Genetic shift in white clover (*Trifolium repens*) after natural selection in a marginal area

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ABSTRACT

Studies of genetic diversity are a prerequisite for any plant breeding programme. Where the primary breeding aims are to combine yield and survival it is important to be able to identify traits associated with these two factors. We compared genetic diversity in bred cultivars of white clover of Nordic origin with that in semi-natural and natural populations adapted to local conditions in Iceland. We also monitored genetic shift in the white clover cultivar Norstar after exposure to natural selection for four years under field conditions in Iceland. The genetic diversity was assessed by AFLP markers and we also measured more conventional morphological and physiological attributes in the field. We found that rapid genetic shift occurred in Norstar for several morpho-physiological traits. Such a cultivar of northern origin seems to contain sufficient genetic diversity to build the base for further selection for improved winter hardiness without sacrificing yielding ability.

Keywords: Genetic diversity, adaptation, AFLP, marginal area, genetic shift, fatty acid composition

YFIRLIT

Erfðabreytingar í hvítsmára (Trifolium repens) eftir náttúruúrval á jaðarslóð

Rannsóknir á erfðafjölbreytni eru forsenda þess að hægt sé að stunda plöntukynbætur. Þegar meginmarkmiðin eru að sameina uppskeru og vetrarþol í kynbættum yrkjum er nauðsynlegt að finna eiginleika sem tengjast þessum tveimur þáttum. Borin var saman erfðafjölbreytni í kynbættum hvítsmárayrkjum af ólíkum uppruna við náttúrulega stofna sem aðlagast hafa erfiðum skilyrðum hér. Við mátum erfðafjölbreytni með AFLP erfðamörkum og einnig mældum við algenga útlits- og lífeðlisfræðilega eiginleika í hefðbundnu hnausasafni. Við fundum að marktækar erfðabreytingar höfðu orðið í norska yrkinu Norstar eftir náttúruval á Íslandi. Norðlæg yrki eins og Norstar virðast því búa yfir nægum erfðabreytileika sem hægt er að byggja á frekara úrval og bæta þannig vetrarþol án þess að fórna þurfi uppskerugetu.

INTRODUCTION

White clover is one of the most important fodder legumes in temperate grasslands (Frame et al. 1998). It is valued for its ability to fix atmospheric nitrogen in symbiosis with *Rhizobium leguminosarum* var. *trifolii* and for its high nutritional value and high voluntary intake by grazing animals. However, successful exploitation of the species in the marginal northern areas is currently limited by the lack of cultivars that combine winter hardiness, yield stability and high production potential

(Helgadóttir et al. 2008). An understanding of the underlying genetic variation in potential gene pools is therefore crucial in order to formulate selection criteria for genetic improvement if the area suitable for white clover cultivation is to be extended.

Both cultivars and natural populations of white clover are generally considered to be highly heterogeneous with a preponderance of highly heterozygous individuals, thus enabling genetic shift in response to natural and artificial selection pressures (Williams 1987a). Many of the small-leaved cultivars used in marginal areas are based on selections from ecotypes rather than controlled crosses. Hence, they maintain high intra-population variability (Aasmo Finne et al. 2000). The environment in which the populations are grown determines the selection pressure (Wedderburn et al. 2005), resulting in either directional or stabilising selection.

Winter hardiness and stability of yield are complex traits influenced by many factors. White clover survives winter as stolons. Hence, the ability of stolon terminal buds to survive and re-grow after winter is crucial (Collins et al. 1991). However, when studying genetic shift in environments at the edge of the range of the species or cultivar in question it is important to include both morphological and physiological traits of relevance to winter survival in cold climates. In white clover both the amount of unsaturated fatty acids and sugar content have been shown to be useful predictors of winter survival in cold climates (Dalmannsdóttir et al. 2001, Frankow-Lindberg 2001). A comprehensive study of survivor populations of two white clover cultivars, collected from a range of sites across Europe, showed that directional selection had occurred in many morphological and reproductive traits but stabilising selection had not operated at any site, even those with extreme climates (Collins et al. 2001). In contrast, there was evidence of stabilising selection operating at the physio-biochemical level, and traits such as the degree of unsaturation of cell membrane lipids showed reduced genetic variation in survivor populations from cold climates (Collins et al. 2002).

