

Patterns of amplified restriction fragment polymorphism in the germination of *Festuca hallii* seeds

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Abstract

Timing of seed germination influences plant lifetime fitness and can affect the ability of plant populations to colonize and persist in changing environments. However, the genetic variation of the seed germination response remains poorly understood. The amplified restriction fragment polymorphism (AFLP) technique was applied to characterize the genetic variation of germinated seeds collected from three *Festuca hallii* populations in the Canadian prairie. Three subpopulations with early, intermediate and late germination were identified from each population, based on germination tests at 10, 15 and 20°C in controlled growth chambers. Three AFLP primer pairs were employed to screen a total of 540 assayed seedling samples and 188 polymorphic AFLP bands were scored for each sample. None of the assayed AFLP bands were significantly associated with seed germination, but marked differences in estimates of mean band frequency were observed for various groups of germinating seeds under different test temperatures. Partitioning of the total AFLP variation showed that 5.9% AFLP variation was present among seeds of the three populations, 0.3% among seeds of three germination subpopulations, and 0.5% among seeds grouped for germination temperature. Genetic differentiation was significant among 27 groups of seeds representing population, germination timing and test temperature. Subpopulations with early and intermediate germination shared similar genetic backgrounds and were genetically differentiated from the late germination subpopulation. These results indicate that seed genotypes respond slightly differently to environmental variation, resulting in significant but

weak genetic differentiation in the germination of *F. hallii* seeds. Implications for plant establishment and fescue restoration are discussed.

Keywords: AFLP, *Festuca hallii*, genetic variation, germination temperature, germination timing

Introduction

Seeds within a population germinate over a period of time even under optimal germination conditions without dormancy. Such characteristics have adaptive value (Donohue, 2002; Mandak *et al.*, 2006) because germination over time also determines the ability of plant populations to colonize and persist in changing environments (Donohue *et al.*, 2005a, b). Consequently, assessment of environmental and genetic influences on seed germination should allow for a better understanding of fundamental processes of population establishment, range expansion and geographic differentiation (Donohue, 2005).

There is considerable evidence that germination timing is under some degree of heritable genetic control (Squire *et al.*, 1997; Cabin *et al.*, 1998; Marshall *et al.*, 2000). For example, Squire *et al.* (1997) showed that subpopulations with early and late germination of an oilseed rape cultivar produced progeny with distinct germination characteristics, indicating genetic control of germination rate. Donohue *et al.* (2005c) detected a significant genotype-by-environment interaction influencing germination timing in recombinant inbred lineages of *Arabidopsis thaliana*. Some studies in natural populations showed that different germination environments favoured particular genotypes and significant genetic differentiation existed between early and late-germinating seeds (Cabin *et al.*, 1997; Mandak *et al.*, 2006). However, the

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extent to which germination timing is genetically or environmentally controlled remains poorly understood (Donohue, 2005).

Plains rough fescue [*Festuca hallii* (Vasey) Piper] is the diagnostic, long-lived perennial species in Fescue Prairie, and a North American endemic tetraploid ($2n = 4x = 28$), presumably outcrossing (Aiken *et al.*, 1996 onwards, 1997; Anderson, 2006). It grows on deep, well-drained soils, and is highly palatable to livestock, particularly during the winter. The fescue plant can propagate with seeds and rhizomes but seed production is erratic, perhaps dependent upon conditions in the previous growing season (Johnston and MacDonald, 1967; Romo *et al.*, 1991). Remnant *F. hallii* stands have been found throughout the Canadian prairies. Efforts have been made to re-establish a network of fescue-dominated patches across its historic range. However, the effectiveness of such restoration efforts remains uncertain, as the biology of dominant fescue species is poorly understood (Romo, 2003) and the germination of *F. hallii* seeds may affect the success of plant establishment in changing environments (Romo *et al.*, 1991).

The objective of this study was to assess the comparative genetic variation of germinated seeds collected from three *F. hallii* populations in the Canadian prairie using the amplified restriction fragment polymorphism (AFLP) technique. The AFLP technique (Vos *et al.*, 1995) is a robust, highly effective molecular tool that has been widely applied to assess genetic variability in grass species (e.g. Larson *et al.*, 2001a, b; Fu *et al.*, 2005a, b; Qiu *et al.*, 2007). Three subpopulations of seeds with early, intermediate and late germination were identified based on the position of the seeds from each population in the time-profile of germination under a given temperature. Specifically, we strived to address the following questions. Is the germination timing of *F. hallii* seeds affected by germination temperature? Does the same germination subpopulation of seeds display a genetic difference under variable germination temperatures? Do different germination subpopulations of seeds show the same pattern of genetic variation under the same germination temperature? And, is there any genetic differentiation among various groups of seeds for surveyed populations?

