also what is specified in the European guidelines for insect pollinated forage crops (Boller *et al.*, 2007).

Supplementary material

To view supplementary material for this article, please visit www.dx.doi.org/10.1017/S1479262115000416

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