It is laborious to measure genetic diversity for morpho-physiological characters and only a limited number of traits can be assessed. Molecular marker analysis offers an efficient alternative since information of genetic structure and genetic diversity between and within populations is obtained on the basis of plant genotype rather than phenotype (Kölliker et al. 2001). Among the variety of molecular marker technologies available, amplified fragment length polymorphism (AFLP) (Vos et al. 1995) is particularly useful for diversity studies (Guthridge et al. 2001). As a result of the fundamental difference between neutral dominant markers on one hand and morpho-physiological markers on the other, AFLP analysis will though, not on its own, be suitable for parental seclection in breeding programmes but it can add valuable information on the genetic structure within the germplasm (Kölliker et al. 2001).

The objectives of the current study were to: (1) compare the pattern of genetic variation in bred cultivars of white clover of contrasting origin and semi-wild and wild populations adapted to northern conditions; (2) monitor genetic shift in a modern white clover cultivar after natural selection in a marginal area; (3) determine whether observed AFLP-based diversity reflects the diversity assessed by morphological and physiological measurements.

MATERIALS AND METHODS

Plant material

Five white clover populations were included in the study (Table 1). The cv. Norstar was released by the Norwegian Crop Research Institute (Bioforsk) in 2002 and is a small leaf type. It is based on an ecotype from Rennebu, Sør Trøndelag (62°50'N, 500 m.a.s.l.) (Rapp & Junttila 2000). It is considered well-adapted to northern conditions (Helgadóttir et al. 2008). A survivor population of Norstar, referred to as Norstar Sel., was collected from an experimental plot at Korpa Experimental Station in Iceland after exposure to four years of natural

Table 1. The type and origin of the white clover populations and number of genotypes included in the field trial, the fatty acid analysis (FA) and the AFLP study.

			No. genotypes		
Name	**Type**	**Origin**	**Field trial**	**FA**	**AFLP**
Norstar	Cultivar	Norway, 62°50'N, 500 m.a.s.l.[1]	24	14	32
Norstar Sel.	Selection from CV Norstar	Iceland, 64°09'N, 30 m.a.s.l.	24	15	32
Korpa	Semi-natural	Iceland, 64°09'N, 30 m.a.s.l.	24	15	NA[2]
Skorradalur	Wild	Iceland, 64°32'N, 70 m.a.s.l.	24	14	32
Ramona	Cultivar	Sweden	24	-	47

[1]Refers to the origin of the ecotype from which cv. Norstar was selected. [2]Not analysed (NA).

selection during which the plots were subjected to standard fertiliser and cutting management (for details see Collins et al. 2012). The remaining populations were Skorradalur (a wild type Icelandic population), Korpa (a locally adapted semi-wild population) and Ramona (a high yielding Swedish cultivar released in 1997 by SW Seed based on a selection from the high yielding medium-sized leaf cultivar Sonja released in 1979 by SW Seed).

The two cultivars were raised from seed in a glasshouse at Korpa in March 2007 and cuttings from the three other populations were collected at random from the field in May. Skorradalur and Korpa were collected with a minimum of two meters' spacing between individuals, whereas Norstar Sel. was visually judged to be separate genotypes upon collection (later confirmed by the molecular genetic analysis). In early June, a total of 32 genotypes from each population were subsequently multiplied as cuttings in the greenhouse to produce three similar-sized clones of each genotype.

Field trial

A spaced plant trial was set up at Korpa Experimental Station in late July 2007. The site is characterised by a cool coastal climate, with cool summers and fluctuating winter temperatures leading to repeated freeze-thaw cycles without stable snow cover. During the years of selection of Norstar Sel. (2004 – 2007) and the experimental years (2007 – 2008) the average annual rainfall was 1142 mm and average temperature was –0.6°C in January and 12.5°C in July (coldest and warmest month, respectively). The trial was located in an old grass field with indigenous low yielding grass species (mainly *Festuca* spp. and *Agrostis* spp.). The white clover plants were transplanted into the sward with a spacing of 0.9 m between plants. The experimental design was a randomised block with three replicates. Within each block there were clones of 24 genotypes randomly selected from the 32 genotypes from each of the five populations cultivated in the greenhouse, resulting in a total of 120 plants per block and a total of 360 plants in the experiment. The field was fertilized with the equivalent of 20 kg N, 25 kg P and 28 kg K ha^{-1} on 14 May 2008. After registration of winter survival on 3 June, dead plants of all populations except Ramona were replaced with duplicate clones which had been maintained in an unheated greenhouse. Ramona was excluded from the field trial as very few plants had survived winter and sufficient material was lacking to replace the dead plants in the field.