Materials and methods

Seed collection, seed germination and subpopulation allocation

Three *F. hallii* populations were selected for this study: Prince Albert National Park (53°35'16"N, 106°02'26"W, located in Boreal Transition Ecoregion), Turtleford (53°27'38"N, 109°03'12"W, in Boreal Transition

Ecoregion) in Saskatchewan, and Riding Mountain National Park (50°48'35"N, 100°14'42"W, in Mid-Boreal Upland Ecoregion) in Manitoba. These populations were abundant in seed production and represented the geographical range of all the surveyed populations. Seeds were collected within 1 week in late July. Collected seeds were kept in paper bags under room temperature for 2 months, then cleaned using a seed blower, and stored at -18°C until use. Benomyl powder (0.05%) was applied to sterilize seeds at the beginning of the germination test.

Seed germination was investigated under 10, 15 and 20°C temperature regimes in growth chambers (Sanyo Versatile Environment Chamber MLR-350H, Sanyo Scientific, USA). A randomized complete block design was used with three replicates, and replicates were put into growth chambers at 1-week intervals. Seeds were randomly selected from each population and germinated on top of two layers of filter paper (Whatman No.1) in separate cells of 96-well plates. The filter paper was moistened with 40 μl of distilled water and one seed was placed on the top of the paper. In total, 288 seeds were germinated for each population and temperature. Seeds were checked and counted daily and the days to germinate for each seed were recorded. Germinated seeds were transplanted into pots and grown in the greenhouse. Seedlings were labelled with population, temperature, replicate and days to germination. Germination tests were terminated when no seeds germinated for 14 consecutive days. Non-germinated seeds were then tested for viability by finger pressing. Viable seeds were firm when pressed with finger tips. After finger pressing, all the soft seeds were cut longitudinally and checked for embryos. The numbers of viable, dead and empty seeds were recorded. Results showed that ungerminated seeds were either dead or empty.

Final germination percentage was calculated based on total seeds incubated minus dead and empty seeds. Since the final germination percentage of each population and temperature was not significantly different among replicates, transplanted seedlings were pooled among replicates for each population and temperature and ranked by the days to germination for subpopulation allocation. The first 0–10% of the seeds germinated were classified as early subpopulations, the middle range of seeds germinated (45–55%) were classified as intermediate subpopulations, and the last (range 90–100%) as late subpopulations (Fig. 1).

AFLP analysis

Twenty resulting seedlings were randomly selected from each germination subpopulation and a total of 540 seedling samples were obtained from 27 groups

