Patterns of Genetic Variation in *Festuca hallii* (Vasey) Piper across the Canadian Prairie

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ABSTRACT

Festuca hallii [(Vasey) Piper] (plains rough fescue) is a dominant native grass species in the Fescue Prairie region of North America that has undergone dramatic range reduction in the past century. This research is undertaken to address the related issues associated with the effectiveness of sampling in capturing genetic diversity, the influence of habitat fragmentation on genetic variation, the geographic variation of seed germination characteristics, and the comparative genetic variation of differential germination. It was found that the tiller samples revealed slightly larger among-population variation than the seed samples. The fescue plant was genetically diverse, as revealed by the proportion of polymorphic bands, the mean band frequency, and the within-population variation. The genetic variation was not highly differentiated with only 6.5% of the total AFLP variation residing among populations. Mantel test revealed a significant correlation between genetic and geographic distances and a spatial autocorrelation up to 60 km among populations was detected. Base temperatures (T_b, minimal or base temperature permitting germination) of the 15 populations fell into a narrow range within 2.2^{o}C with an average of 1.1^{o}C. High final germination percentage was reached at a wide temperature range from 5 to 20^{o}C with the highest germination percentage at 10^{o}C. Germination rate index increased with increasing temperature from 5 to 20^{o}C. T_b was positively correlated with latitude and negatively with longitude and the thermal time requirement for 50% germination was negatively correlated with latitude. The AFLP variation and germination responses were significantly associated with environmental attributes related to moisture, indicating local adaptation. However, the AFLP variation and germination was not significantly associated with the estimated population size and geographic distance to the nearest neighbor, suggesting that fragmentation has not generated considerable genetic and germination impact on the fescue populations. Marked differences in estimates of mean band frequency were observed for various groups of germinating seeds under different test temperatures. Comparisons of AFLP variation among 27 groups of seeds representing population, germination timing and test temperature indicates seed genotypes respond slightly differently to environmental variation, resulting in significant but small impact of germination timing and temperature on the genetic diversity of populations. These findings are significant not only for understanding and predicting

the ecological adaptation of the species, but also for formulating effective restoration strategies for remnant populations.

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1. INTRODUCTION

Anthropogenic fragmentation of the formerly continuous natural ecosystems consists of reduced population size and increased isolation, leading to remnant plant populations (Saunders et al., 1991; Hooftman et al., 2004; Banks et al., 2005). Habitat fragmentation can result in genetic erosion and differentiation due to founder effects, random genetic drift, inbreeding, and reduced gene flow, and in the long term, these small populations can have degraded ecological fitness and reduced ability to adapt to changing environments, and potentially become extinct (Templeton et al., 1990; Ellstrand and Elam, 1993; Young et al., 1996). Maintaining adequate levels of genetic diversity within and among populations is critical for plant populations to persist in the face of increasing habitat fragmentation (Ellstrand and Elam, 1993; Falconer and Mackay, 1996; Frankham and Ralls, 1998). Doing so requires effective conservation, restoration and management strategies, which demands a better understanding of the relative levels of within- and among-population genetic diversity.

Fescue Prairie, bordering the boreal forest to the south and centred in Saskatchewan, Alberta, Manitoba, Montana, and North Dakota (Aiken et al., 1996, 1997; Anderson, 2006), is one of the most threatened ecosystems in the Canadian Prairies (World Wildlife Fund, 1988). Due largely to agricultural conversion, Fescue Prairies have been threatened over the last 100 years (World Wildlife Fund, 1988). Less than 5% of the Fescue Prairie remains in Saskatchewan (Grilz and Romo, 1995; Gerry and Anderson, 2002) and in Manitoba (Grilz and Romo, 1994), and about 15% in Alberta (Alberta Environmental Protection, 1997). Much of what remains of Fescue Prairie occurs in small isolated patches, surrounded by cultivation or hay fields (Archibold and Wilson, 1980; Grilz and Romo, 1995). Efforts have been made to protect Fescue Prairie remnants within national parks, provincial parks and provincial natural areas. However, the effectiveness of these conservation practices remains largely unknown.

Plains rough fescue [*Festuca hallii* (Vasey) Piper] is the diagnostic, perennial species in Fescue Prairie, and a North American endemic tetraploid ($2n = 4x = 28$), presumably outcrossing (Aiken et al., 1996, 1997; Anderson, 2006). *F. hallii* has been found widely distributed throughout the Canadian Prairies, including Moist Mixed Grassland, Mixed Grassland, Aspen Parkland, and Boreal Transition (Ecological Stratification Working Group, 1996). Understanding

genetic variation and seed germination response of *F. hallii* is important not only for understanding and predicting the evolutionary potential and the ecological adaptation of the species, but also for formulating the effective strategies of conserving and restoring remnant plant populations (Groom et al., 2006). While a few biological studies have been reported on this genus (Coupland and Brayshaw, 1953; Pavlick and Looman, 1984; Romo et al., 1991), ecological and genetic research on this species is limited.

Sampling plant materials from natural stands represents an important component in plant genetic diversity research, germplasm conservation and germplasm improvement for pasture seeding. However, little attention has been paid to assessments of the effectiveness of sampling in capturing genetic diversity in native plant species (Sackville Hamilton and Chorlton, 1995; Johnson et al., 2004). A recent study of little bluestem (*Schizachyrium scoparium* (Michx.) Nash) revealed that a greater genetic diversity was captured in tiller than seed samples (Fu et al., 2004a). Similar to little bluestem, *F. hallii* is a densely tufted bunchgrass with short rhizomes, and can propagate via seeds and rhizomes. However, its seed production is erratic, largely dependent upon conditions in the previous growing season (Johnston and MacDonald, 1967; Romo et al., 1991). These features suggest possible sampling differences in capturing genetic diversity.

Genetic diversity can be shaped by the interaction between habitat fragmentation and evolutionary processes, such as gene flow, genetic drift, and selection. For a widespread species such as *F. hallii*, different environmental factors may exert selection pressure for local adaptation. Temperature, moisture availability, longitude and latitude are important variables controlling the distribution of grassland species in southern Alberta (Hill et al., 2000). A number of environmental factors, such as precipitation, temperature and geographic gradient may exert selection pressure thus leading to genetic divergence (Still et al., 2005). Limited information is available on the genetic and ecological impacts of habitat fragmentation on fescue plants. Little is known about the interactions of habitat fragmentation with habitat and environmental attributes to influence the long-term survival of small, isolated fescue populations.

The ecological restoration of altered natural systems is a major action for conservation (Krauss et al., 2007). The collection of seeds and successful seed germination and seedling establishment are critical components of ecological restoration (Eriksson and Ehrlen, 1992; Pendleton and Meyer, 2004; Krauss et al., 2007). Seed germination is a complex trait influenced by both genetic determinants and environmental factors (Pendleton and Meyer, 2004; Schmuths

et al., 2006; Boyd et al., 2007). Seed germination varies with the environmental conditions of the mother plants during seed development, such as temperature (Lacey, 1996; Sugiyama, 2003) and soil moisture conditions (Luzuriaga et al., 2006). The within-population variation in germination has shown interactions between seed genotypes and environments (Baskin and Baskin, 1989; Evans and Cabin, 1995; Cabin et al., 1997; Donohue, 2005; Donohue et al., 2005c). The identification of geographic pattern in germination thresholds can help us understand species distribution range, predict possible changes in its distribution range as a result of global warming, and develop suitable conservation strategies. The study of genetic and environmental control on germination timing is important for the conservation of genetic diversity, particularly under the climate change scenarios.

Advances in molecular genetics have offered a wide array of molecular techniques, that can be applied to address questions of conservation significance and catalog diversity at multiple ecological scales (Wayne and Morin, 2004). Molecular analysis of genetic variation among individuals of a population can offer a means of monitoring the genetic variability of a declining population and assess genetic consequences of fragmentation on remnant populations (Cruzan, 2001; Wallace, 2002). The AFLP technique (Vos et al., 1995) is a robust, highly effective tool and 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). The demonstrated effectiveness of AFLP markers in detecting and monitoring genetic variability in *F. hallii* will open a new research avenue to address challenging conservation issues and facilitate the conservation of declining populations of other plant species, particularly residing within the Fescue Prairie of western Canada.

The objectives of this study were 1) to assess the comparative genetic diversity of six *F. hallii* populations in Manitoba and Saskatchewan and their corresponding seed collections using AFLP markers; 2) to assess genetic diversity within and among 30 *F. hallii* populations and the correlations of genetic diversity with geographical, environmental and ecological attributes; 3) to assess the germination characteristics of seeds collected from 15 populations across the Canadian Prairie and the correlations of germination characteristics with genetic, geographic, demographic and climatic variables; and 4) to evaluate genetic variability within three seed *F. hallii* populations under different temperatures.

2. LITERATURE REVIEW

2.1 *Festuca hallii* (plains rough fescue) in the Fescue Prairie of Canada

Fescue Prairie is bordering the boreal forest to the south and centred in Saskatchewan, Alberta, Manitoba, Montana, and North Dakota (Aiken et al., 1996, 1997; Anderson, 2006). Currently it is one of the most threatened ecosystems in the Canadian Prairies (World Wildlife Fund, 1988). Since the time of Europeans settlement, widespread destruction of Fescue Prairie has created a landscape with small, isolated patches of remnant native vegetation embedded in a dissimilar matrix of cultivated fields, grazed pastures, roads, and human settlements (Archibold and Wilson, 1980; Romo, 2003). The landscape was once covered by as much as 121,000 km^2 of fescue grasslands in Saskatchewan alone when European just settled, but now it has been reduced to an estimated 5% of its original area due to cultivation and development (Gerry and Andersen, 2002). Less than 5% of the Fescue Prairie remains in Manitoba (Grilz and Romo, 1994), and 15% in Alberta (Alberta Environmental Protection, 1997). Some remnant fescue stands have been protected within national parks and conservation efforts have been made with the hope to re-establish the fescue grassland. However, most of these fragments are unprotected and vulnerable to stochastic processes and environmental changes (Schemske et al., 1994; Postma and van Noordwijk, 2005); therefore, plant species remaining in the fragmented prairie habitats are of conservation concern due to the inevitability of extinction of these remnants through inbreeding within the patch, complete eradication of patches or reduced ability to recover from catastrophic events (Gilpin, 1990).

Plains Rough Fescue [*Festuca hallii* (Vasey) Piper] is the distinctive characteristic species in the Fescue Prairie. It is an endemic tetraploid (2n = 4x = 28), presumably outcrossing (Aiken et al., 1996, 1997; Anderson, 2006). It is an erect, densely tufted perennial bunchgrass that produces short rhizomes. Vegetative growth begins in early May, with a maximum biomass achieved by the end of July. Seed production begins in late June, and seeds are released by mid-July. Flowering and seed production show considerable year to year variation, perhaps dependent upon conditions in the previous growing season (Johnston and MacDonald, 1967; Romo et al., 1991). *F. hallii* plants can grow up to 20 – 80 cm tall. Panicles are 6 - 16 cm long, branches are

stiffly erect and spikelets have two fertile florets and 0 - 2 sterile florets. Anthers are 4 - 6 mm long, with tops lying towards apex of paleas.

This species is widely distributed in the Fescue Prairie region of North America. Remnant *F. hallii* stands have been found throughout the Canadian Prairies, including Moist Mixed Grassland, Mixed Grassland, Aspen Parkland, and Boreal Transition Ecoregions (Ecological Stratification Working Group, 1996). Within Parks Canada holdings, *F. hallii* can be found in Riding Mountain and Prince Albert National Parks, and Batoche and Fort Walsh National Historical Sites, with remnant patches found in Elk Island National Park and Fort Battleford National Historical Site. Conserving the remaining *F. hallii* populations is imperative as the number of populations is declining due to land conversion, which can result in genetic erosion and isolation. Re-establishing a network of fescue dominated patches across its historic range is challenging, as the ecology of Fescue Prairie and the biology of dominant fescue species are poorly understood (Romo, 2003) and few biological studies have been conducted on this genus (Coupland and Brayshaw, 1953; Pavlick and Looman, 1984; Romo et al., 1991). Knowledge about the genetic diversity and structure of *F. hallii* is required for Fescue Prairie restoration, but is largely lacking (Fu et al., 2006).

2.2 Plant conservation and habitat fragmentation

It is critical to conserve the native plants facing reduced population size and isolation in the fragmented habitat. The study and practice involved in plant conservation include proper management, such as fire management to protect grasslands from encroachment and exotic invasion; *ex situ* conservation, the conservation of target species outside of their native habitat, with hope of reintroducing them, *in situ* conservation, the conservation of target species within their natural habitat; and reintroductions/habitat restoration, the study and practice of restoring an target species to its native lands (Heywood and Iriondo, 2003). It is important to protect sites not currently protected, to manage sites for persistence and to re-establish sites where they have disappeared. Developing effective conservation, restoration and management strategies requires the understanding of the genetic, demographic, and environmental effects on plant persistence, such as population viability and fitness components (Oostermeijer et al., 2003).

Habitat fragmentation is commonly defined as the reduction of continuous habitat into several smaller, spatially isolated remnants, primarily by human disturbances such as land

clearing and conversion of vegetation from one type to another (Young et al., 1996; Franklin et al., 2002). Franklin et al. (2002) proposed a situational definition with four requisites: 1) what is being fragmented, which requires the understanding of a species' habitat, 2) the scale of fragmentation as to where inferences are being made and the level of habitat description being considered, 3) the extent and pattern of fragmentation, providing a description of the magnitude and type of habitat fragmentation, and 4) disturbance mechanism putting habitat fragmentation into a temporal scale, and an ecological and conservation context. The pattern or the situational definition for habitat fragmentation forms the basis of most quantitative measures of fragmented habitat.

The most direct effect of the process of fragmentation is habitat loss. Fahrig (2003) stated that habitat loss led many researchers to measure the degree of fragmentation as the amount of habitat remaining on the landscape. Few researchers quantified the degree of habitat fragmentation in a natural landscape using the amount of habitat loss, which requires measuring the original habitat area. It is possible that populations and communities take many years or even decades to respond to habitat loss-induced changes, so this prediction requires long-term observation proving very difficult to perform.

Most researchers measure habitat fragmentation as a change in habitat configuration (Fahrig, 2003), focusing on the reduced size (Cruzan, 2001; Vergeer et al., 2003; Llorens et al., 2004; Hooftman et al., 2004; Oostermeijer and de Knegt, 2004; Byers et al., 2005; Gonzales and Hamrick, 2005; Honnay et al., 2007) and increased spatial isolation (Lammi et al., 1999; Brown et al., 2000; Torres et al., 2003; Darling et al., 2004; Gonzalez-Astorga et al., 2004; Sheng et al., 2005) of populations occupying the fragmented habitat (Young et al., 1996). Population size is counted as the number of individuals in a population. Different measures of population isolation were used, such as distance to the nearest other population, distance to the nearest large population, and the mean distance to all other populations (Young et al., 1999). The most common measure of spatial isolation is the distance to the next-nearest neighbor (Fahrig, 2003; Young et al., 1999).