Morpho-physiological traits

Clover metamers (see Collins et al. 2001) were collected from the main stolon of all plants in the field trial on 5-6 August 2008. A metamer consists of the youngest fully expanded leaf according to the Carlson score (Carlson 1966), the corresponding petiole and the stolon between the youngest and second youngest fully expanded leaf. The following characters were measured: stolon diameter, stolon (internode) length, petiole length, leaflet length (defined as the length of the central leaflet of

the trifoliate leaf of the metamer), and the leaf area was measured using a leaf area meter (LI-COR Inc., Nebraska, USA. Model LI3000C, LI3050-C). Metamer components (stolon, petiole, leaf and lateral shoots) were dried at 100 °C overnight and their dry matter recorded. Dry matter per stolon length (DMSL) and dry matter per leaf area (DMLA) were calculated and used to estimate the allocation of resources to the stolons and leaves, respectively (Helgadóttir et al. 2001).

Fatty acid content was analysed in 15 randomly selected genotypes (out of 24) in each of the four populations Norstar, Norstar Sel., Skorradalur and Korpa. Meristematic stolons were collected from the field trial on 5 November 2008, after exposure to frost. On average, approximately five stolon apices of each genotype were used as bulked samples, excluding all leaves, petioles and roots. All samples had a dry weight of 20.0 – 20.5 mg. The stolons were instantly frozen in liquid nitrogen, freeze dried and ground in a ball mill according to Collins et al. (2002). Each sample was analysed for fatty acid composition by gas chromatography (GS) (Varian 3900 GC equipped with a fused silica capillary column (HP-88, 100m X 0.25mm X 0.20 μm film), split injector and flame ionisation detector fitted with Galaxie Chromatography Data System, Version 1.9.3.2 software). The extraction method was a direct methylation procedure (Browse et al. 1986), where methanolic HCl was used as a reagent (Dalmannsdottir et al. 2001). Fatty acids were identified by their retention times compared with the internal standard. The fatty acids measured were 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3.

AFLP analysis

An amplified fragment length polymorphism (AFLP) analysis (Vos et al. 1995) was performed on Norstar, Norstar Sel., Skorradalur and Ramona. Samples for the first three populations were taken from all 32 genotypes, kept in the greenhouse at Korpa, prior to the establishment of the field trial, whereas samples for Ramona were obtained from 47 genotypes grown at IBERS, Aberystwyth in UK (for details, see Collins et al. 2012). A sample consisting of one fresh actively-expanding leaf per plant (approximately 50 mg fresh weight) was placed in an Eppendorf tube, submerged in liquid nitrogen or kept on ice (Ramona) and subsequently stored at -80 °C until freeze-dried. The AFLP analysis was carried out at IBERS according to the methods of Skøt et al. (2005). Total genomic DNA was extracted from the leaf samples with a DNeasy™ 96 plant kit (QIAGEN GmbH, Hilden, Germany). AFLP analysis was carried out on 100 ng of genomic DNA using the two restriction enzymes EcoRI and MseI. Four combinations of primer pairs were used: *Eco*RI-ACA/*Mse*I-CAG, *Eco*RI-ACA/*Mse*I-CCT, *Eco*RI-ACT/*Mse*I-CCT and *Eco*RI-ACA/*Mse*I-CGA. The electropherograms were analysed using Gene Mapper v3.7 software program with manual editing. Only peaks above a threshold intensity value of 100 were scored. AFLP bands below a frequency level of 5% were removed from the data set prior to analysis.