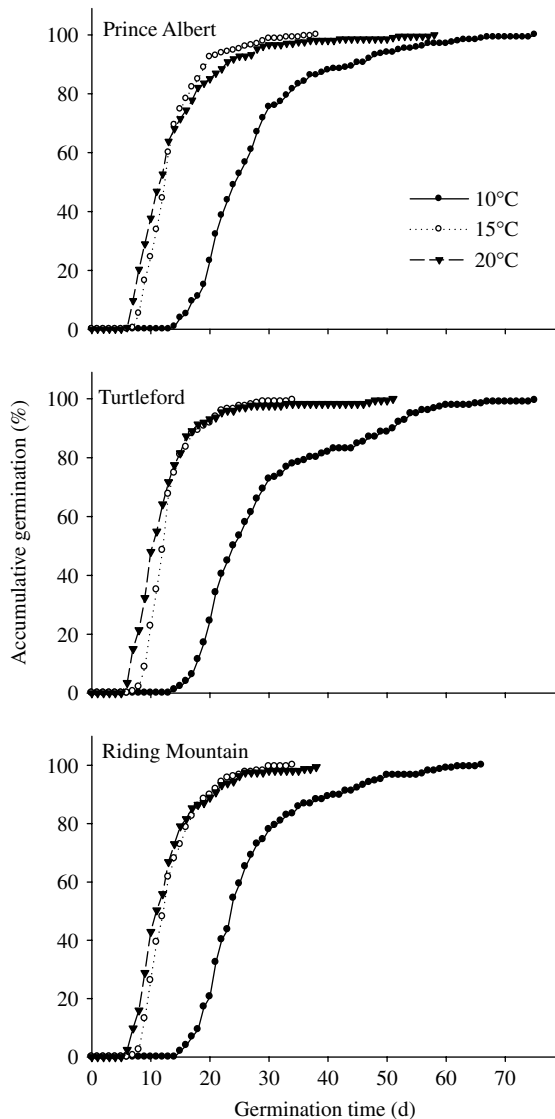


Figure 1. The time-course of seed germination at 10, 15 and 20°C for three *F. hallii* populations. Germination percentages, based on the total number of seeds incubated minus dead and empty seeds, were used to identify germination subpopulations. The first 0–10% of the seeds germinated were classified as early germination subpopulations, the middle range of the seeds germinated (45–55%) were classified as intermediate germination subpopulations, and the last (range 90–100%) as late germination subpopulations.

representing germination subpopulations, temperature regimes and seed-source populations. Young leaf tissue of selected seedlings was individually harvested after 45 d in the greenhouse from transplanting, freeze-dried and stored at -80°C until use. Genomic DNA was extracted from the leaf samples using the DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, Ontario, Canada) according to the manufacturer's instructions. Extracted DNA was quantified by fluorometry using Hoechst 33,258 stain (Sigma Chemical Co., St. Louis,

Missouri, USA), and diluted to $25\text{ ng }\mu\text{l}^{-1}$ for AFLP analysis.

The AFLP™ Analysis System 1 (Life Technologies, Burlington, Ontario, Canada) was applied following the protocol described by Vos *et al.* (1995) with a modification of using $\gamma^{33}\text{P}$ to label *EcoRI* selective primers. After electrophoresis of amplified DNA fragments, the gel was transferred to Whatman 3MM paper, dried on a gel dryer for 2 h at 80°C , and exposed to Kodak BIOMAX film at -80°C for 1 to 7 d, depending on the signal intensity. Three *EcoRI*:*MseI* primer pairs (E + AAC/M + CAG, E + ACG/M + CTA, and E + AGG/M + CTC) were used to screen all samples, based on the previous AFLP analysis of *F. hallii* (Qiu *et al.*, 2007). To minimize technique-related and scoring errors, three duplicated samples of one individual were arranged across all gels as a control.

Automated analysis of banding patterns on 18 gels was conducted using GelComparII™ (Applied Maths, Belgium). The gel images were automatically scored as 1 (present) or 0 (absent). The duplicated samples were used to assess the consistency of AFLP reactions. Only the bands either present or absent in all the 18 control samples for each primer pair were output for data analysis.

Data analysis

A three-way analysis of variance (ANOVA) was conducted for the days to germinate (in log scale) with respect to source population, germination subpopulation and temperature. Since the population displayed significant interactions with the other two factors, a two-way ANOVA was then conducted for subpopulation and temperature. Significance of group means was assessed with *post hoc* multiple comparison tests.

The presence/absence data from the AFLP samples were analysed for the level of polymorphism by counting the total number of bands and the number of polymorphic bands and calculating their band frequencies with respect to source population, germination subpopulation and temperature. To identify the chromosome regions or segments associated with seed germination, a χ^2 -test was performed following Fu *et al.* (2005b) to determine the significance of the AFLP differences at each AFLP band between early and late germination subpopulations. This was repeated for all polymorphic AFLP bands for each temperature regime and each population.

Analysis of molecular variance (AMOVA) was performed with respect to source population, germination subpopulation and temperature regime. This analysis not only allows partitioning of the total AFLP variation into within- and among-group components,

Table 1. Days to completion of germination with respect to germination subpopulation and test temperature in three *F. hallii* populations. The numbers followed by different letters are statistically different within a population at $P \leq 0.05$

Population	Early			Intermediate			Late		
	10°C	15°C	20°C	10°C	15°C	20°C	10°C	15°C	20°C
Prince Albert	16 c	8 e	7 e	25 b	13 cd	12 d	52 a	25 b	27 b
Turtleford	16 d	9 fg	6 g	24 b	12 e	10 ef	52 a	20 c	23 b
Riding Mountain	16 d	8 f	7 f	24 b	12 e	11 ef	43 a	21 c	24 b

but also provides a measure of inter-group distances as a proportion of the total AFLP variation residing between any two groups (the Phi statistic; Excoffier *et al.*, 1992; Huff *et al.*, 1998). The significance of variance components and inter-group distances was tested with 10,098 random permutations. Three models of genetic structuring for germinated seeds were assessed: source population, subpopulation of germination and temperature group.