2.3 Genetic diversity and genetic differentiation

Genetic diversity is a measure of genetic variation within a population or species. The amount of genetic variation within a population provides insight into the demographic structure and

evolutionary history of a population. Genetic differentiation is genetic divergence among populations, indicative of the amount of genetic exchange that has occurred among populations. Genetic diversity and genetic differentiation play important roles in the conservation, restoration, and management of native plant species.

2.3.1 Molecular methods for studying genetic diversity and differentiation

Genetic diversity and genetic differentiation in plant populations have been detected using allozyme electrophoresis (Gonzalez-Astorga et al., 2004) and DNA markers, such as Restriction Fragment Length Polymorphisms (RFLP) (Botstein et al., 1980; Melchinger et al., 1991; Gauthier et al., 2002), Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990; Vucetich et al., 2001; Gonzalez-Rodriguez et al., 2005), Amplified Fragment Length Polymorphism (AFLP) markers (Vos et al., 1995; Ajmone-Marsan et al., 1997; Arroyo Garcia et al., 2002; Guo et al., 2005), and microsatellite markers (Edwards et al., 1991; Schlotterer and Tautz, 1992; Vosman and Arens 1997; Fu et al., 2003). The first molecular markers were allozyme markers based on the different mobility of differently charged protein that are coded for by different alleles at the same locus with the same enzymatic function on the gel. Although allozyme markers can detect heterozygotes and have functions, they have limited genome coverage and numbers. Allozyme variation is widespread in almost all natural populations and allozyme markers are widely used for analyzing genetic diversity and genetic differentiation in plant species (Oostermeijer and de Knegt, 2004; Lopez-Pujol et al., 2004).

RFLP markers are DNA-based markers, which involves restriction of genomic DNA followed by electrophoresis. The fragments are then transferred to a membrane by Southern blotting and hybridized with a radioisotope-labelled probe (Young et al., 1988). The polymorphisms detected by RFLP rely on the specific and characteristic nucleotide sequence which is recognized and cut by restriction enzymes. RFLP markers are codominant and very informative, but RFLP technique is labour intensive and time consuming and requires a relatively large amount of DNA.

RAPD analysis is a polymease chain reaction (PCR) application, which uses arbitrary oligonucleotide primers that bind to homologous base sequences in the genome, thus detecting differences in the nuclear genome (Welsh and McClelland, 1990; Williams et al., 1990). RAPD analysis does not require any prior knowledge of the genome as in RFLP studies (Rowe et al.,

1997). RAPDs are simple, quick and avoid the use of radioisotopes (Rowe et al., 1997). The availability of RAPDs for use with anonymous genomes and their relative lack of expense make them potentially valuable genetic markers for population studies (Pearson et al., 2002). The RAPD technique has the potential to reveal large numbers of polymorphisms, as it screens large numbers of alleged noncoding regions of the genome (Comes and Avbott, 2000). RAPD markers have been shown to be useful for investigating clonal genetic diversity and population structure in various plant species (Bush and Mulcahy, 1999; Muller-Scharer and Fischer, 2001; Vucetich et al., 2001; Torres et al., 2003; Gonzalez-Rodriguez et al., 2005). However RAPDs are very sensitive even to slight changes in the amplification conditions and make the RAPD bands difficult to reproduce. In addition, RAPD markers are dominant and therefore not very informative for some applications (Ajmone-Marsan et al., 1997).

Simple sequence repeats (SSRs) or microsatellites are short (mostly 2–4 bp) tandem repeats of DNA sequences. It is hypothesized that the variation or polymorphism of SSRs is a result of polymerase slippage during DNA replication or unequal crossing-over (Schlotterer and Tautz, 1992). SSRs are not only very common but also hypervariable among the types of tandem repetitive DNA in the genomes of eukaryotes (Edwards et al., 1991; Vosman and Arens, 1997). DNA polymorphism due to microsatellites is based on the number of repeat units in a defined region of the genome (Weber and May, 1989). Microsatellite repeats occur in both coding and noncoding region of the genomes (Blair et al., 2003). The DNA sequences flanking microsatellites are conserved and used to design specific polymerase chain reaction primers, which will amplify the microsatellite locus and enable the detection of different alleles of a locus (Sun et al., 1998). The advantages of SSR markers are their codominant mode of inheritance, abundance, reproducibility, and hypervariability, which make them ideal for a wide range of applications including diversity study (Xiao et al., 1996; Li et al., 2000; Metais et al., 2002; Fu et al., 2003; Harper et al., 2003). However, the development of an SSR assay requires the laborious processes of library construction, DNA sequencing, and primer synthesis (Li et al., 2000; Gaitan-Solis et al., 2002; Gao et al., 2004).

The AFLP® is a new PCR-based DNA fingerprinting technique that could replace RFLP and RAPD. It is based on selective PCR amplification of restriction fragments from a total digest of genomic DNA without knowledge of nucleotide sequences of the target organism (Vos et al., 1995). AFLP markers are generated from restriction fragments ligated to double-strand adapters

and are selectively amplified with primers, complementary to the adapters, which carry additional random nucleotides at their 3' end that probe the internal sequence of restriction fragments (Vos et al., 1995). AFLP simultaneously screens high numbers of loci for polymorphism and detects a greater number of polymorphic DNA markers than any other PCR-based detection systems (Vos et al., 1995). Many genetic studies have utilized AFLP markers to detect genetic diversity and genetic structure between and within populations in plants (Besse et al., 1998; Schmidt and Jensen, 2000; Arroyo Garcia et al., 2002; Juan et al., 2004; Kim et al., 2005; Wilson et al., 2005; Abbott et al., 2007). Regarding detection of genetic diversity of grass species, this technique has also been successfully applied on bluebunch wheatgrass (*Pseudoroegneria spitcata* [Push] A. Love) (Larson et al., 2000), crested wheatgrass (*Agropyron* spp. Gaerth.) (Mellish et al., 2002), and little bluestem (*Schizachyrium scoparium* [Michx.] Nash) (Fu et al., 2004a). These studies showed that AFLPs, although behaving as dominant markers, can be used as informative, robust, and highly effective markers for diversity analyses (Fu et al., 2005a, b).

2.3.2 Measurement and analysis of genetic variation within and among populations

Population genetic variation consists of the sum of all genetic variation among individuals within a population (Young et al., 1996). Several standard genetic parameters are used to describe genetic diversity, such as the heterozygosity (*H*), allelic richness (A), and the percentage of polymorphic loci per population (P) (Hooftman et al., 2004; Gonzales and Hamrick, 2005; Allendorf and Luikart, 2007). Heterozygosity ($H = 1 - \sum Pi^2$, where *Pi* is the frequency of alleles at a locus) measures genetic variation at the individual level within a population. Nei (1973, 1977) referred to this measure as gene diversity. It can be the average proportion of heterozygotes per locus in a randomly mating population or the expected proportion of heterozygous loci in a randomly chosen individual. Average heterozygosity is a valuable measure for genetic variation since it is insensitive to sample size and it can be used for genes of different ploidy levels and in organisms with different reproductive systems (Allendorf and Luikart, 2007). However, it is difficult to compare among plants if using AFLP markers only, because AFLP markers are scored without reference to dominance (i.e., without distinction between homozygotes and heterozygotes), and co-migrating AFLP bands are not always homologous (Koopman, 2005). Loveless and Hamrick (1984) reported that the predominantly

allogamous plants had a higher allozyme gene diversity (H = 0.214) than the species with mixed mating (H = 0.174) or autogamy (H = 0.128). Allelic richness (A) is estimated as the total number of alleles at a locus. It is more sensitive to the loss of genetic variation due to small population size than heterozygosity, but unlike heterozygosity, it is highly dependent on sample size, which makes it meaningless for comparisons between samples unless sample sizes are similar (Allendorf and Luikart, 2007). The number and frequency of alleles at a locus determine the level of heterozygosity within a population. Polymorphism or the proportion of loci that is genetically variable can measure the level of heterozygosity among populations (Young et al., 1996). Natural populations of bluegrass (*Poa* spp.) had a proportion of polymorphic bands P = 0.84 (Larson et al., 2001a). The gene diversity (H) observed in cultivars of nordic meadow fescue (*F. pratensis*) ranged from 0.125 to 0.215 (Fjellheim and Rognli, 2005), and it was relative higher in natural populations of buffalograss [*Buchloe dactyloides* (Nutt.) Englem] (H = 0.35) (Budak et al., 2004). Proportion of polymorphic bands, mean band frequency and within population variation were used for measuring genetic diversity with AFLP markers (Fu et al., 2004a, b; 2005 a, b) and within population variation with allozymes (Quiroga and Premoli, 2007). The mean band frequency of natural populations of fringed brome (*Bromus ciliatus* L.) was 0.53 (Fu et al., 2005a) and it was 0.56 for mountain rough fescue (*F. campestris* Rydb.) (Fu et al., 2005b).

Increased spatial isolation due to habitat fragmentation results in spatial genetic structure by variation in genotype frequencies among the populations (Heywood, 1991). Genetic differentiation associated with habitat fragmentation is commonly evaluated as genetic variation and spatial genetic structure. In recent years the genetic differentiation of populations or the genetic structure of populations is regarded as allelic frequency variation over populations (Weir and Hill, 2002). Wright (1943, 1946, 1951, 1965) introduced F-statistics for describing the properties of hierarchically subdivided natural populations. Three parameters were proposed in terms of total population (T), subpopulations (S), and individuals (I) (Wright, 1951). F_{IT} (Wright, 1965) is the among-individual sample differentiation within populations and is considered indicative of the level of in- or out-breeding within populations (Weir and Cockerham, 1984; Hooftman et al., 2004). F_{IS} is the correlation of genes within individuals within subpopulations (Wright, 1965; Weir and Cockerman, 1984). F_{ST} is the correlation of alleles within subpopulations in the same population (Weir and Cockerham, 1984). It is considered indicative

of genetic drift (Hooftman et al., 2004) and also used for estimation of gene flow (Wright, 1951; Vucetich et al., 2001; Gonzalez-Astorga et al., 2004). For a single allele, frequency differences among the populations can be summarized as an allele-frequency variance among populations, σ^2_P. The normalized parameter $F_{ST} = \sigma^2_P/P(1-P)$ measures the degree of differentiation of subpopulations relative to complete fixation, subject to a constant global allele frequency, P. The numerator and denominator of F_{ST} are both variance components that can be estimated from AMOVA (Heywood, 1991), in which the total amount of genetic variation within a species can be partitioned into genetic differences among individuals within a population and among different populations.

Nei (1973) regarded F_{ST} as a statistic, but not a parameter, and proposed a statistic G_{ST}, which estimates the proportion of the total genetic diversity found among populations for each polymorphic locus. Nei (1977) demonstrated that G_{ST} is equivalent to a multiallelic F_{ST}. Weir and Cockerham (1984) described a moment estimator of θ, which is related to Wright's F_{ST}. However, it is not affected by the numbers of alleles observed per locus, numbers of individuals sampled per population or numbers of populations sampled (Weir and Cockerham, 1984). Chakraborty and Danker-Hopfe (1991) showed that these two indices, F_{ST} and G_{ST}, are empirically equivalent to θ (Weir and Cockerhams, 1984) when sample sizes are equal and a large number of populations are analyzed. Both indices are used widely in the analysis of genetic structure. For example, Lammi et al. (1999) and Hooftman et al. (2004) applied the protocol of Weir and Cockerham (1984) to analyze genetic structure using F-statistics (Wright, 1951). Cruzan (2001) and Gonzales and Hamrick (2005) adopted Nei's G_{ST} to assess the genetic variation among fragmented populations. The degree of genetic differentiation among populations is considered high when F_{ST} or G_{ST} value is greater than 0.1 (Godt and Hamrick, 1993; Hirao and Kudo, 2004) which is equivalent to 10% variation among populations (Llorens et al., 2004) for species with wind-dispersed pollen.

Since most researchers measure habitat fragmentation focusing on the reduced size and increased spatial isolation, the relationship between population size and geographical distance and spatial genetic structure can help recognize the response of plant species in small and isolated populations to habitat fragmentation. Relationship between population size and genetic variation can be tested using Pearson's correlation coefficient (Llorens et al., 2004; Oostermeijer and de Knegt, 2004; Quiroga and Premoli, 2007), and stepwise multiple linear regression (Young

et al., 1999; Schmidt and Jensen, 2000; Zhao et al., 2006). In order to test the relationship between isolation by distance and genetic diversity, pairwise genetic distances, Φ_{ST} (Nei, 1972; Lammi et al., 1999; Muller-Scharer and Fischer, 2001; Harper et al., 2003; Sheng et al., 2005; Honnay et al., 2007) among populations are obtained to produce matrices of genetic distances. Mantel permutation test (Mantel, 1967) is then used to test whether genetic distances between pairs of populations are significantly correlated with corresponding geographical distances (usually quantified as the logarithm of metres that separate pairs of populations (Cruzan, 2001; Muller-Scharer and Fischer, 2001; Vucetich et al., 2001; Gonzales and Hamrick, 2005; Sheng et al., 2005; Wang et al., 2005). The interpopulation distance matrices can be clustered with the algorithm of unweighted pair-group method using the arithmetic averages (UPGMA) or neighbour joining (Rohlf, 1997). To evaluate the genetic associations of individual plants or populations, a principal coordinate analysis (PCO) is usually performed based on the Euclidean square distance matrices from Arlequin (Peakall and Smouse, 2005).

2.3.3 Sampling considerations in assessment of genetic diversity

Sampling plant materials from natural populations is an important component in plant genetic diversity research, germplasm conservation, and germplasm improvement. However, little effort has been made to assess the effectiveness of sampling on capturing patterns of genetic variation in native plant species. Sampling can be accomplished with collecting seeds and/or tillers. But most sampling strategies commonly depend on the ease and accessibility of samples, rather than the effectiveness in capturing genetic diversity. Most studies about genetic variation in plant species exhibiting both sexual and asexual reproduction have been conducted by sampling vegetative tissues (Ivey and Richards, 2001, Wang et al., 2005) or seeds (Larson et al., 2000, Garnier et al., 2002) without knowing which sampling method was more efficient for revealing genetic diversity. For plant species that reproduce both sexually and vegetatively, the magnitude and extent of spatial genetic structure of clonal individuals might be different from those of sexual individuals. Significant genetic divergence between seeded and vegetative buffalograsses [*Buchloe dactyloides* (Nutt.) Englem.] was revealed (Budak et al., 2004). Greater diversity was detected in tiller samples than in seed samples by comparing AFLP variation in little bluestem [*Schizachyrium scoparium* (Michx.) Nash] (Fu et al., 2004a). Geert et al. (2007) investigated genetic variation and structure of adult and seedling populations in the self-incompatible long-

lived perennial herb *Primula vulgaris* to examine whether the old (adults) and young (recently germinated seedlings) generations differ in genetic diversity and structure. They found that the recent generation (seedlings) has lower genetic diversity and higher genetic differentiation compared to the adult generation.