Statistical analyses

Genstat v13.3 was used for statistical analyses of morpho-physiological traits. Analysis of variance (ANOVA) was carried out using the residual maximum likelihood (REML) defining blocks as fixed and genotypes as random effects. Levels of fatty acids in each population were analysed using one-way ANOVA; population means were based on values for 15 replicate genotypes. The level of genetic variation for morphological traits within each population was estimated by calculating the coefficient of variation (% CV = √(genetic variance/mean) × 10^2). For levels of fatty acids the variance was used, as differences in % CV can be assumed to be primarily expressed by genetic variation for these traits (Collins et al. 2002). To test for significant differences in % CV between the populations a χ^2 test with 3 degrees of freedom was used. Pearson's correlation coefficients based on pairwise correlations were calculated for all populations and all morphological traits. A principal compo-

nent analysis (PCA) was performed on standardized data in a correlation matrix based on the means of selected morpho-physiological traits available for 15 genotypes from the three populations included in both morpho-physiological and AFLP- analysis.

The binary AFLP data matrix produced in GeneMapper was analysed using the program ALP-SURV 1.0 (Vekemans et al. 2002) (distributed by Laboratoire de Génétique et Ecologie Végétale, Université Libre de Bruxelles, Belgium). The following parameters were calculated using the method of Lynch and Milligan (1994): number and proportion of polymorphic loci at the 5% level; expected heterozygosity within a population (*Hj*) and its variance components; total gene diversity in the overall sample (*Ht*); average gene diversity between populations in excess of that observed within populations (genetic differentiation between populations) (*Hb*); the proportion of the total gene diversity that occurs between (as opposed to within) populations ($F_{st} = Hb/Ht$). A t-test was used for comparisons of *Hj* values between populations. To assess molecular genetic variation within and between populations, an analysis of molecular variance (AMOVA) and principal co-ordinates analysis (PCoA), using data from all primer pairs, were carried out using the program GenAlEx v6.41 (Peakall and Smouse 2006) in Microsoft Excel on the matrix of the presence or absence of amplified bands produced by GeneMapper. The PCoA was restricted to the same genotypes as used in the PCA for morpho-physiological traits. A Bayesian clustering method in the software STRUCTURE v2.3.3 (Pritchard et al. 2000; Falusch et al. 2007) was used to assign genotypes to K (unknown number) populations. We slightly modified the method of Hargreaves et al. (2010) using an admixture model with a burn-in period of 10^4 for 10^4 iterations. We assessed a range of values of *K* from 2 to 7 to cover the actual number of populations assessed in the study. All simulations for each *K*-value were repeated 15 times. We selected a K value of 4 based on the rate of change of the log probability between successive values of *K* (ΔK) according to the method of Evanno et al. (2005).

RESULTS

Variation in morpho-physiological traits

Less than 30% of the Ramona population survived the winter and it was subsequently discarded from further measurements in the field. Winter survival was significantly higher in Norstar Sel. than in Norstar, or 88% and 74% respectively, but comparable to Skorradalur although Skorradalur and Norstar Sel. differed significantly in a number of phenotypic traits (Table 2). The total yield of the metamer was

Table 2. Means and standard error of the difference (SED) for winter survival (WS), total dry matter of metamer (DM), stolon diameter (SD), internode length (IL), dry matter per leaf area (DMLA), dry matter per stolon length (DMSL), saturated fatty acid content (SFA), content of linoleic acid (18:2) and linolenic acid (18:3), and proportion of UFA in total fatty acid content (% UFA) of four populations of white clover grown in the field at Korpa Experimental Station.

	WS	DM	SD	IL	DMLA	DMSL	SFA	18:2	18:3	%UFA
	%	mg	mm	mm	mg cm^{-2}	mg mm^{-1}	mg gDM^{-1}			
Norstar	73.6^{b}	35.8^{a}	1.71^{b}	20.33^{ab}	3.52^{a}	0.51^{b}	4.09^{a}	2.87^{a}	1.19^{b}	54.5^{b}
Norstar Sel.	87.5^{a}	34.3^{a}	1.82^{a}	18.14^{b}	3.35^{a}	0.58^{a}	3.97^{a}	3.67^{b}	1.62^{a}	61.1^{a}
Korpa	81.9^{ab}	34.1^{a}	1.76^{ab}	22.52^{a}	3.41^{a}	0.51^{b}	4.22^{a}	2.83^{a}	1.21^{b}	55.7^{b}
Skorradalur	88.9^{a}	20.1^{b}	1.45^{c}	22.20^{a}	3.15^{b}	0.38^{c}	3.47^{b}	2.92^{a}	1.20^{b}	60.1^{a}
Mean	83.0	31.1	1.68	20.79	3.36	0.49	3.94	3.07	1.30	57.8
SED	3.7^{6}	1.97	0.044	1.191	0.100	0.025	0.144	0.296	0.188	2.1
Significance	***	***	***	***	**	***	***	*	*	**