A group-specific AMOVA analysis was also performed to assess the differences in AFLP variation among 27 groups representing source population, germination subpopulation and temperature regime. The resulting group–distance matrix was analysed using NTSYS-pc 2.3 (Rohlf, 1997) and unrooted trees were obtained from distance matrices using the neighbour-joining procedure (Saitou and Nei, 1987) to assess the genetic differentiation of the 27 representative groups of the fescue seeds.

Results

The germination timing of *F. hallii* seeds was affected by germination temperature. The interactions between germination timing and temperatures were significant for the days to germination (Table 1). Differences in germination time between early and intermediate subpopulations were smaller than those between intermediate and late germination subpopulations. Seeds took many more days to germinate at 10°C than at 15 and 20°C. No significant difference in the days to germinate was found between 15 and 20°C, except for seeds of late germination subpopulations from Riding Mountain and Turtleford populations.

Three germination subpopulations in each population displayed variation in mean band frequency under three temperature regimes (Fig. 2). The same germination subpopulation of seeds displayed genetic differences under variable germination temperatures. Germinating seeds in all three populations had higher estimates of mean band frequency at 20°C than at 10°C. Different germination subpopulations of seeds showed different patterns of genetic variation under the same germination temperature. Early germination subpopulations had consistently lower estimates

of mean band frequency than late germination subpopulations at 10°C but had consistently higher estimates of mean band frequency than late germination subpopulations at 15°C. At 20°C, the estimate of mean band frequency was higher in early germination subpopulations than the others from the Prince Albert

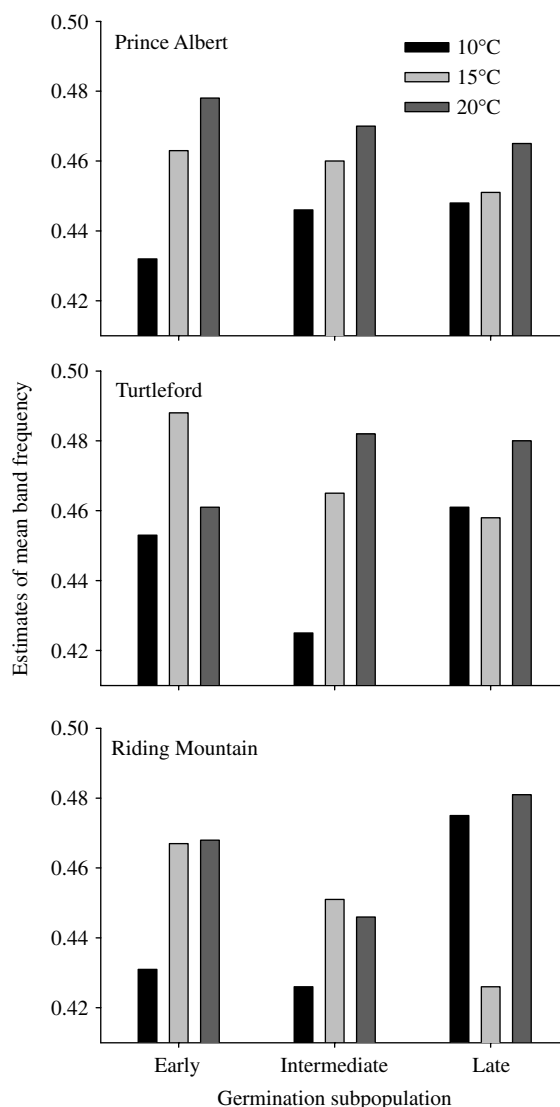


Figure 2. Patterns of estimates in mean AFLP band frequency with respect to source population, germination subpopulation and temperature regime.