Unequal dispersal of pollen and seeds has been reported in many species (Ennos, 1994). In wild barley, pollen dispersal is four times greater than seeds (Ennos, 1994; Streiff et al., 1998). More gene flow via pollen dispersal in the seeds could result in weaker differentiation among populations (Fu and Thompson, 2006). Pollens that can disperse over longer distance than seeds can result in higher gene flow in seedlings, which can explain the lower genetic differentiation in seed samples. On the other hand, it can be explained by the long-lived perennial life trait if lower genetic differentiation is detected in adults. Adult populations likely contain multiple cohorts of seedlings and overlapping generations. The structure of genetic variation at the adult stage is therefore from different reproductive gene pools, which result in lower genetic differentiation in adults compared to a single seedling generation (Geert et al., 2007).

Levels of overall population differentiation may be related to the age of populations. Many grass species do not produce seeds in the first several years of growth or only a portion of the population sets seed every year, and the young tillers often die before setting seeds. If mortality is non-random, then the genotype composition of the mature plants with seed set may then well be different from pre-reproductive tillers, resulting in distinct genetic composition of the reproductive vs. vegetative components of a population. Consequently, the genetic diversity of seeds from mature plants will be biased against genotypes present as pre-reproductive tillers (Sackville Hamilton and Chorlton, 1995).

2.4 Effect of habitat fragmentation on genetic variation

Habitat fragmentation results in decreased population size and increased isolation, which can lead to the erosion of genetic variation through genetic drift, increased inbreeding and reduced gene flow between populations (Young et al., 1996). Reductions in population size at the time of fragmentation create genetic bottlenecks because remaining individuals contain only a small portion of the original gene pool (Young et al., 1996). Landergott et al. (2001) identified extraordinarily low diversity of 280 individuals from 14 populations of *Dryopteris cristata* using RAPD marker, suggesting an ancient bottleneck in the species' history. Subsequently remnant

populations that remain small and isolated for several generations continue to lose alleles due to random genetic drift (Young et al., 1996). Genetic drift is the random changes in allele frequency. It occurs because gametes transmitted from one generation to the next carry only a sample of the alleles present in the parental generation (Ellstrand and Elam, 1993). In small populations, changes in allele frequencies may be large and unpredictable. Genetic drift is selectively neutral, both deleterious and advantageous alleles can get lost, which results in the loss of heterozygosity and allelic richness. Reduced genetic diversity may decrease the potential of a species to adapt to environmental change. In the short term, the loss of heterozygosity can reduce individual fitness through inbreeding depression (Ellstrand and Elam, 1993) and lower remnant population viability, and in the longer term, reduced allelic richness may limit a species' ability to respond to changing selection pressure (Young et al., 1996).

Habitat fragmentation also leads to increased genetic differentiation among populations due to founder effects, increased random genetic drift, and reduced interpopulation gene flow (Young et al., 1996). Gene flow in plants is the successful movement of genes among population by mating or by migration of seeds or vegetative propagules (Ellstrand and Elam, 1993). In the absence of gene flow through seeds and/or pollen between fragmented populations, random genetic drift increases genetic differentiation among populations (Ellstrand and Elam, 1993; Schaal and Leverich, 1996). However, Young et al. (1999) found increased levels of gene flow among fragmented populations.

Habitat fragmentation causing reduced population size and isolation-by-distance results in low genetic diversity and high genetic differentiation in many plant species, especially in rare and endangered species. For example, Xiao et al. (2004) detected low genetic diversity (P = 14%, H = 0.06) and high genetic differentiation (G_{ST} = 0.43) in the endangered *Cycas guizhouensis* (K. M. Lan & R. F. Zou) endemic to the southwest of China. Wang et al. (2005) revealed low genetic diversity (P = 23%, H = 0.08) and high genetic differentiation (38.7% among-population variation) in a woody, outcrossing and insect-pollinated endangered *Neolitsea sericea* ([Blume] Koidz) in the Zhoushan archipelago in China. Hooftman et al. (2004) detected lower genetic diversity and higher genetic differentiation in small than large habitat islands in common species of Swiss fern meadows.

The predicted consequences of habitat fragmentation on genetic variation within and among populations, however, are not found in many plant species. Genetic analysis in three very

rare, threatened *Ballota* species in Egypt demonstrated that high levels of genetic diversity (H = 0.195-0.317) were maintained and no genetic differentiation (G_{ST} = 0.045-0.099) were detected (Zaghloul et al., 2006). Kang et al. (2005) found low level of genetic diversity (P = 35%, H = 0.11) was not correlated with population size and high degree of genetic differentiation (F_{ST} = 0.535, G_{ST} = 0.608) was not correlated with geographical distance in endangered *Isoetes sinensis* (Palmer) in eastern China. Non-significant correlation between population size and genetic variation within populations was also found in *Pedicularis palustris* (L). in Germany (Schmidt and Jensen, 2000), endangered shrub *Grevillea caleyi* (R. Br.) in Australia (Llorens et al., 2004), endangered calcareous grassland species *Globularia bisnagarica* L. in Belgium (Honnay et al., 2007), and in three native plant species across the State of Minnesota (Moncada et al., 2007). This suggests that habitat fragmentation has not manifested itself in the population genetics of these species, which is common in plant populations, especially in long-lived perennials (Young et al., 1999; Culley et al., 2007; Honnay et al., 2006, 2007; Geert et al., 2007). The lack of a genetic response to habitat fragmentation may be primarily due to insufficient time having elapsed since fragmentation. It may also be due to environmental, geographical, and ecological attributes, rather than to habitat fragmentation.

2.5 Geographical patterns of genetic variation

Genetic variation can be shaped by the interaction between landscape characteristics and evolutionary processes, such as gene flow, genetic drift, and selection. Landscape genetics combining landscape ecology and population genetics can provide information about the interaction between landscape features and spatial pattern of genetic variation (Manel et al., 2003). Mantel test has been used to test the effects of local environment on genetic differentiation by measuring the association between genetic distance and local environmental variable, such as moisture or temperature (Keyghobadi et al., 1999; Manel et al., 2003; Vandepitte et al., 2007). A partial Mantel test by holding the effect of the third matrix constant was used to determine relationships between multivariate genetic attributes and environmental factors (Hirao and Kudo, 2004; Still et al., 2005; Vandepitte et al., 2007).

Landscape features influence genetic variation through affecting gene flow and genetic drift by isolating mechanisms such as spatial barriers, spatial location, isolation-by-distance, and variation in other traits that limit gene flow. Schmidtling and Hipkins (1998) found genetic

diversity in longleaf pine (*Pinus palustris*) populations was significantly related to longitude and western sources tended to have more allozyme diversity. Fu et al. (2005a) observed that the AFLP variability was significantly associated with the geographic origins of fringed brome (*Bromus ciliatus* L.) populations. Still et al. (2005) found genetic variation of *Echinacea angustifolia* populations along a north-south climatic gradient, indicating the associations with climatic parameters. A clear east-west genetic structure was found across large geographic scales in *Arabidopsis thaliana* (Beck et al., 2008). Grivet et al. (2008) explored spatial patterns of multivariate genotypes and genetic diversity throughout the range of California valley oak (*Quercus lobata* Née) and found clear geographical patterns of genetic variation for both chloroplast and nuclear markers. The observed latitudinal genetic trend was probably due to different historical gene movement through pollen and seed in different regions or selection pressure associated with environmental change (Grivet et al., 2008).

Environment-mediated processes can also shape the plant population genetics through strong selective forces in a heterogeneous environment, such as reduced seedling recruitment in less suitable habitats (Eisto et al., 2000; Manel et al., 2003; Vergeer et al., 2003; Pendleton and Meyer, 2004). A number of environmental factors, such as precipitation, temperature and geographic gradient may exert selection pressure thus leading to genetic divergence (Still et al., 2005). Several studies have provided evidence that environmental or habitat variability has the potential to influence the genetic variation within and among populations (Still et al., 2005; Assogbadjo et al., 2006; Quiroga and Premoli, 2007; Vandepitte et al., 2007).

Spatial genetic structure within plant populations is determined by many processes, including gene flow and local selection (Gehring and Delph, 1999; Smouse and Peakall, 1999; Parker et al., 2001; Ally and Ritland, 2007; Yamagishi et al., 2007). Under restricted gene flow and neutral selection the population develops a patchy distribution of genotypes (Smouse and Peakall, 1999; Wilson et al., 2005). The weight of the spatial structure on genetic diversity is estimated and the critical spatial dimensions are outlined using constrained ordinations considering the spatial location of each individual (Smouse and Peakall, 1999; Torres et al., 2003; Jones et al., 2007). Shape of the spatial genetic structure at each population and the testing for the presence of spatial autocorrelation are estimated using the multivariate spatial autocorrelation method and Moran's *I* and Mantel correlograms (Smouse and Peakall, 1999; Gehring and Delph,

1999; Parker et al., 2001; Torres et al., 2003; Wilson et al., 2005; Ally and Ritland, 2007; Jones et al., 2007; Zamudio and Wieczorek, 2007).

2.6 Germination and genetic variation in seed populations

2.6.1 Seed germination and sources of variation in seed populations

Seed germination as one of the components of offspring fitness is a critical stage of the life cycle reflecting adaptation to local habitats (Probert, 2000). Maximal germination can occur over a range of temperatures and germination declines sharply on either side of the range (Kebreab and Murdoch, 2000; Probert, 2000). Germination rate usually increases linearly with increasing temperature from a minimal or base temperature (T_b) up to an optimum and decreases linearly to a ceiling temperature (Garcia-Huidobro et al., 1982; Steinmaus et al., 2000; Bradford, 1990; 2002; Rowse and Finch-Savage, 2003). T_b is the most sensitive parameter that varies between species and genotypes, and seed treatment for dormancy release can modify T_b (Welbaum and Bradford, 1991; Del Monte and Tarquis, 1997; Pritchard et al., 1999). Thermal time (Degree-day or hour), the heat unit for plant development, is a firmly established developmental principle for plants (Fry, 1983), including seed germination (Garcia-Huidobro et al., 1982; Ellis et al., 1986).

The ecological restoration of altered natural ecosystems is a major action for conservation and the successful seed germination and seedling establishment are critical components of ecological restoration (Eriksson and Ehrlen, 1992; Pendleton and Meyer, 2004; Krauss et al., 2007). Seed germination is affected by environmental, demographic and genetic effects (Ouborg and Van Treuren, 1995; Morgan, 1999; Eisto et al., 2000; Sugiyama, 2003; Vergeer et al., 2003; Holzel and Otte, 2004; Pendleton and Meyer, 2004; Luzuriaga et al., 2006; Schmuths et al., 2006; Boyd et al., 2007; Krauss et al., 2007). Understanding seed germination responses related to these factors are beneficial for ecological restoration.

Seed germination is known to be highly plastic in response to environmental conditions experienced both during seed maturation and after dispersal (Donohue et al., 2005 a, b, c). Environmental factors are known to control resource partitioning to developing seeds and regulate seed dormancy release, seed germination rate and percentage, and seed deterioration and mortality (Luzuriaga et al., 2006; Boyd et al., 2007). In particular, ambient environmental conditions of the mother plants during seed development, such as temperature (Lacey, 1996;

Sugiyama, 2003), soil moisture conditions (Luzuriaga et al., 2006), and photoperiod (Donohue et al., 2005 a, b, c), strongly influence traits expressed early in the life history, such as seed mass and germination rate (Roach and Wulff, 1987; Luzuriaga et al., 2006; Boyd et al., 2007). Seed mass and germination rate are important seed features that are likely targets of natural selection and have high adaptive implications for seedling survival (Luzuriaga et al., 2006; Boyd et al., 2007).

Demographic and genetic processes, which are all strongly related to population size, fragmentation and isolation, can determine population persistence through affecting seed germination (Vergeer et al., 2003; Krauss et al., 2007). Only a few studies have tried to assess the consequences of habitat fragmentation in terms of fitness components in perennials. Menges (1991) indicated that the reduced germination success in the small, isolated populations was related to reduced population size but not isolation. Similarly, Heschel and Paige (1995) demonstrated that small, isolated populations have reduced seed size and reduced germination success due to genetic drift and/or inbreeding depression. Cases have also been reported in which population size did not affect seed germination (Ouborg and Van Treuren, 1995; Lammi et al., 1999; Morgan, 1999; Eisto et al., 2000; Hooftman et al., 2003; Krauss et al., 2007).

Seedbed conditions directly surrounding seeds affect total germination percentage, the absolute time and the spread of time for seed germination, seedling establishment, and eventually population dynamics (Squire et al., 1997; Donohue et al., 2005a, b, c). Temperature, moisture, gases and light are the four major environmental factors affecting germination and emergence. Temperature is one of the most important environmental factors affecting seed germination (Vleeshouwers and Kropff, 2000). For most species, soil temperature determines seed germination percentage and germination rate (GR) (Garcia-Huidobro et al., 1982; Ellis et al., 1986; Kebreab and Murdoch, 2000).

The global temperature increase at 3-5°C by the end of the century is predicted and the increasing temperature and drought has been identified recently as one of the biggest potential conservation problems (Thomas et al., 2004). Recent years have seen the increasing efforts to address the possible shift of species distribution under predicted climate change (Hogg and Schwarz, 1997; Graae et al., 2008; Levine et al., 2008; Morin et al., 2008; Petru and Tielborger, 2008). Grasslands in North America are expected to expand northward as the climate becomes warmer and drier under climate change scenarios (Sargent, 1988; Rizzo and Wiken, 1992; Hogg,

1994). Seed germination response to environmental conditions is important for understanding and predicting the ecological adaptation of plant species under future climate change.

2.6.2 Genetic variation in seed populations

Seeds within a population germinate over a period of time even under optimal germination conditions after dormancy release. The within-population variation in germination is complicated by the complex interactions between seed genotypes and environments (Baskin and Baskin, 1989; Evans and Cabin, 1995; Cabin et al., 1997; Donohue, 2005; Donohue et al., 2005c). Seed genotypes responded differentially to environmental variation through the germination process (Cabin, 1996; Cabin et al., 1997; Mandak et al., 2006). This genetic sorting under different environmental conditions significantly affected the genetic structure of sub-populations (Cabin et al., 1997; Mandak et al., 2006). Squire et al. (1997) found that early and late germinators of an oilseed rape cultivar produced progeny with distinct germination characteristics, indicating genetic control of the position of a seed in the time-profile of germination. Marshall et al. (2000) indicted that the non-germinators and the secondary dormant seeds at low temperature appeared to originate mainly from the late germinating seeds at optimal temperature and confirmed the existence of genetically discrete subpopulations. Donohue et al. (2005c) found significant genotype-by-environment interaction on germination timing in recombinant inbred lineages of *Arabidopsis thaliana*. Studies on seed germination in natural populations showed that different germination environments favored particular genotypes and consequently resulted in significant genetic differentiation (Cabin et al., 1997; Mandak et al., 2006). Assessment of genetic variation in seed populations is important for a better understanding of fundamental processes of population establishment, range expansion and geographic differentiation.