*, **, ***: significant at P < 0.05, P < 0.01 and P < 0.001, respectively. For WS (N=288); SD, IL, DWLA and DWSL (N=261); and UFA, SFA, 18:2, 18:3 and % UFA (N=58). Means with the same superscript within columns do not differ significantly at P = 0.05 in the analysis of variance.

Table 3. Coefficient of variation (% CV) for metamer total dry matter (DM), stolon diameter (SD), internode length (IL), dry matter per leaf area (DMLA), dry matter per stolon length (DMSL), saturated fatty acid (SFA), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3) and proportion of UFA in total fatty acid content (% UFA) measured in four white clover populations grown in the field at Korpa Experimental Station.

	DM	SD	IL	DMLA	DMSL	SFA	18:2	18:3	% UFA
Norstar	30.9	12.6	27.1	11.0	23.8	9.4	32.4	47.3	13.9
Norstar Sel.	26.3	11.4	23.3	8.4	23.2	9.9	21.8	30.5	7.6
Korpa	21.8	10.1	23.1	15.0	14.0	9.1	19.8	30.4	8.4
Skorradalur	23.5	11.2	21.1	12.8	22.1	9.5	30.5	46.6	10.1
χ^2 (3 d.f.)	7.95	3.00	3.94	19.59	16.72	0.10	4.04	3.85	6.25
Significance	**	n.s.	n.s.	***	***	n.s.	n.s.	n.s.	n.s.

*, **, ***: significant at P < 0.05, P < 0.01 and P < 0.001, respectively, and n.s. is non-significant in the Chi-square test.

the same for all populations, except Skorradalur, where it was significantly lower. The dry matter per leaf area (DMLA) was not significantly different between Norstar, Norstar Sel. and Korpa whereas it was significantly lower for Skorradalur. The dry matter per stolon length (DMSL) was highest in Norstar Sel., significantly lower in Norstar and Korpa, and lowest in Skorradalur. There was a positive correlation (r=0.15) between winter survival and DMSL (P<0.05). DMSL and DMLA were also highly correlated (r=0.46; P<0.001). The content of 18:2, 18:3 and degree of unsaturation of fatty acids (% UFA) were significantly higher in Norstar Sel. compared with Norstar. No significant difference was found in saturated fatty acid content (SFA) between the two populations. The coefficient of variation (% CV) tended to be lower in Norstar Sel. compared to Norstar even though this was not significant for any of the traits (Table 3).

Molecular variation

Total number of loci scored by the four AFLP primer pairs was 376 and the number of polymorphic loci at the 5% level ranged from 213 to 272 (mean for all populations was 245). The proportions of polymorphic loci were 72 % and 70 % for Norstar and Norstar Sel. respectively (Table 4). Gene diversity (*Hj*) , estimating the expected heterozygosity was highest in Norstar (0.232), somewhat lower in Norstar Sel. (0.226; P< n.s.) and significantly lower in both Ramona (0.202; P < 0.025) and in Skorradalur (0.188; P < 0.001) (Table 4). Analysis of molecular variance showed that 73 % of the total variation was distributed within and 27 % between populations. The pairwise genetic differentiation (F_{st}) was lowest between Norstar and Norstar Sel., and highest between Skorradalur and Ramona (Table 5).

An inferred grouping of the genotypes (Figure 1) resulted in distinct clusters of Ram-

Table 5. Pairwise genetic differentiation (Fst) between four white clover populations based on data from an AFLP analysis. A value of 0 indicates identical populations and 1 indicates no alleles in common.

	Norstar	Norstar Sel.	Skorradalur
Norstar Sel.	0.022		
Skorradalur	0.169	0.136	
Ramona	0.189	0.202	0.305

Table 4. Proportion of polymorphic loci and gene diversity averaged across loci (Hj) of four populations of white clover, based on an AFLP-analysis with four primer pairs (n=376).