Table 2. Analysis of molecular variance (AMOVA) within and among groups of germinating seeds of the three *F. hallii* populations under variable test temperatures. Three models of genetic structuring were assessed with respect to source population, germination subpopulation and test temperature

Source of variation	df	Variance components	% Variation
Overall			
Among populations	2	1.48	5.72
Among germination subpopulations and temperatures within populations	24	0.49	1.88
Within germination subpopulations and temperatures	483	23.99	92.40
Group for germination timing			
Among germination subpopulations	2	0.07	0.26
Within germination subpopulations	507	25.41	99.74
Group for test temperature			
Among groups for temperature	2	0.12	0.48
Within groups for temperature	507	25.37	99.52

All the variance components were statistically significant at $P < 0.001$ level, as calculated from 10,098 random permutations.

population, but it was lower than the others from the Riding Mountain and Turtleford populations. None of the 188 polymorphic AFLP bands displayed a significant AFLP difference between the early and late germination subpopulations under any temperature in any population assayed, after Bonferroni correction.

There was genetic differentiation among various groups of seeds for surveyed populations. Partitioning of the total AFLP variation showed that 92.4% of the total AFLP variation was held among seeds of any given population, 5.7% among seeds of different populations, and 1.9% among seeds grouped for germination timing and temperatures (Table 2). These variation fractions were significantly different from zero at $P < 0.0001$ based on the permutation test.

Analysis of molecular variance with respect to germination timing and temperature regime revealed that only 0.3% AFLP variation resided among seeds grouped for germination timing and 0.5% AFLP variation among seeds grouped for temperature regime.

Assessment of group-wise genetic differences with the proportional AFLP variations showed significant genetic differences among three germination subpopulations (Table 3). The largest difference was present between intermediate and late germination subpopulations and the smallest between early and intermediate germination subpopulations. For specific populations and germination temperatures, the AFLP differences among germination subpopulations could be up to 3.7%. The differences between early and

Table 3. Genetic differentiation between germination subpopulations of *F. hallii* seeds with respect to source population and test temperature, as measured by the proportion of the total AFLP variation

Population/temperature	Subpopulation pairs		
	Early versus Intermediate	Early versus Late	Intermediate versus Late
Prince Albert			
10°C	0.0000	0.0203*	0.0038
15°C	0.0095	0.0367**	0.0306**
20°C	0.0000	0.0213*	0.0288**
Turtleford			
10°C	0.0000	0.0065	0.0256**
15°C	0.0141	0.0266**	0.0119
20°C	0.0022	0.0070	0.0037
Riding Mountain			
10°C	0.0110	0.0076	0.0145
15°C	0.0090	0.0181*	0.0095
20°C	0.0248**	0.0283**	0.0095
All populations and temperatures	0.0017*	0.0029**	0.0031***

*, ** and ***, significant at $P \leq 0.05$, 0.01 and 0.001, respectively.

Table 4. Genetic differentiation within the same group of *F. hallii* seeds under variable test temperatures with respect to source population and germination subpopulation, as measured by the proportion of the total AFLP variation

Population/germination subpopulation	Germination temperature pairs		
	10°C versus 15°C	10°C versus 20°C	15°C versus 20°C
Prince Albert			
Early	0.0042	0.0169	0.0042
Intermediate	0.0233**	0.0260**	0.0243**
Late	0.0000	0.0334**	0.0195
Turtleford			
Early	0.0166*	0.0308***	0.0126
Intermediate	0.0164*	0.0232**	0.0074
Late	0.0419***	0.0250**	0.0050
Riding Mountain			
Early	0.0000	0.0010	0.0085
Intermediate	0.0219*	0.0298**	0.0104
Late	0.0276**	0.0320**	0.0261**
All populations and subpopulations	0.0050***	0.0087***	0.0004

*, ** and ***, significant at $P \leq 0.05$, 0.01 and 0.001, respectively.

late germination subpopulations were found in all populations and temperatures, and those between early and intermediate germination subpopulations were found only in the Riding Mountain population under the germination temperature of 20°C.

Early germination subpopulations from both Prince Albert and Riding Mountain populations did not display significant AFLP differences under variable germination temperatures (Table 4). Intermediate germination subpopulations from the Prince Albert population and late germination subpopulations from the Riding Mountain population showed significant AFLP variation under germination temperatures between 15 and 20°C. The largest AFLP difference was observed for germinating seeds under temperatures between 10 and 20°C and the least AFLP variation between 15 and 20°C.