3. PATTERNS OF AMPLIFIED RESTRICTION FRAGMENT POLYMORPHISM IN PLAINS ROUGH FESCUE AS REVEALED BY TILLER AND SEED

Abstract

Plains rough fescue [*Festuca hallii* (Vasey) Piper] is a dominant native grass species in the Fescue Prairie region of North America that has undergone dramatic range reduction in the past century. Little is known about the genetic diversity of this species. The amplified restriction fragment polymorphism (AFLP) technique was applied to assess the comparative genetic diversity of six *F. hallii* populations in Manitoba and Saskatchewan and their corresponding seed collections. Three AFLP primer pairs were employed to screen 529 samples, representing about 30 samples each of reproductive tiller, vegetative tiller and seed collected from each population. A total of 330 polymorphic AFLP bands were scored for each sample; their occurrence frequencies ranged from 0.01 to 0.99 and averaged around 0.47. Analysis of molecular variance revealed more than 90% of the total AFLP variation resided within natural populations (reproductive and vegetative tillers) and within seed samples. Four populations sampled from protected areas appear to have relatively lower within-population variation than two unprotected populations. Only 0.2% AFLP difference was revealed among the three tissue types examined. The tiller samples revealed slightly larger among-population variation than the seed samples, and captured significant associations of AFLP variation with population geographic distances. These findings are significant for germplasm sampling for *ex situ* conservation, are useful for germplasm development for pasture seeding, and should facilitate the management of fragmented fescue populations.

3.1 Introduction

Plains rough fescue [*Festuca hallii* (Vasey) Piper] is a native, tetraploid ($2n = 4x = 28$), presumably outcrossing, perennial species (Aiken et al., 1996, 1997; Anderson, 2006). This species is widely distributed in the Fescue Prairie region of North America, bordering the boreal forest to the south and centred in Saskatchewan, Alberta, Manitoba, Montana, and North Dakota (Aiken et al., 1996, 1997; Anderson, 2006). Due largely to agricultural conversion, Fescue

Prairies have been threatened over the last 100 years (World Wildlife Fund, 1988). Less than 5% of the grassland dominated by *F. hallii* remains in Saskatchewan (Grilz and Romo, 1995; Gerry and Anderson, 2002), about 15% of the Fescue Prairie in Alberta (Alberta Environmental Protection, 1997), and less than 5% in Manitoba (Grilz and Romo, 1994). Much of what remains of fescue grassland occurs in small isolated patches, surrounded by cultivation or hay fields (Archibold and Wilson, 1980; Grilz and Romo, 1995). Efforts have been made to protect Fescue Prairie remnants within national parks, provincial parks and provincial natural areas, but nothing is known about the genetic status of these protected populations. Re-establishing a network of fescue dominated patches across its historic range is challenging, as the ecology of Fescue Prairie and the biology of dominant fescue species are poorly understood (Romo, 2003) and few biological studies have been conducted on this genus (Coupland and Brayshaw, 1953; Pavlick and Looman, 1984; Romo et al., 1991). Knowledge about the genetic diversity and structure of *F. hallii* is required for Fescue Prairie restoration, but is largely lacking (Fu et al., 2006).

Sampling plant materials from natural stands represents an important component in plant genetic diversity research, germplasm conservation and germplasm improvement for pasture seeding. However, little attention has been paid to assessments of the effectiveness of sampling in capturing genetic diversity in native plant species (Sackville Hamilton and Chorlton, 1995; Johnson et al., 2004). A recent study of little bluestem (*Schizachyrium scoparium* (Michx.) Nash) revealed that a greater genetic diversity was captured in tiller, than seed, samples (Fu et al., 2004a). Like little bluestem, *F. hallii* is a densely tufted bunchgrass with short rhizomes, and can propagate via seeds and rhizomes. However, its seed production is erratic, largely dependent upon conditions in the previous growing season (Johnston and MacDonald, 1967; Romo, 1991). These features suggest possible sampling differences in capturing genetic diversity.

The amplified restriction fragment (length) polymorphism (AFLP) technique (Vos et al., 1995) is a robust, highly effective method for molecular assessments of genetic variability. AFLP markers are scored without reference to dominance (i.e., without distinction between homozygotes and heterozygotes), and co-migrating AFLP bands are not always homologous (Koopman, 2005; Mechanda et al., 2004a, b). Despite these limitations, the technique has been successfully applied to characterizing genetic variation in many native grass species, including bluebunch wheatgrass (*Pseudoroegneria spicata* [Push] A. Love) (Larson et al., 2000), crested wheatgrass (*Agropyron* spp. Gaerth.) (Mellish et al., 2002), little bluestem (Fu et al., 2004a), blue

grama [*Bouteloua gracilis* (Willd, ex Kunth) Lab. Ex Griffiths] (Fu et al., 2004b) and fringed brome (*Bromus ciliatus* L.) (Fu et al., 2005a). Recently, Fu et al., (2005b) revealed large AFLP variation in mountain rough fescue (*Festuca campestris* Rydb.). No specific diversity analysis has been made for *F. hallii* (Fu et al., 2006).

The objective of this study was to assess the comparative genetic diversity of six *F. hallii* populations in Manitoba and Saskatchewan and their corresponding seed collections using AFLP markers. It is our hope that this assessment will generate some baseline information not only for understanding the effectiveness of sampling grass germplasm from natural stands, but also for conserving fragmented fescue populations.

3.2 Materials and methods

3.2.1 Plant materials

Fescue samples used in this study were collected in August 2005 from six *F. hallii* populations in Saskatchewan and Manitoba: Prince Albert National Park (PA), Batoche National Historical Site (Batoche), Riding Mountain National Park (RM), University of Saskatchewan Kernen Research Farm (Kernen), Macrorie, and Turtleford (Table 3.1; Fig. 3.1). The dominant area of *F. hallii* in these six sites ranged largely from 1 to 17 acres. PA, Batoche, RM, and Kernen are four populations currently under protection from development or cultivation. From each population, 30 individual reproductive plants with seed set and that were at least 5 m apart were randomly selected and seeds (or more precisely grass fruit, or caryopses, plus surrounding reproductive bracts) from each plant and plugs of 'reproductive tillers' (i.e., the vegetative portions of tillers bearing seeds) were collected separately. On the same day, another 30 single 'vegetative tillers' (i.e., plants without fruit set), also at least 5 m apart, were also randomly sampled for plugs of tissue.

The collected samples were transported to Plant Gene Resources of Canada, Saskatoon Research Centre, Agriculture and Agri-Food Canada (Saskatoon, SK, Canada). The collected tiller plugs were grown immediately in a greenhouse at the centre. Young leaf tissue was harvested from the growing tillers, stored in a paper envelope, freeze-dried, and kept at -20^{o}C for later molecular analysis. Ten seeds were randomly selected from the seed sample of each plant, scarified (after pealing off the lemma and palea), and germinated on two layers of filter papers in

Petri dishes in a growth chamber (25/15°C, 12/12h light/darkness). Two of the growing seedlings were randomly selected, transplanted into pots and grown in the greenhouse. Young leaf tissue from one surviving seedling per seed sample was harvested after 45 days. Seedlings from 5 reproductive tillers, 1 vegetative tiller, and 5 seed samples did not survive (Table 3.1).

Table 3.1 Patterns of amplified restriction fragment polymorphism (AFLP) in six plains rough fescue populations as reflected in three tissue types.

Population and sample type	NPA	PPB	MBF	WPV
Batoche (52°45'05"N, 106 ° 08'25"W)				
Reproductive tiller	29	80.3	0.451	86.1
Vegetative tiller	30	79.7	0.468	85.6
Seed	30	84.5	0.449	89.2
Kernen (52°09'54"N, 106°31'46"W)				
Reproductive tiller	30	81.5	0.437	85.8
Vegetative tiller	30	82.4	0.455	87.2
Seed	28	87.0	0.456	93.1
Macrorie (51°13'47"N, 107°13'50"W)				
Reproductive tiller	29	82.4	0.455	89.8
Vegetative tiller	30	81.5	0.455	88.3
Seed	30	91.2	0.454	98.8
Prince Albert (53°35'16"N, 106°02'27"W)				
Reproductive tiller	28	78.8	0.462	85.4
Vegetative tiller	30	79.4	0.466	86.5
Seed	27	80.9	0.464	92.0
Riding Mountain (50°48'36"N, 100°14'45"W)				
Reproductive tiller	29	77.6	0.430	81.2
Vegetative tiller	29	79.4	0.442	83.8
Seed	30	80.9	0.432	87.0
Turtleford (53°27'40"N, 109°03'12"W)				
Reproductive tiller	30	85.2	0.455	90.6
Vegetative tiller	30	86.1	0.458	92.5
Seed	30	87.0	0.453	93.7

Note: NPA = the number of plants assayed; PPB = the percentage of polymorphic AFLP bands scored; MBF = the mean band frequency; and WPV = the within population variation calculated as the average number of pairwise differences from the analysis of molecular variance (Excoffier et al., 2005).

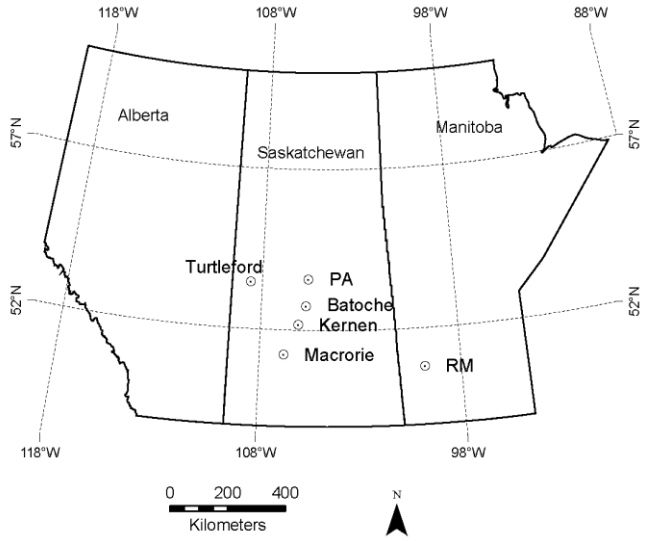


Fig. 3.1 Geographic locations of six *Festuca hallii* sample sites: Prince Albert National Park (PA), Batoche National Historical Site (Batoche), Riding Mountain National Park (RM), University of Saskatchewan Kernen Research Farm (Kernen), Macrorie, and Turtleford.

3.2.2 DNA extraction and AFLP analysis

Genomic DNA was extracted from the 529 leaf samples using the DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's directions. Extracted DNA was quantified by fluorometry using Hoechst 33258 stain (Sigma Chemical Co., St. Louis, MO, USA), diluted to 25 ng μL^{-1} for AFLP analysis. The AFLP™ Analysis System 1 (Life Technologies, Burlington, ON, Canada) was applied following the protocol described by Vos et al. (1995) with the exception of using $\gamma^{33}P$ to label *Eco*RI 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.

Based on the previous AFLP analysis of *F. campestris* (Fu et al., 2005b), three most informative *Eco*RI: *Mse*I primer pairs (E+AAC/M+CAG, E+ACG/M +CTA, and E+AGG/M+CTC) were selected to screen all the samples. To minimize technique-related and scoring errors, all the individual plants of three tissue types from one population plus four duplicated samples of one individual were arranged on one gel.

3.2.3 Data analysis

Automated analysis of banding patterns on 18 gels was conducted using GelComparII™ (Applied Maths, Belgium). The TIF format gel images were processed from the autoradiographs using a digital camera. The conversion, normalization, and background subtraction of the gel images were conducted following the GelCompar II user's guide. The image within one gel was normalized using the four duplicate samples as reference lanes. Image alignment among multiple gels produced from the same primer pairs was performed using an external reference (a 30-330 base pair AFLP DNA ladder; Promega, Madison, WI) and an internal reference (bands that were monomorphic across gels). The aligned gel images were automatically scored as 1 (present) or 0 (absent). The duplicated samples were used to assess the consistency of AFLP reactions.

The scored bands were analyzed for the level of polymorphism by counting the total number of bands and the number of polymorphic bands, calculating the proportion of polymorphic bands, and generating summary statistics on the band frequencies with respect to primer, population and sample type. For each sample type, the within-population variation was also calculated from an analysis of molecular variance (AMOVA; Excoffier et al., 2005) as the average number of pairwise differences within a population.

To assess AFLP variation components and test their statistical significance levels, AMOVA was performed with respect to population and sample type. This analysis not only allows partitioning of the total AFLP variation into within- and among- group components, 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. A population specific AMOVA analysis was also performed to assess the differences in AFLP variation among the three tissue types from a given population. This analysis not only allows the partition of the total AFLP variation into within- and among- sample components from single populations, but also provides tests on the significance of the AFLP difference between any tissue types.

The following three analyses were made for each sample type. First, the inter-population distance matrices were analyzed using NTSYS-pc 2.3 (Rohlf, 1997) and unrooted trees were obtained from the distance matrices using the neighbour-joining procedure (Saitou and Nei, 1987) to assess genetic associations of the assayed populations. In case of ties a warn option was used

in the neighbour-joining analysis. Second, the association between inter-population distance and geographical distance matrices was assessed with the Mantel test (Mantel, 1967) using GenAIEx 6 with 9,999 random permutations (Peakall and Smouse, 2005). Third, the genetic associations of the individual plants were evaluated with principal coordinate analysis (PCO), as this performs well with a binary data set. Euclidean square distances were first computed from Arlequin (Excoffier et al., 2005) and then a PCO of 529 individual plants was performed based on the Euclidean square distance matrices using GenAIEX 6 (Peakall and Smouse, 2005).

3.3 Results

3.3.1 AFLP profile

Three AFLP primer pairs amplified a total of 330 DNA fragments and more than 93% of these AFLP bands were polymorphic in the samples of reproductive tiller, vegetative tiller or seed (Table 3.2). The reproducibility of the AFLP bands observed for duplicated samples ranged from 97 to 99%. The band frequencies in each tissue type ranged from 0.006 to 0.994 and averaged from 0.466-0.483. The percentages of polymorphic bands for each primer pair ranged from 85.0 to 100%. The primer pair E+ACG/M+CTA amplified the most DNA fragments (114). The primer pair E+AAC/M+CAG had the largest percentages of polymorphic bands (>96%). The primer pair E+AGG/M+CTC revealed the polymorphic bands with the lowest mean band frequencies, ranging from 0.45 to 0.46 (Table 3.2). Thus, a wide range of AFLP variation was detected in this grass species.