Population	Proportion of polymorphic loci	Gene diversity averaged across loci (H_j) ± S.E.	Comparison of Hj for Norstar vs. others (t-test)
Norstar	0.72	0.232±0.0085	
Norstar Sel.	0.70	0.226±0.0087	n.s.
Skorradalur	0.57	0.188±0.0096	P<0.001
Ramona	0.63	0.202±0.0087	P<0.025

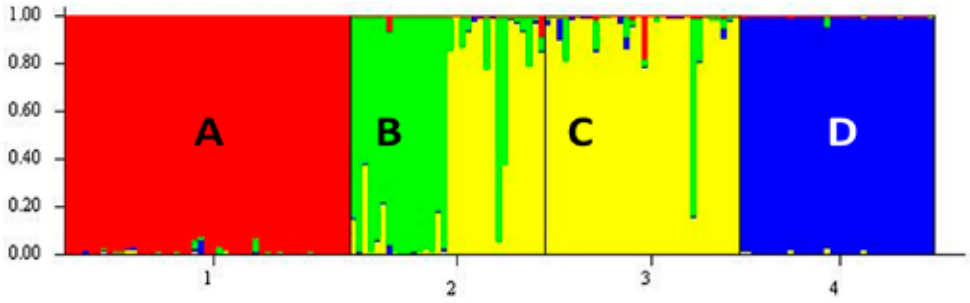

Figure 1. Bar plot of molecular genetic structure with four inferred populations (A, B, C and D marked in red, green, yellow and blue, respectively); the white clover populations Ramona (n=47), Norstar (n=32), Norstar Sel. (n=32) and Skorradalur (n=32) are marked below the figure (each bar indicates one genotype), the y-axis indicates the proportion of molecular genetic variation in each genotype that is assigned to the respective cluster.

ona (Cluster A) and Skorradalur (Cluster D) whereas Norstar was split into two clusters (B, C) one of which was common with Norstar Sel. (Cluster C). The virtual disappearance of cluster (B) from Norstar Sel. indicates that the selection pressure had acted against genotypes of this particular type.

Comparison of morpho-physiological and molecular variation

The biplot display of a principal component analysis (PCA), based on means of morpho-physiological data, shows a reduction in genotypic variation of Norstar Sel. compared to Norstar, primarily in response to increased proportion of unsaturated fatty acids (Figure 2a). Skorradalur is clearly separated from the Norstar populations on the basis of dry matter (DMSL, DMLA) and stolon diameter. A PCoA, based on AFLP data for the same genotypes, shows a similar pattern (Figure 2b).

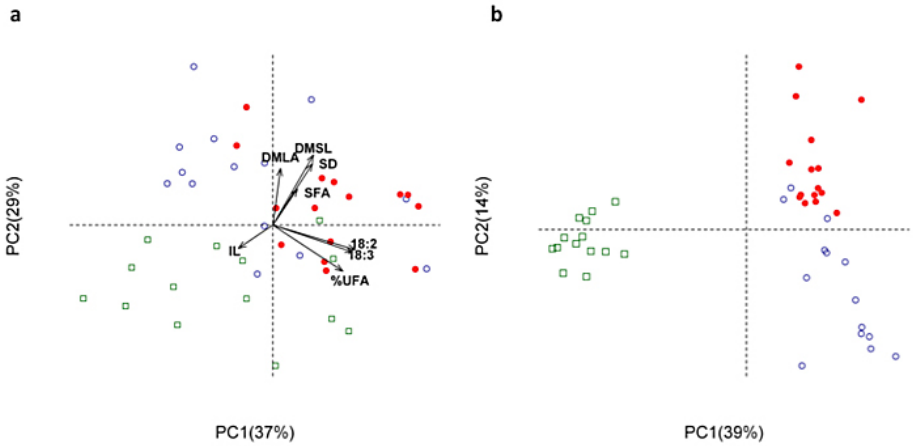

Figure 2. Principal components analysis based on (a) means of morpho-physiological data in a biplot display indicating the weight (arrow length) and action (direction of arrow) of the following traits: dry matter per leaf area (DMLA), dry matter per stolon length (DMSL), stolon diameter (SD), saturated fatty acid content (SFA) content of linoleic acid (18:2) and linolenic acid (18:3), proportion of unsaturated fatty acids in total fatty acid content (% UFA) and internode length (IL), and (b) AFLP data for Norstar (∘), Norstar Sel. (•) and Skorradalur (▫) grown in the field at Korpa Experimental Station.