Assessment of the genetic differentiation among 27 groups of germinating seeds revealed three distinct clusters with each population grouped together (Fig. 3). The most genetically distinct cluster consisted of various groups of germinating seeds from the Riding Mountain population. Within each distinct group, early and intermediate subpopulations tended to be clustered together.

Discussion

The present AFLP analysis of germinating fescue seeds revealed several interesting patterns of genetic

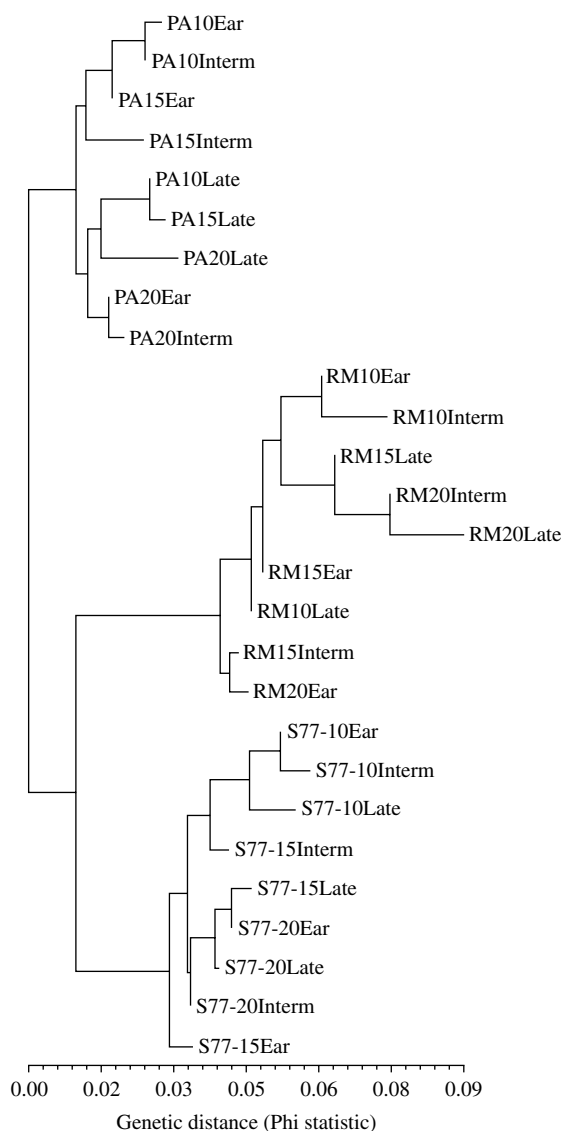


Figure 3. A neighbour-joining tree of the 27 groups of germinating seeds representing source population, germination subpopulation and temperature regime, which was based on pairwise genetic distances (Phi statistics) derived from the analysis of AFLP variation. Each group is labelled with source population (PA, Prince Albert National Park; RM, Riding Mountain National Park; S77, Turtleford) and test temperature (10, 10°C; 15, 15°C; 20, 20°C) and germination subpopulation (Ear, Interm and Late for early, intermediate and late subpopulations, respectively).

variation with respect to seed germination. First, non-random patterns of mean band frequency were observed with variable germination temperature. Second, no chromosomal regions or segments were identified to be significantly associated with seed germination. Third, the proportions of the total AFLP variation explained by germination timing and due to test temperatures were 0.3% and 0.5%, respectively. Fourth, early and intermediate germination

subpopulations shared similar genetic backgrounds and were genetically differentiated from late germination subpopulations. These findings clearly indicate a weak genetic basis of adaptive seed germination and provide the first molecular evidence for genetic differentiation in the germination of *F. hallii* seeds.