3.3.2 Variation patterns in reproductive tiller samples

Genetic variation for six *F. hallii* populations was quantified in this study by determining the percentage of polymorphic bands, the mean band frequency, and the within-population variation as calculated from the AMOVA sum of squares (Table 3.1). Within a population, the percentage of polymorphic bands ranged from 77.6 to 85.2; the mean band frequency ranged from 0.430 to 0.462; and the within-population variation ranged from 81.2 to 90.6. The estimate of within-population variation took into account both the number of bands and their frequencies in each population and thus is relatively more informative. Based on this estimate, the Turtleford population displayed the largest AFLP variation (90.6), followed by Macrorie (89.8), Batoche

(86.1), Kernen (85.8), PA (85.4) and RM (81.2) (Table 3.1). Note that the last four populations are sampled from areas protected for at least 50 years.

Table 3.2 Patterns of amplified restriction fragment polymorphism (AFLP) in six plains rough fescue populations with respect to AFLP primer pair and sample type.

Primer and sample type	Number of AFLP bands scored	Percentage of polymorphic bands	Frequency of polymorphic bands	
			Mean	Range
E+AAC/M+CAG				
Reproductive tiller	109	96.3	0.486	0.017-0.994
Vegetative tiller	109	100.0	0.506	0.028-0.994
Seed	109	98.2	0.482	0.023-0.994
E+ACG/M+CTA				
Reproductive tiller	114	96.5	0.476	0.011-0.994
Vegetative tiller	114	96.5	0.479	0.011-0.994
Seed	114	97.4	0.469	0.029-0.994
E+AGG/M+CTC				
Reproductive tiller	107	85.0	0.448	0.011-0.994
Vegetative tiller	107	90.7	0.459	0.011-0.994
Seed	107	90.7	0.458	0.023-0.994
All primers				
Reproductive tiller	330	93.9	0.466	0.006-0.994
Vegetative tiller	330	95.8	0.483	0.011-0.994
Seed	330	96.4	0.467	0.006-0.994

Note: Three *Eco*RI primers (E+ three selective nucleotides; 5'-GACTGCGTACCAATTC+AAC, ACG, and AGG) and three *Mse*I primers (M+ three selective nucleotides; 5'-GATGAGTCCTGAGTAA+CAG, CTA, and CTC) are shown for the primer pair combinations applied in this study.

Partitioning of the total AFLP variation into within and among population components by AMOVA showed that 90.4% of the total variation resided within populations and 9.6% was present among the six populations (Table 3.3). The largest between-population difference measured by the Phi statistic was observed between the RM and Turtleford populations (0.169), followed by population pairs of RM vs. PA (0.147), RM vs. Kernen (0.133), RM vs. Macrorie (0.107), and RM vs. Batoche (0.106). These differences can also be seen in the inferred genetic distances of the six populations in a dendrogram (Fig. 3.2a). Two distinct groups were revealed and no ties were found in the neighbour-joining analysis. The PA and Turtleford populations

were in one group. The Kernen, Macrorie, Batoche and RM populations were in a second group in which Batoche and RM were most genetically similar. Turtleford and RM had the greatest genetic distance from each other.

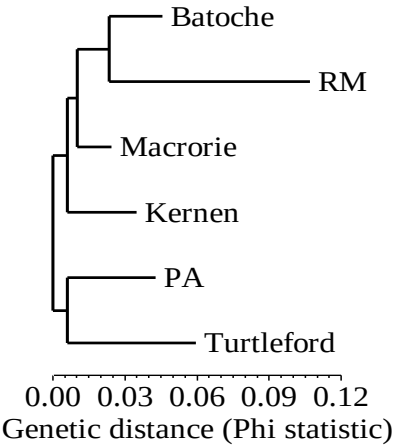
Table 3.3 Analysis of molecular variance (AMOVA) within and among the samples of six plains rough fescue populations with respect to sample type.

Model and source of variation	df	SS	Variance component	% variation
Reproductive tiller				
Among populations	5	888.4	4.6	9.6
Within populations	169	7309.5	43.3	90.4
Vegetative tiller				
Among populations	5	857.2	4.3	8.9
Within populations	173	7555.0	43.7	91.1
Seed				
Among populations	5	771.2	3.7	7.4
Within populations	169	7797.4	46.1	92.6
Overall				
Among sample type	2	132.2	0.1	0.2
Within sample type	526	25064.5	47.7	99.8

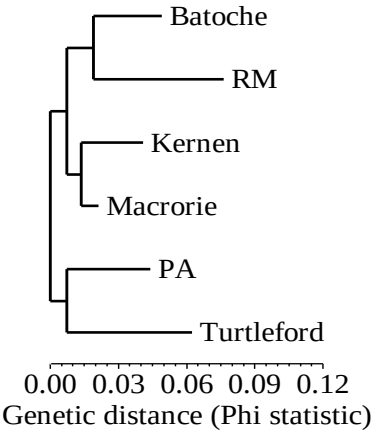
Note: all the variance components were statistically significant at $P < 0.0001$ level, as calculated from random permutations.

The correlation between the population-wise genetic differences and their geographical distances was significant, but not very strong ($R^2 = 0.75$; $P < 0.001$) (Fig. 3.3a). The genetic structures observed above were further supported by the principal coordinate analysis based on the Euclidean distance matrix of individual plants (Fig. 3.4a). The Turtleford and RM populations each formed a separate group with almost no overlap between RM and other populations, while the other four populations overlapped. Note that the first two principal coordinates accounted for only 48.5% of the AFLP variation.

a: Reproductive tiller



b: Vegetative tiller



c: Seed

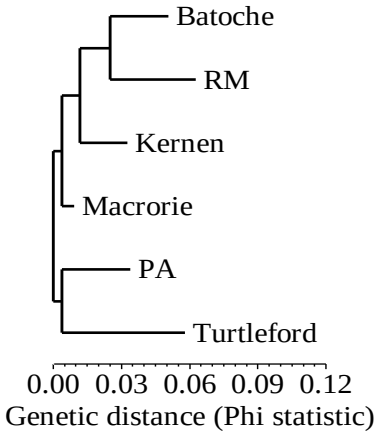


Fig. 3.2 Neighbour-joining trees of six plains rough fescue populations based on pairwise genetic distances (Phi statistics) derived from analysis of molecular variance, as reflected in the samples of reproductive tiller (a), vegetative tiller (b), and seed (c).

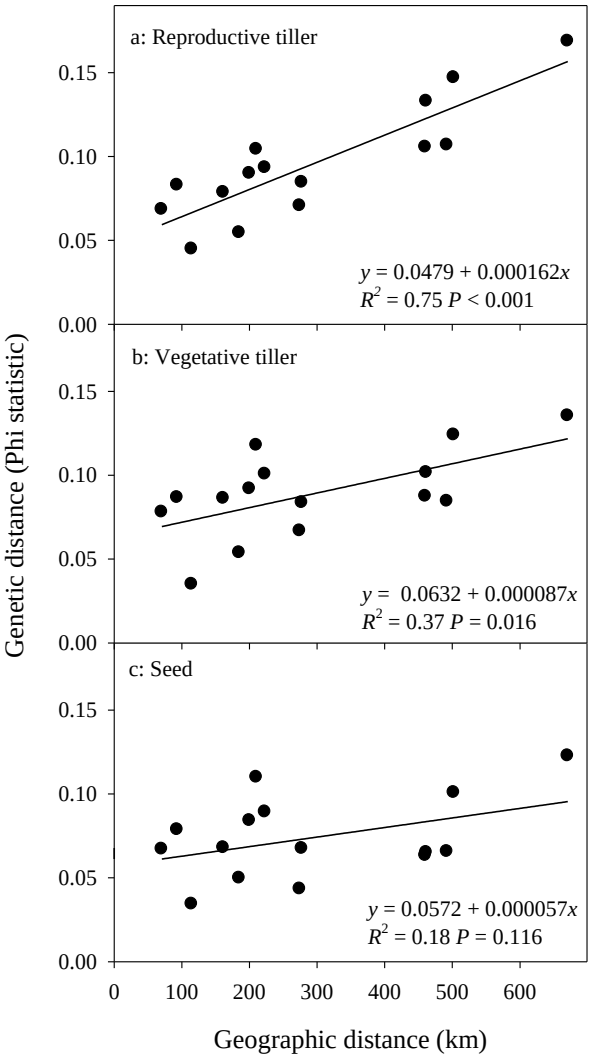


Fig. 3.3 The relationships between geographic and genetic distances (Phi statistic) among six plains rough fescue populations, as reflected from the samples of reproductive tiller (a), vegetative tiller (b), and seed (c).

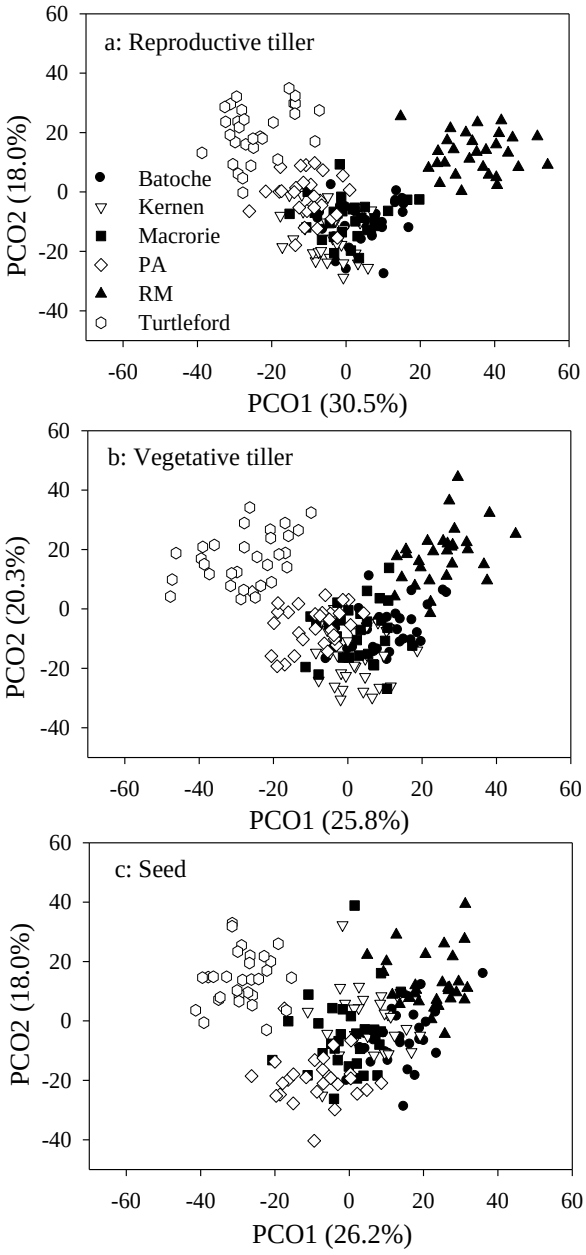


Fig. 3.4 Principal coordinate analysis of 529 individual plants from six plains rough fescue populations using pairwise individual Euclidean distances, as reflected in the samples of reproductive tiller (a), vegetative tiller (b), and seed (c).

3.3.3 Comparisons of AFLP variation among tissue types

Comparisons of the three tissue types in each population revealed more polymorphic AFLP bands in the seed samples than the tiller samples, while the average band frequencies were similar (Table 3.1). Thus, not surprisingly, relatively higher within-population variation was

observed in the seed samples than in tiller samples (Table 3.1). In spite of these differences, the four protected populations (RM, PA, Batoche, Kernen) consistently displayed relatively lower within-population AFLP variation than the two unprotected populations (Table 3.1).

Analysis of molecular variance with respect to sample type revealed on average only 0.2% AFLP variation resided among the three tissue types (Table 3.3). Specifically, no difference was observed between two tiller tissue types, and only 0.2-0.4% AFLP differences were found between seed and tiller tissue types (Table 3.4). For specific populations, the differences among tissue types could be up to 1.5%. Differences were also found between seed and vegetative tiller samples in five populations and between reproductive and vegetative tiller samples in three populations, but not between seed and reproductive tiller samples of any fescue populations (Table 3.4). Assessments of genetic structure pattern among the three tissue types showed seed samples captured marginally less among-population, and marginally more within-population, variation than the other two tissue types (Table 3.3). For example, the proportion of among-population variance component was 7.2% for the seed samples and 8.9% for the vegetative tiller samples. This may explain the slightly different patterns in the inferred genetic associations of the six populations observed for these three tissue types (Fig. 3.2). Significant correlations between geographical and genetic distances were observed for the tiller samples, but not in the seed samples (Fig. 3.3). These sampling differences can also be visualized in the genetic associations of individual plants shown in Fig. 3.4, in which populations were somewhat more distinct using the tiller samples than seed samples.

Table 3.4 Sampling differences as measured by the proportion of the total variation residing between any two samples of six plains rough fescue populations.

	Sample pair		
Population	R-tiller vs. V-tiller	R-tiller vs. Seed	V-tiller vs. Se
Batoche	0.006 ns	0.000 ns	0.015 ***
Kernen	0.008 **	0.003 ns	0.014 ***
Macrorie	0.000 ns	0.004 ns	0.009 **
PA	0.000 ns	0.004 ns	0.009 **
RM	0.010 **	0.000 ns	0.012 ***
Turtleford	0.007 *	0.000 ns	0.006 ns
All populations	0.001 ns	0.002 ***	0.004 ***

Note: R-tiller = reproductive tiller. V-tiller = vegetative tiller. ns, *, **, and *** for a significant level of $P > 0.05$, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3.4 Discussion

This study represents the first attempt using AFLP markers to measure the genetic variability and structure of plains rough fescue populations. Our goal is to produce baseline data relevant to the conservation and management of fragmented fescue populations. This AFLP assessment revealed several interesting patterns. First, a large proportion (>93%) of polymorphic bands with a wide range (0.01 to 0.99) of occurrence frequencies were observed for this species. Second, more than 90% of the AFLP variation resided within fescue populations and only about 9% among them. Third, four populations sampled from protected areas appear to have lower AFLP variation than two unprotected populations. Fourth, very small differences in levels of variation were found among the samples of reproductive tiller, vegetative tiller and seed, with the tiller samples revealing slightly larger among-population variation and (in contrast to the seed samples) capturing a low but significant association of AFLP variation with population geographic distances.

3.4.1 Patterns of AFLP variation among fescue populations

The large number of polymorphic DNA fragments detected by three AFLP primer pairs shows the utility of AFLP markers in assessments of genetic variability in *F. hallii* plants, despite the issues of dominance and homology in the use and interpretation of AFLP data (Koopman, 2005). These fragments were presumably sampled from the whole *F. hallii* genome, but their exact genomic coverage is unknown. In spite of these limitations, this study revealed more than 90% of the AFLP variation residing within a population. Such a high level of variation may indicate a high level of outcrossing in this plant, although no studies have been perfromed on the mating system of this species. This level of variation appears to be higher than the 81% of the AFLP variation within populations in its closely related species mountain rough fescue (Fu et al., 2005b), but is compatible with those findings reported for other highly outcrossing grass species such as bluebunch wheatgrass (Larson et al., 2000), bluegrasses (*Poa* spp.) (Larson et al., 2001a), smooth bromegrass (*Bromus inermis* Leyss.) and meadow bromegrass (*Bromus riparius* Rehmann) (Ferdinandez and Coulman, 2002), crested wheatgrass (Mellish et al., 2002), little bluestem (Fu et al., 2004a), and fringed brome (Fu et al., 2005a).