DISCUSSION

Sufficient intra-population variation in white clover is a prerequisite for populations to adapt to challenging environmental conditions (Williams 1987b). The present study showed that there is ample marker-based genetic variation present in the Norwegian cultivar Norstar (72% of all loci polymorphic) even though greater variation was found in a survey of Dutch white clover populations or 94% (van Treuren et al. 2005). An explanation for the greater inter-population molecular genetic variation that was observed in this study compared to previous studies (van Treuren et al. 2005; Kölliker et al. 2001) could be the highly diverse background of the populations in this study. The variation for morpho-physiological traits in Norstar was comparable to that found in the semi-wild population from Korpa. On the other hand, the wild population Skorradalur contained significantly less variation than the other populations, both at the molecular level and for some of the morpho-physiological traits. It would be tempting to speculate that this is the result of a founder effect, as the population probably originates from only a few individuals, which have since been exposed to natural selection. The effect of genetic drift may further have reduced the genetic variation within the population.

Both morpho-physiological variation and molecular genetic variation in Norstar has clearly made it possible for the cultivar to adapt to the environmental conditions prevailing at Korpa Experimental Station, Iceland. The multivariate analyses demonstrate that there has been a genetic shift in the survival population Norstar after surviving for four years in the field in a marginal environment. The direction of the change in both physiological variation for UFA and molecular genetic variation was towards locally adapted and wild material. The inferred population structure based on molecular genetic variation showed clusters in Norstar which were not present in its survivor population, indicating that a selection had acted against genotypes with this genetic structure.

Norstar Sel. had levels of winter hardiness comparable to those of the wild Icelandic population after only four years of natural selection, yet at the same time its ability to produce harvestable dry matter was unchanged. Similarly, the proportion of unsaturated fatty acids, primarily 18:2 and 18:3, had increased in the survivor population compared with the original population sown. These results are in line with previous studies (Collins et al. 2002, Dalmannsdóttir et al. 2001). The significant increase in the proportion of unsaturated fatty acids in the survival population indicates that these fatty acids are of particular importance in the acclimation process in the autumn (Dalmannsdóttir et al. 2001).

Based on the assessment of morpho-physiological traits, changes in the survival population could be explained as directional selection in traits associated with persistence (stolon morphology and content of unsaturated fatty acids) and stabilising selection in traits associated with yield. The study supports results by Helgadóttir et al. (2008) in that there seems to be no conflict in simultaneous breeding for high winter survival and large leaves, and hence, high yielding capacity under northern conditions. The wild Icelandic population included in the study did not have morphological traits of sufficient agronomic importance in spite of good winter survival and a low level of molecular genetic diversity and, hence, can be excluded as a potential contributor in future breeding programmes.

Attempts have been made to compare estimates of genetic variability based on molecular markers and morpho-physiological traits and generally a poor correlation between the data types has been found (Kölliker et al. 2001). As morpho-physiological variation can result from the action of only a small number of genes, such divergence may have little relationship with the overall degree of molecular genetic variation within and between populations (Arroyo-García et al. 2001). However, in a genetic diversity study with white clover from the Netherlands there were indications that AFLP markers might be linked with loci

for agronomic traits that are influenced by natural selection (van Treuren et al. 2005). Comparable patterns of population structure emerged from the two multivariate analyses, based on either morpho-physiological traits or AFLP data. Although this may be a result of population genetic structuring, it could also indicate that marker-based variation can reflect the genetic diversity assessed by either morphological or physiological measurements. Molecular genetic variation assessed by neutral markers can therefore add valuable information on the genetic structure within the germplasm and can be used in conjunction with data on morpho-physiology in plant breeding programmes with white clover.

In conclusion, the present study has demonstrated that rapid genetic shift can occur in a white clover cultivar grown under marginal growing conditions. A cultivar of northerly origin seems to contain sufficient genetic diversity to build the base for further selection for improved winter hardiness without sacrificing yielding ability.

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