Significant changes detected in mean band frequency between early and late germination subpopulations under increased temperature indicate that *F. hallii* seed genotypes respond differently to environmental variation during the germination process. This finding accords well with those from related studies comparing allele frequencies using allozyme markers between seed-bank and above-ground populations under different soil water regimes (Cabin *et al.*, 1997), in optimal and suboptimal conditions (Mandak *et al.*, 2006) and across multiple populations (Cabin *et al.*, 1998). Patterns of change in mean band frequency in this study appear to imply that the germination timing of *F. hallii* seeds was involved with a genetic sorting of seed genotypes influenced by germination temperatures. If this is true, the AFLP variation observed in this study should reflect (or is genetically linked to) the variation residing among individual seeds in physiological, biochemical or molecular processes required for germination. First, variation among individual seeds in hormone sensitivity (Liptay and Davidson, 1971) and enzyme activity such as endo- β -mannanase (Still and Bradford, 1997; Still *et al.*, 1997) was found to be associated with the variation in germination time among seeds within a population. The hormone sensitivity and enzyme activity can be affected by different temperatures. Second, seed germination potential is genetically controlled in dry seeds, while the germination rate is controlled by changes of gene expression initiated upon imbibition (Rajjou *et al.*, 2004; Holdsworth *et al.*, 2008). Zhang (2008) found differences in gene expression pattern between 50% germinated and 50% ungerminated canola seeds imbibed in water, gibberellic acid (GA), saline and abscisic acid (ABA) solutions. These differences may be due to genetic sorting during imbibition in different solutions.

Assessing genetic differentiations among *F. hallii* seeds grouped for germination timing and test temperature further confirms that early germination subpopulations are genetically differentiated from late germination subpopulations. Thus, the germination of *F. hallii* seeds was under weak genetic control. A quantitative trait locus (QTL) has been identified in *Arabidopsis* for germination rate (Clerkx *et al.*, 2004), which ties in with the observed differences in genetic variation for early and late germination. A weak genetic differentiation of the germination of *F. hallii* seeds is not surprising. First, germination timing is a composite trait that results from mechanisms of dormancy induction and maintenance, dormancy

breaking and germination after dormancy breaking (Bewley, 1997). Thus, many genes, probably of small effects each, are expected to be involved and stimulated to influence germination under variable environmental conditions. Second, the inability to detect significant chromosomal regions associated with germination may be due to the use of limited sample size (i.e. 20) per germination subpopulation and the limited genomic coverage of the assayed AFLP fragments. It might also reflect the true small effects of those genes involved with germination responses. Third, the smaller proportion of the total AFLP variation explained by germination timing than test temperature signals that environmental factors, or their interactions with genetic factors, may play a more important role in initiating germination. This reasoning also gains support from the finding of increased mean band frequencies with higher test temperatures, particularly for early subpopulations (Fig. 2).

Different patterns of seed germination in different temperature regimes observed here may enhance the ability of fescue plants to adapt to changing environments. The warmer and drier future climate as predicted for the Canadian prairie may favour earlier germination of subpopulations, as these groups of seeds could germinate in early spring. Different habitats with different environmental conditions could favour different alleles and select different seed genotypes during a certain favourable period of time for germination. Thus, the chance to succeed in restoration of remnant fescue stands with seeds may vary across the Canadian prairie. The patterns of seed germination revealed in the three populations should provide a rough guide to secure a higher germination rate in the restoration effort. However, the temperatures tested in this study may not realistically reflect real soil temperatures (Romo *et al.*, 1991). Also, other environmental factors, such as moisture, may contribute more to the genetic sorting than temperature, as genetic and germination variation among *F. hallii* populations were more correlated with mean annual precipitation than mean annual temperature of sites (Qiu, 2009; Qiu *et al.*, 2009). Moreover, maternal environmental effects on seed germination have been widely identified in many species (Roach and Wulff, 1987; Lacey, 1996; Pendleton and Meyer, 2004; Luzuriaga *et al.*, 2006; Boyd *et al.*, 2007) and were not considered in this study.

This study has demonstrated the existence of subtle genetic control for seed germination and establishment of seed genotypes in response to environmental variation, which offers the potential for seeds to respond to spatial and temporal variation. This information should be helpful in explaining how fescue seeds germinate, how fescue plants adapt to a changing environment, and how substantial patterns of genetic variation are formed among fescue stands,

which has been demonstrated in Fig. 3. However, this study provides only a preliminary glance at the genetics of seed germination in a native grass. Further research is needed to determine the number, effect and location of genes controlling germination timing. With the development of informative molecular markers, such research will offer many insights into the behaviours of germination genes in natural populations (Donohue, 2005). These insights will facilitate the development of reliable thermal time and hydrothermal time models to predict germination variability and quantify the impacts of temperature and water on germination (Garcia-Huidobro *et al.*, 1982; Kebreab and Murdoch, 1999a, b; Wang *et al.*, 2004; Qiu *et al.*, 2006) and consequently enhance our understanding of plant establishment in changing environments.

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