This study also revealed that only 9% of the AFLP variation present is partitioned among the assayed populations. Such weak population differentiation may reflect extensive gene flow via pollen and seed among populations. If this is true, habitat fragmentation may have not significantly hindered genetic exchange among fescue remnants. Further studies are needed to provide information on gene flow between fragmented fescue populations and its effect on genetic diversity (Hutchison and Templeton, 1999).

Significant correlations of the between-population AFLP variation with population geographic distances may reflect isolation by distance or local adaptations in these populations, particularly for the Turtleford population in Saskatchewan and the Riding Mountain population in Manitoba. A similar phylogeographic pattern was observed in *Nassella pulchra* (Hitchc.) Barkworth (Larson et al., 2001b), *Elymus* spp. (Larson et al., 2003), little bluestem (Fu et al., 2004a), and fringed brome (Fu et al., 2005a). However, the generality of this association remains to be determined, as the populations assessed in this study were largely located in the northern fringe of the species range. Analysis of more populations with an adequate coverage of the species range is needed.

The finding of somewhat lower within-population variation in four protected populations is interesting, and we speculate on the possible causes of this. PA and RM are located in Boreal Transition and Aspen Parkland ecoregions, respectively, that have more tree encroachment than the other sites. The surrounding forests, by acting as physical barriers, could restrict dispersal of external pollen. The lack of proper grazing effects and high litter build-up with impeded recruitment in the protected populations might also reduce within-population variation (Fu et al., 2005b). The lower within-population variation in the protected populations could also be due to the local adaptation of populations growing in the northern fringe of the species range. *F. hallii* is associated with moist black soil throughout the prairies. Such environmental conditions might have affected the distribution of *F. hallii* populations. However, the lower within-population variation may simply reflect historical bottlenecks. Further analysis of the relationship of genetic diversity with geographic origin would permit a more detailed characterization of genetic diversity of populations in protected and adjacent unprotected areas (Fu et al., 2005b).

3.4.2 Effectiveness of genetic sampling

This study applied three sampling strategies for each population, i.e., using reproductive tiller, vegetative tiller and seed. These methods yielded significant, although small, AFLP differences for all the six *F. hallii* populations. This finding, along with those findings on little bluestem (Fu et al., 2004a), appears to suggest that different sampling methods could differ in the effectiveness of capturing genetic variation from natural stands. However, different sampling methods captured slightly different population structures. For example, more polymorphic AFLP bands were observed in the seed samples than the tiller samples, while the average band frequencies were similar (Table 3.1). Higher within-population variation was found in the seed samples than the tiller samples (Table 3.1). The seed samples captured less among-population, but more within-population variation than the other two tissue types (Table 3.3). These differences were also reflected in the inferred genetic distances of the six populations (Fig. 3.2), the genetic associations of individual plants (Fig. 3.4) and the significant associations of geographical and genetic distances observed for the tiller samples only (Fig. 3.3). *F. hallii* sets seed infrequently, but its sporadic flowering does not seem to affect the genetic diversity. Unequal gene flow via long-distance pollen dispersal and short-distance seed dispersal reported in many other species (Ennos, 1994) may count for the difference of genetic diversity between seeds and tillers. The results in this study indicate less gene flow among populations exists in the tillers than in the seeds. More gene flow via pollen dispersal in the seeds could result in weaker differentiation among populations (e.g., Fu et al., 2005b).

3.4.3 Implications for plant conservation

The patterns revealed with AFLP assessment in this study are significant for germplasm sampling for *ex situ* conservation and should facilitate the management of fragmented fescue populations. Small sample differences reflected in AFLP markers imply that both seed and tiller samples are adequate for germplasm conservation and genetic diversity research of this species. However, the tiller sampling appears to be more informative for germplasm development for pasture seeding, as it captured more among-population variation and revealed significant correlations of genetic and geographic distances. The finding of large within-population, or small among-population AFLP variation in this species might suggest that monitoring regional genetic diversity would be better served by sampling more populations (and perhaps less samples per

population). Also, significant associations of AFLP variation with geographic distance suggest more distant populations should be considered to increase the potential of capturing more adaptive genetic diversity. Moreover, it could be argued that efforts to conserve fescue populations should not be limited to the populations currently under protection, which are slightly less genetically variable than the two unprotected populations at Turtleford and Macrorie. Restoring genetically declining fescue populations with genetically diverse seed sources identified from molecular assessments of this nature would also be enhanced by considering the unprotected populations.

4. GENETIC DIVERSITY AND GENETIC STRUCTURE OF REMNANT POPULATIONS OF PLAINS ROUGH FESCUE IN WESTERN CANADA

Abstract

Genetic diversity is essential for predicting plant evolutionary potential and for formulating conservation strategies. However, little is known about the genetic variation of plains rough fescue [*Festuca hallii* (Vasey) Piper], a widespread and diagnostic grass in the Fescue Prairie. We applied AFLP technique to assess the genetic variation of 30 fragmented populations of *F. hallii* across the northern Canadian Prairie and its associations with 12 geographic, fragmentation, and environmental attributes. Three AFLP primer pairs were employed to screen 840 samples, representing about 30 samples from each population, and 246 polymorphic bands were scored. The fescue plant was genetically diverse, as revealed by the proportion of polymorphic bands (0.870 - 0.967), the mean band frequency (0.364 - 0.457), and the within-population variation (69.4 - 85.4). The genetic variation was not highly differentiated with only 6.5% of the total AFLP variation residing among populations. Mantel test revealed a significant correlation between genetic and geographic distances ($r = 0.39$; $P < 0.004$) and a spatial autocorrelation up to 60 km among populations was detected. The AFLP variation displayed a longitudinal decline and was significantly associated with environmental attributes related to moisture, indicating local adaptation. However, the AFLP variation was not significantly associated with the estimated population size and geographic distance to the nearest neighbor, suggesting that fragmentation has not generated considerable genetic impact on the fescue populations. Implications for fescue conservation, restoration and management are discussed.

4.1 Introduction

Understanding genetic variation of plant species is important not only for predicting the evolutionary potential of the species, but also for formulating the effective strategies of conserving remnant plant populations (Groom et al., 2006). Remnant populations can be theoretically predicted with greater chance to deteriorate in genetic variation and become increasingly differentiated due to (1) founder effects at the time of fragmentation, (2) increased

random genetic drift, (3) elevated inbreeding, and (4) reduced interpopulation gene flow (Templeton et al., 1990; Ellstrand and Elam, 1993; Young et al., 1996). In the long term, these small populations may have reduced ability to adapt to changing environments and potentially become extinct. Consequently, remnant populations have gained attention in conservation and subjected to research. However, empirical studies of small, isolated populations have yielded variable results (Young et al., 1996; Groom et al., 2006). Cases have been reported in which fragmented populations of some widespread plant species promoted gene flow and were not detrimental (Honnay et al., 2006, 2007; Culley et al., 2007; Geert et al., 2007). Several factors responsible for such discrepancy have been proposed such as interspecific longevity differential, pre-fragmentation abundance, different mating system, the presence of seed banks, and interaction of animal population and seed dispersal (Young et al., 1996). Less understood are the influences of these factors on plant population viability and adaptation under habitat fragmentation (Schmidt and Jensen, 2000). Further research efforts are warranted to assess variable genetic effects of habitat fragmentation in plants, particularly of endemic grass species.

Fescue Prairie of North America bordering the southern boreal forest and centred in Saskatchewan, Alberta, Manitoba, Montana, and North Dakota (World Wildlife Fund, 1988; Aiken et al., 1996, 1997; Anderson, 2006) has become increasingly fragmented into small isolated patches, due to the conversion of once continuous grasslands for agricultural, industrial, and domestic use over the last century. The current coverage has greatly reduced to 15% of the formerly extensive Fescue Prairie in Alberta (Alberta environmental Protection, 1997) and less than 5% in Saskatchewan (Grilz and Romo, 1995; Gerry and Anderson, 2002) and in Manitoba (Grilz and Romo, 1994). Thus, some remnant fescue stands have been protected within national parks and conservation efforts have been made with the hope to re-establish the fescue grassland. However, the effectiveness of these conservation practices remains largely unknown. Limited information is available on the genetic and ecological impacts of habitat fragmentation on fescue plants. Little is known about the interactions of habitat fragmentation with habitat and environmental attributes to influence the long-term survival of small, isolated fescue populations.

Plains rough fescue [*Festuca hallii* (Vasey) Piper] is the diagnostic, perennial species in Fescue Prairie, and a North American endemic tetraploid ($2n = 4x = 28$), presumably outcrossing (Aiken et al.,1996, 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 (Romo et al., 1991; Johnston and MacDonald, 1967). Remnant *F. hallii* stands have been found throughout the Canadian Prairies, including Moist Mixed Grassland, Mixed Grassland, Aspen Parkland, and Boreal Transition (Ecological Stratification Working Group, 1996). While a few biological studies have been reported on this genus (Coupland and Brayshaw, 1953; Pavlick and Looman, 1984; Romo et al., 1991), ecological and genetic researches on this species have hardly been conducted. Recently, a comparative diversity study of seed and tiller tissue of six *F. hallii* populations revealed that *F. hallii* was genetically diverse and that little genetic changes were found between seedling and adult plants, either in natural and reserved populations (Qiu et al., 2007).

To facilitate the re-establishment of fragmented fescue communities, we investigated the genetic diversity of *F. hallii* using amplified fragment length polymorphism (AFLP) technique. The AFLP technique (Vos et al., 1995) is a robust, highly effective tool and has been widely applied to assess genetic variability in grass species (e.g., Larson et al., 2001a; Fu et al., 2005; Qiu et al., 2007). The specific objectives of this study were: 1) to assess the genetic variation and genetic structure of 30 *F. hallii* populations and 2) to evaluate the associations of genetic diversity with 12 geographic, fragmentation and environmental attributes.

4.2 Materials and methods

4.2.1 Site sampling and tiller collection

Thirty *F. hallii* populations within the Fescue Prairie region of Western Canada covering Saskatchewan, Alberta and Manitoba, were sampled in summer 2005 and 2006 (Table 4.1; Fig. 4.1). The sampled *F. hallii* populations were distributed in five ecoregions (Ecological Stratification Working Group, 1996): Mixed Grassland, Moist Mixed Grassland, Aspen Parkland, Boreal Transition, and Mid-Boreal Upland, belonging to two ecozones: Prairie and Boreal Plain. Five of the collection sites were protected in National Parks or National Historic Sites. Land management practice of each site was recorded. The management practice of most non-protected sites was grazed by livestock, except a few that were grazed by bison or not grazed for up to 10 years.

Table 4.1 Major site characteristics and three estimates of genetic variation of the 30 *F. hallii* populations.

Site (label)	Eco-zone	Eco-region	Land management	Soil type	Latitude (N)	Longitude (W)	Population size	GDN (Km)	Genetic diversity estimates		
									PPB	MBF	WPV
Rumsey Natural Area (AB1)	P	MM	P	L	51°52'50"	112°41'41"	343,527	1.1	0.890	0.456	77.8
Cadogan (AB2)	P	MM	U	L	52°11'05"	110°48'40"	1,754	0.6	0.943	0.457	80.9
Horseshoe lake (AB3)	P	AP	U	SL	52°20'49"	110°54'19"	3,064	2.1	0.927	0.418	78.6
Holden (AB4)	P	AP	U	SL	53°15'03"	112°00'49"	1,373	1.7	0.967	0.397	82.2
Paradise valley (AB5)	P	AP	U	L	53°10'30"	110°41'40"	10,639	2.9	0.947	0.442	85.4
Kitscoty (AB6)	P	AP	U	L	53°18'29"	110°27'52"	20,281	1.0	0.911	0.426	77.4
Batoche (Ba)	B	BT	P	S	52°45'05"	106°08'25"	2,118	0.8	0.902	0.412	74.3
Hague (Hag)	P	AP	P	L	52°32'22"	106°33'17"	12,426	2.3	0.927	0.436	80.8
Kernen (Ker)	P	MM	P	SC	52°09'55"	106°31'41"	16,754	3.7	0.931	0.398	74.0
Macrorie (Mac)	P	MG	U	L	51°13'48"	107°13'48"	7,669	0.6	0.935	0.414	80.1
Monet (Mon)	P	MM	U	SL	51°08'35"	107°56'11"	2,260	0.7	0.902	0.430	79.4
PAJonasson (PAJ)	B	BT	P	SL	53°38'36"	106°38'11"	63,912	9.0	0.919	0.446	81.1
PASouthend (PAS)	B	BT	P	SL	53°35'16"	106°02'26"	11,117	2.5	0.915	0.430	77.7
PAWasstrom (PAW)	B	BT	P	SL	53°36'08"	106°31'01"	55,910	9.0	0.927	0.443	76.5
Riding Mountains (RM)	B	MB	P	SC	50°48'35"	100°14'42"	118,598	6.2	0.870	0.364	69.4
Aylesbury (S13)	P	MM	U	L	50°46'58"	105°51'19"	104	1.1	0.947	0.441	78.4
Hearts Hill (S31)	P	MM	U	SL	51°55'20"	109°59'43"	1,606	0.5	0.919	0.394	77.9
Wolverine (S32)	P	AP	U	L	51°57'55"	105°19'40"	124,882	1.4	0.927	0.418	73.7
Biggar (S34)	P	MM	U	SL	52°05'11"	107°43'10"	29,460	1.2	0.951	0.412	77.3
Bodo (S37)	P	AP	U	SL	52°08'12"	110°00'13"	2,730	6.0	0.935	0.428	80.0
Scott (S47)	P	MM	U	SL	52°18'42"	108°47'15"	2,156	1.7	0.947	0.445	80.3
Unity (S52)	P	MM	U	SL	52°20'56"	109°11'44"	4,795	3.0	0.947	0.424	82.8
Macklin (S54)	P	AP	U	S	52°20'56"	109°53'02"	420	1.2	0.931	0.423	79.8
Cando (S57)	P	MM	U	L	52°22'28"	108°25'47"	47,260	0.5	0.894	0.417	73.5
Denzil (S59)	P	MM	U	L	52°27'17"	109°33'24"	1,977	2.4	0.911	0.426	80.8
Hafford63 (S63)	P	AP	U	L	52°48'13"	107°32'56"	8,208	2.7	0.931	0.422	81.4
Hafford65 (S65)	P	AP	U	SL	52°50'35"	107°39'37"	9,098	1.0	0.963	0.424	81.9
Glaslyn (S72)	P	AP	U	L	53°12'03"	108°22'20"	539	0.8	0.955	0.442	80.5
Turtleford (S77)	B	BT	U	S	53°27'38"	109°03'12"	8,690	1.3	0.890	0.435	75.3
Zhner (S8)	P	AP	U	L	50°34'21"	104°29'14"	1,108	1.3	0.907	0.407	76.3

Note: Population: Monet = Monet PFRA community pasture; PAJonasson = Prince Albert National Park Jonasson's Flat; PASouthend = Prince Albert National Park Southend meadows; PAWasstrom = Prince Albert National Park Wasstrom's Flat (or Westrom Flats or Westrum Flats); Hearts Hill = Hearts Hill PFRA community pasture; Wolverine = Wolverine PFRA community pasture
Ecoregion: MM = Moist Mixed Grassland; AP = Aspen Parkland; BT = Boreal Transition; MG = Mixed Grassland; MB = Mid-Boreal Upland.
Ecozone: B = Boreal Plain; P = Prairie.
Land management: P = protected; U = grazed and oil gas.
Soil type: L = Loam; SL = Sandy loam; S = Sand; SC = Silt and clay loam.
GDN = geographic distance to the nearest neighbor with *F. hallii.* (km).
Genetic diversity estimates: PPB = proportion of polymorphic bands; MBF = mean band frequency; WPV = within population variation.

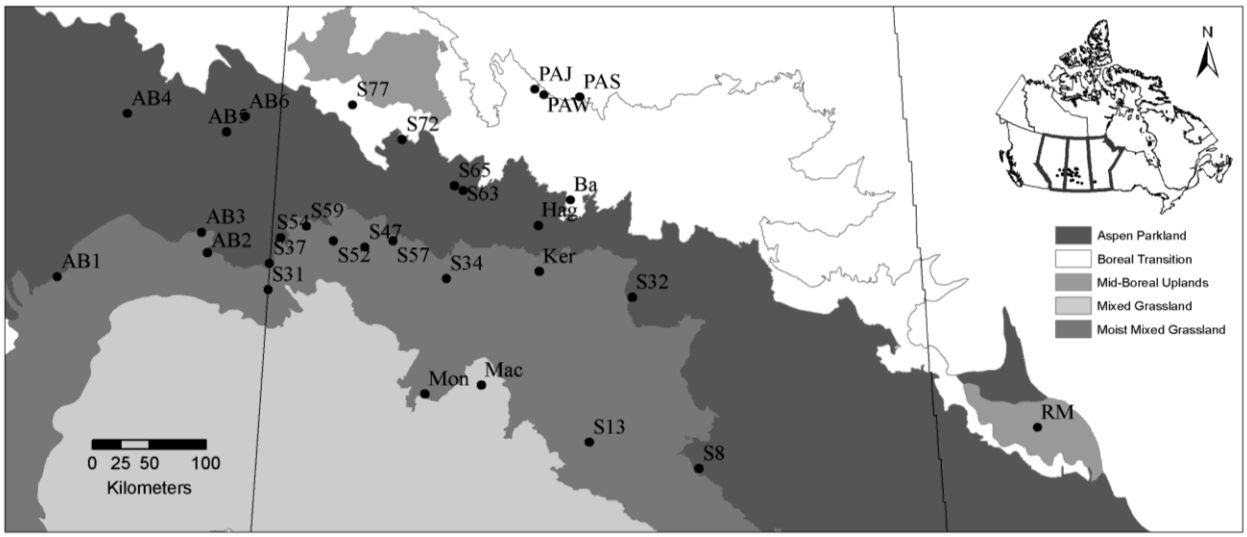


Fig. 4.1 Geographic locations of 30 *F. hallii* populations across the Canadian Prairie. Population abbreviation follows those in the text and Table 4.1.

Two measures of population fragmentation were estimated: (1) population isolation, and (2) population size. GPS positions of site boundary were recorded using a Trimble GeoXT™ GPS receiver (submeter accuracy) (Trimble, Sunnyvale, California, USA) and used to calculate site area and interpopulation geographic distances. Geographic distance to the nearest neighbor with *F. hallii* was used as the measure of population isolation. The coverage of *F. hallii* at each site was estimated using frequency frames (0.5 × 0.5 m) (Vogel and Masters, 2001). Each frame contained 25 squares (10 x 10 cm each). The number of squares in which at least one plant occurred was recorded. The process was repeated 20 times at each site. The relative population size at each site was estimated by multiplying the coverage of *F. hallii* with the site area.

At each sampled site, 30 individual plants that were at least 50 m from the site boundary and at least 5 m apart each other were randomly selected and tiller plugs were collected. Areas with extensive shrubs and invasive species were also avoided. Samples were transported to Plant Gene Resources of Canada, Saskatoon Research Centre, Agriculture and Agri-Food Canada (Saskatoon, SK, Canada). Tiller plugs were transplanted to plastic containers in the greenhouse at the Centre.

4.2.2 Environmental attributes of sampling sites

To characterize environmental conditions of the sites, three soil attributes were assessed and they were soil bulk density, soil type, and C:N ratio of the rooting zone. Four soil samples were

collected from 0 to 15 cm depth for each site using an 8 cm (in diameter) metal cylinder. Two replicate samples were dried at 80^{o}C for 48 hrs and then weighed for measuring soil bulk density. Two other samples were bulked and used for soil type determination. Soil type was determined by the percentage of clay, silt and sand (Canada Department of Agriculture, 1976) calculated from soil texture class and density readings (corrected for water temperature at the time of reading). Soil texture analysis was conducted with the Bouyoucos Hydrometer Procedure (Dodd et al., 2000). For carbon and nitrogen analysis, five soil samples were collected from 0 to 30 cm depth for each site using a push soil probe. Soil samples were air dried at room temperature and then ground to fine powder and sub-samples of 0.15 g were used. Total soil carbon and nitrogen concentration were analyzed using a LECO® Carbon/Nitrogen Analyzer (Leco Corp., St. Joseph, MI, U.S.A.), and C:N ratio was calculated.

Six climatic attributes corresponding to the ecodistrict (climate normals from 1961 to 1990) where each population was collected were extracted from the attribute data of the National Ecological Framework for Canada (Marshall et al., 1999). They were temperature parameters: mean annual temperature (MAT), average April to October temperature (T4-10), growing degree days above 5^{o}C (GDD5), and growing season length (GSL) and moisture parameters: mean annual precipitation (MAP) and the potential evapotranspiration and water deficit (PE).

4.2.3 AFLP analysis

Young leaf tissue from the growing tillers was harvested, stored in a paper envelope, freeze-dried, and kept at -20^{o}C for AFLP analysis. Genomic DNA was extracted from the leaf samples from each plant collected using the DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's instructions. Extracted DNA was quantified by fluorometry using Hoechst 33258 stain (Sigma Chemical Co., St. Louis, MO, U.S.A.), diluted to 25 ng μL^{-1} for AFLP analysis. The AFLP™ Analysis System 1 (Life Technologies, Burlington, ON, Canada) was applied following the protocol described by Vos et al. (1995) with exception of using γ^{33}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. Based on the previous AFLP analysis of *F. campestris* (Fu et al., 2005; Qiu et al., 2007), three most informative EcoRI: MseI primer pairs (E+AAC/M+CAG, E+ACG/M +CTA, and E+AGG/M+CTC) were selected to

screen all samples. To minimize technique-related and scoring errors, three duplicated samples of one individual were placed on each gel as control.

Automated analysis of banding patterns on 30 gels was conducted using GelComparII™ (Applied Maths, Belgium). The TIFF format gel images were processed from the autoradiographs using Epson Expression 10000XL scanner (Epson America, Long Beach, CA, U.S.A.). The conversion, normalization, and background subtraction of the gel images were conducted following the Gelcompar II User's Guide. The image of one gel was normalized using the three duplicate samples as reference lanes. Image alignment among multiple gels produced from the same primer pairs was performed using an external reference (a 30-330 base pair AFLP DNA ladder; Promega, Madison, WI, U.S.A.) and an internal reference (bands that were monomorphic across gels). The aligned gel images were automatically scored as 1 (presence) or 0 (absence). The duplicated samples were used to assess the consistency of AFLP reactions. Only the bands either present or absent in all the 30 control samples for each primer pair were output for further analysis.

4.2.4 Data analysis

The presence/absence data from the AFLP samples were analyzed for the level of polymorphism by counting the total number of bands and the number of polymorphic bands, calculating the proportion of polymorphic bands (PPB), and generating mean band frequencies (MBF) with respect to primer and population. The within-population variation (WPV) (the average number of pairwise differences within a population) was estimated using Arlequin 3.0 (Excoffier et al., 2005).

To assess AFLP variation components and test their statistical significance levels, analysis of molecular variance (AMOVA) was performed with respect to population, ecoregion, ecozone, soil type, and land management. This analysis not only allows partitioning of the total AFLP variation into within- and among- group components, but also provides a measure of inter-group genetic distances as a proportion of the total AFLP variation residing between any two groups (the Phi statistic; Excoffier et al., 1992). The significance of variance components and inter-group genetic distances was tested with 9,999 random permutations.

At the population level, the inter-population genetic distance matrices were analyzed using NTSYS-pc 2.3 (Rohlf, 1997) and clustered with the algorithm of unweighted pair-group

method using the arithmetic averages (UPGMA) to assess genetic associations of the assayed populations. Where ties occurred, a warn option was used in the UPGMA analysis. The patterns of regional genetic structure were assessed using GenAlEx v6 software (Peakall and Smouse, 2005) to determine the population-scale genetic spatial autocorrelation. As the average physical distances of the pairwise plants sampled among 30 populations varied greatly from 9 to 870 kilometres with an average of 249 kilometres, the autocorrelation analysis was arbitrarily limited to a maximum distance of 40 kilometres with 21 equal distance classes.

The association between inter-population genetic distance and geographic distance matrices was also assessed with the Mantel test (Mantel, 1967) using NTSYS-pc 2.3 (Rohlf, 1997) with 9,999 random permutations. Mantel test and partial Mantel test were used to evaluate the effects of fragmentation (population size and geographic distance to the nearest neighbor), geographic position (latitude and longitude), and local environment (MAT, T4-10, GDD5, and GSL; MAP and PE; soil bulk density and C:N ratio) on genetic differentiation. Since moisture parameters and geographic distances were correlated, the correlation between genetic and moisture attributes (Volis et al., 2004) was assessed using a partial Mantel association test (MXCOMP module of NTSYSpc; Smouse et al., 1986; Legendre and Fortin, 1989; Hirao and Kudo, 2004; Roseman, 2004; Still et al., 2005; Vandepitte et al., 2007). The relationship between pairwise genetic distance (phi statistic) was first tested separately for dissimilarity matrix of each moisture factor and geographic distance among populations. All moisture factors and the geographic distance were then added as control variables in a partial Mantel test. Each moisture dissimilarity matrix was constructed by subtracting the differences in values between populations.

4.3 Results

Major site characteristics including geographic and fragmentation characteristics of the 30 *F. hallii* populations are listed in Table 4.1 and environmental characteristics in Appendix Table 4.2. The study area of *F. hallii* populations ranged from 173 to 412,893 m^2 and the estimated relative population size ranged from 104 to 343,527 individuals per sampling site. The geographic distance to the nearest neighbor ranged from 0.5 to 9.0 km. Most populations were found on loam to sandy loam soils with soil bulk density ranged from 0.75 to 1.43 g/cm^3 and C:N ratio ranged from 9.3 to 13.4.

Table 4.2 Environmental characteristics of the 30 studied *F. hallii* populations. MAT = mean annual temperature, T4-10 = mean temperature from April to October, GSL = growing season length, GDD5 = growing degree days above 5°C, MAP = mean annual precipitation, and PE = potential evapotranspiration and water deficit.

Population	MAT (°C)	T4-10 (°C)	GSL (day)	GDD5 (°C day)	MAP (mm)	PE (mm)	Bulk density (g/cm^3)	C:N ratio
Rumsey Natural Area	2.9	11.2	181	1392.7	428.5	-265.0	1.12	10.2
Cadogan	2.4	11.5	179	1472.1	367.3	-415.4	1.04	9.8
Horseshoe lake	2.4	11.5	179	1472.1	367.3	-415.4	0.97	10.1
Holden	2.8	11.4	183	1437.3	432.7	-249.1	1.32	11.2
Paradise valley	1.7	10.8	178	1350.7	428.8	-253.0	0.94	10.8
Kitscoty	1.7	10.8	178	1350.7	428.8	-253.0	0.98	10.3
Batoche	0.6	10.8	173	1374.5	412.4	-281.3	1.36	10.4
Hague	1.1	11.1	175	1439.3	397.5	-339.0	0.84	10.2
Kernen	2.2	12.0	180	1584.9	362.2	-394.0	1.14	10.3
Macrorie	3.2	12.5	185	1661.1	322.4	-513.8	0.96	9.3
Monet	3.2	12.2	184	1612.3	324.5	-579.1	1.13	10.4
PAJonasson	0.5	10.4	172	1293.7	441.0	-219.3	1.04	10.6
PASouthend	0.5	10.4	172	1293.7	441.0	-219.3	1.06	10.8
PAWasstrom	0.5	10.4	172	1293.7	441.0	-219.3	1.01	11.0
Riding Mountains	1.2	10.9	173	1406.4	503.5	-95.7	0.75	10.2
Aylesbury	3.1	12.3	183	1636.2	351.7	-508.4	0.98	10.2
Hearts Hill	2.4	11.5	179	1472.1	367.3	-415.4	1.10	10.5
Wolverine	1.2	11.2	174	1451.2	414.1	-264.2	1.20	11.8
Biggar	2.0	11.6	178	1519.3	350.5	-417.4	1.03	11.4
Bodo	2.4	11.5	179	1472.1	367.3	-415.4	1.26	11.3
Scott	1.7	11.3	177	1444.3	367.5	-361.0	1.43	13.4
Unity	1.7	11.3	177	1444.3	367.5	-361.0	1.29	10.8
Macklin	2.1	11.4	178	1473.8	407.6	-344.9	1.25	10.7
Cando	1.7	11.3	177	1444.3	367.5	-361.0	1.24	12.5
Denzil	1.9	11.4	179	1463.0	398.3	-333.8	1.15	10.4
Hafford63	0.8	10.5	172	1325.4	392.6	-293.6	1.07	10.0
Hafford65	0.8	10.5	172	1325.4	392.6	-293.6	1.20	10.1
Glaslyn	0.8	10.5	172	1325.4	392.6	-293.6	0.98	10.7
Turtleford	1.2	10.5	174	1298.4	421.6	-251.5	1.43	11.8
Zhner	2.1	11.7	177	1531.3	407.5	-331.3	1.12	10.4

Note: Population: Monet = Monet PFRA community pasture; PAJonasson = Prince Albert National Park Jonasson's Flat; PASouthend = Prince Albert National Park Southend meadows; PAWasstrom = Prince Albert National Park Wasstrom's Flat (or Westrom Flats or Westrum Flats); Hearts Hill = Hearts Hill PFRA community pasture; Wolverine = Wolverine PFRA community pasture.

4.3.1 AFLP variation

The three AFLP primer combinations detected a total of 330 DNA fragments among the 840 individuals, of which 246 bands were polymorphic. The primer pair E+ACG-M+CTA amplified the most DNA fragments (87). The primer pair E+AAC-M+CAG had the highest mean band frequency of 0.528, ranging from 0.024 to 0.983, while E+AGG-M+CTC revealed the

polymorphic bands with the lowest mean band frequency of 0.350, ranging from 0.014 to 0.930.

Genetic variation for the 30 *F. hallii* populations was quantified by the proportion of scored polymorphic bands, the mean band frequency, and the within-population variation (Table 4.1). Within a population, the proportion of scored polymorphic bands over the three primer pairs ranged from 0.870 to 0.967 ($\overline{X}$ = 0.926), the mean band frequency ranged from 0.364 to 0.457 ($\overline{X}$ = 0.424), and the within-population variation ranged from 69.4 to 85.4 ($\overline{X}$ = 78.5). The Riding Mountain population (RM) consistently had the lowest proportion of polymorphic bands, mean band frequency, and within-population variation. The Holden population from Alberta had the highest proportion of polymorphic bands, the Cadogan population in Alberta had the highest mean band frequency, and the Paradise Valley population in Alberta had the most within-population variation.

4.3.2 Population structure

Partitioning of the total AFLP variation into within- and among- population components by AMOVA showed that 93.5 % of the total variation was held within populations and 6.5% resided among populations (Table 4.3). These fractions were significantly different from zero at $P <$ 0.0001 based on the permutation test. The AMOVA with respect to ecozone and ecoregion revealed only 1.3% AFLP variation between two ecozones, 1.3% among the three ecoregions, whereas 98.7% of AFLP variation resided within ecozones and within ecoregions. Only 1.3% AFLP variation was found among four soil types and 0.6% AFLP variation resided between two land management categories of fescue samples. Interestingly, protected fescue stands displayed relatively less AFLP variation than those unprotected.

Table 4.3 The results for the analysis of molecular variance (AMOVA) within and among the samples of 30 *F. hallii* populations with respect to population, ecozone (Boreal Plain and Prairie), ecoregion (Moist Mixed Grassland; Aspen Parkland; Boreal Transition; Mixed Grassland; Mid-Boreal Upland), soil type (Loam, Sandy loam, Sand, and Silt and clay loam), and land management (protected and unprotected).

Model and source of variation	df	Variance components	% variation
Population			
Among populations	29	2.7	6.5
Within populations	810	39.3	93.5
Ecozone			
Between ecozones	1	0.6	1.3
Within ecozones	838	41.7	98.7
*Ecoregion**			
Among ecoregions	2	0.5	1.3
Within ecoregions	837	41.5	98.7
Soil type			
Among soil types	3	0.6	1.3
Within soil types	836	41.5	98.7
Land management			
Between land managements	1	0.3	0.6
Within land managements	838	41.8	99.4

Note: all the variance components were statistically significant at $P < 0.0001$ level, as calculated from random permutations.

*Macrorie (Mixed Grassland) was added to Moist Grassland Ecoregion, and Riding Mountain (Mid-Boreal upland) was combined into Aspen Parkland Ecoregion.

The largest between-population difference measured by interpopulation genetic distance was observed between the Kistscoty population in Alberta and the Riding Mountain population in Manitoba (0.184), and the lowest between-population difference measured was between Unity and Macklin populations in Saskatchewan (0.004). Genetic clustering of the 30 populations revealed three major groups and four distinct populations at the genetic distance of 0.06 (Fig. 4.2). The first group on the top of the dendrogram consisted of three populations (two from Alberta and one from Prince Albert National Park), the second group had four populations (two from Prince Albert National Park), and the third large group consisted of 19 populations. Four distinct populations on the bottom of the dendrogram were Kernen (or Ker), Aylesbury (or S13), Batoche (or Ba), and Riding Mountain (or RM). Also, the genetic clustering did not appear to be associated with the ecoregions and ecozones in which these populations were located.

A significant correlation was found between the pairwise genetic distances and their

corresponding geographic distances (r = 0.39; P < 0.004) (Fig. 4.3a). The autocorrelation analysis revealed a weak population differentiation among the 30 *F. hallii* populations (Fig. 4.3b). The autocorrelogram, however, showed significant positive genetic correlations among geographically close populations and the autocorrelogram of the genetic correlation coefficient tended to decrease at increasing distance classes. The x-intercept for the autocorrelation coefficient was approximately 60 km where the coefficient was statistically significant (i.e., outside of the 95% confidential interval), which corresponds to the distance among populations where genetic correlations expect to cease.

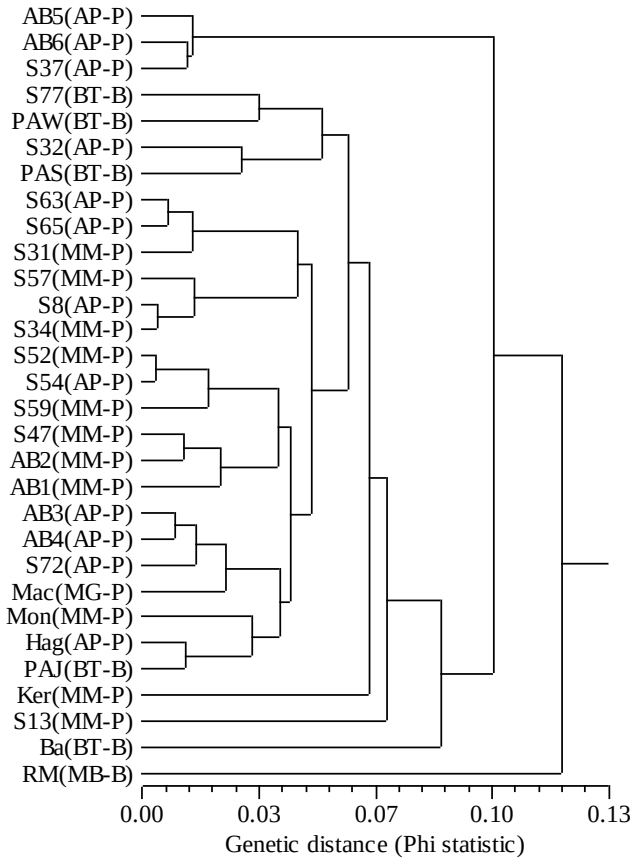


Fig. 4.2 Genetic associations of 30 *F. hallii* populations revealed by UPGMA cluster analysis of pairwise genetic distances (Phi statistics) derived from analysis of molecular variance. A population is labelled, followed by its ecoregion and ecozone labels in parenthesis. The labels for population, ecoregion, and ecozone are given in Table 4.1.

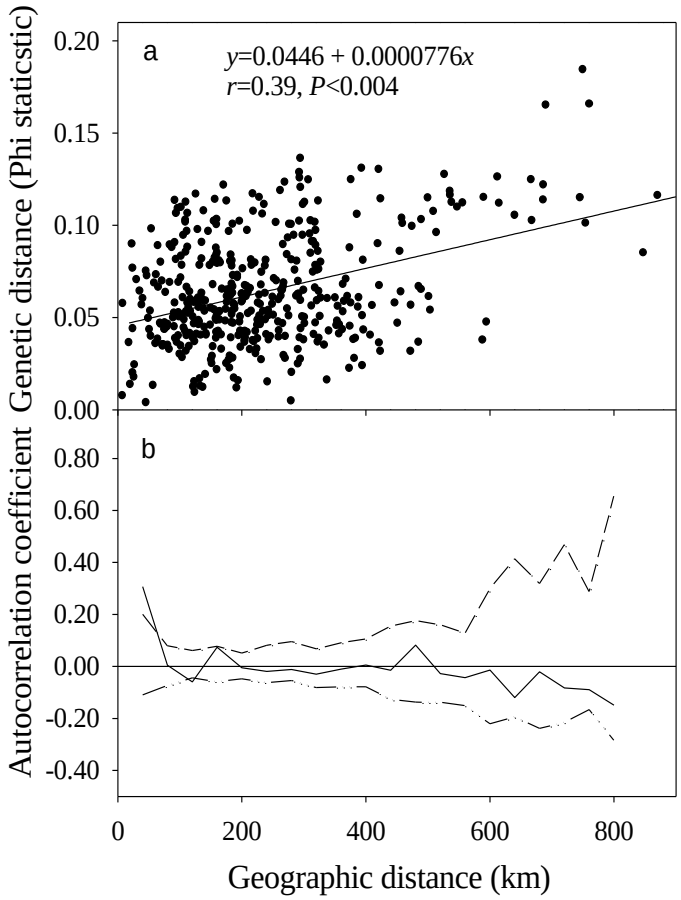


Fig. 4.3 Associations between geographic distances and genetic distances (Phi statistics) assessed by Mantel test (a) and correlograms of genetic correlation coefficient as a function of geographic distance inferred by a spatial structure analysis (b) in 30 *F. hallii* populations. The dashed lines in B show the permuted 95% confidence intervals.

4.3.3 Associations with geographic, fragmentation, and environmental variables

Genetic variation was not correlated with latitude but was significantly correlated with longitude, with more genetic variation in the western than in the eastern populations. Genetic differentiation was also significantly correlated with the differentiation of longitude based on Mantel test (Table 4.4). Genetic variation was not correlated with population size or isolation measured as geographic distance to the nearest neighbor (Table 4.4).

Temperature parameters including mean annual temperature, mean April to October temperature, growing season length and growing degree days above 5^{o}C and soil characteristics including soil bulk density and C:N ratio were not significantly correlated with genetic variation (not shown). Based on the Mantel test, genetic differentiation was significantly correlated with

the differentiation of moisture attributes (Table 4.4). As a correlation between the differentiation of the moisture attributes and geographic distances was also detected, the partial Mantel test was made conditional on either geographical distance or moisture attribute. It was found that genetic differentiation was more associated with geographical distance than the differentiation of the moisture attributes.

Table 4.4 Significant correlations of genetic differentiation with the differentiation of geographic, fragmentation, and environmental variables of the 30 *F. hallii* populations, as assessed by Mantel and Partial Mantel tests. GED = pairewise interpopulation genetic distances (phi statistics) among 30 *F. hallii* populations; GD = pairewise interpopulation geographic distances; MAP = mean annual precipitation; PE = potential evapotranspiration and water deficit (mm).

Matrix 1	Matrix 2	Matrix 3	Correlation	*P-value*
GED	Longitude		0.359	0.009
GED	MAP		0.292	0.015
GED	PE		0.256	0.029
GED	GD		0.390	0.004
MAP	GD		0.468	0.001
PE	GD		0.401	0.001
GED	GD	MAP	0.299	0.008
GED	GD	PE	0.324	0.006
GED	MAP	GD	0.135	ns
GED	PE	GD	0.119	ns

Note: under the partial Mantel test as described in Smouse et al. (1986), the pattern of association in the dependent matrix 1 was compared with the predictor matrix 2, while controlling for the effects of matrix 3.
ns: Not significant ($P > 0.05$).

4.4 Discussion

This study represents the first comprehensive diversity analysis of a fescue grass species across the Canadian Prairie. The analysis not only demonstrates that *F. hallii* was genetically highly diverse, but also that the AFLP variation in remnant fescue populations was weakly differentiated but geographically associated. Geographic position and moisture gradient, rather habitat fragmentation and attributes, displayed considerable genetic influence on the remnant fescue populations. These patterns of genetic variation are compatible with the interesting biology of this fescue species with outcrossing, tetraploid, and perennial. The findings obtained in this study are significant not only for understanding the long-term survival and adaptation of this fescue species, but also facilitate the efforts of restoring the fragmented fescue communities.

4.4.1 Genetic variation and differentiation

This study revealed an extremely high level of AFLP variation harboured in the remnant fescue populations, despite the reduction and fragmentation in its geographic distribution, when compared with other grass species. For example, these fescue populations had the higher level of proportion of polymorphic bands (ranged from 0.870 to 0.967) than natural populations of bluegrasses, in which the proportion of polymorphic bands was 0.84 (Larson et al., 2001a). It is not surprising for this tetraploid and perennial fescue species with outcrossing to have a high level of genetic polymorphism. Comparable high levels of AFLP variation have been reported for other highly outcrossing grass species such as bluebunch wheatgrass (Larson et al., 2000), smooth and meadow brome (Ferdinandez and Coulman, 2002), crested wheatgrass (Mellish et al., 2002), little bluestem (Fu et al., 2004), and mountain rough fescue (Fu et al., 2005).

However, the fescue populations were weakly differentiated with only about 6.5% of the total AFLP variation spread over various populations. A large majority (93.5%) of the total AFLP variation resided within the fescue populations. This level of variation partition represents the high end of genetic variation observed for outcrossing perennial grass species, in which the within-population marker variation ranges generally from 65-95% of the total variation (Casler et al., 2007). It is possible that the assayed fescue populations may have consisted of several generations maintained from rhizome propagation and seed banks. The persistence of overlapping generations within populations would greatly contribute to the maintenance of high genetic variation within the natural stands and to dilute the genetic differentiation among populations. The weak differentiation may also reflect extensive gene flow through seed and pollen dispersal, particularly among the nearby fescue stands (Hamrick and Godt 1989; Culley et al., 2007).

4.4.2 Geographic variation

The AFLP variation in remnant fescue populations was found to be geographically associated across the Canadian Prairie. Mantel test revealed a positive correlation between the pairwise genetic distances and their geographic distances (Fig. 4.3a), pointing to the pattern of isolation-by-distance for this fescue species. A gradual decrease in spatial autocorrelation with increasing geographic distance between populations was found (Fig. 4.3b). The correlogram showed that a pair of fescue populations originating 60 km or less apart would have a positive genetic

autocorrelation and the correlation coefficient declined slowly when the geographic distance was beyond 60 km. However, these correlation analyses also seem to suggest that the spatial genetic structure across the Canadian Prairie was relatively weak, given the findings of high within-stand variation and strong gene flow within a short distance.

The geographic variation of fescue plants was further confirmed by the finding of the correlation between the AFLP variation and longitudes of the fescue stands. The western populations had more genetic variation than the eastern populations (Table 4.4). A similar east-west pattern of genetic variation was also found in *Pinus palustris* (Schmidtling and Hipkins, 1998) and in *Arabidopsis thaliana* (Beck et al., 2008). This east-west pattern may be related to Pleistocene glaciation and post-glacial colonization, as many species became restricted or retreated into glacial refugia (Schmidtling and Hipkins, 1998; Elderkin et al., 2007; Beck et al., 2008). As glaciers retreated, areas of suitable habitat to the east were colonized by individuals from adjacent western refugia. The post-glacial colonization had resulted in reduced genetic diversity in newly-founded populations (Beck et al., 2008). In North America, glaciers from the Rocky Mountains and north-central Canada met each other at the center of the continent. It is possible that this fescue species had a single refugium in the foothills of Alberta during the recent glaciations and migrated northward and eastward during the post-glacial colonization. However, this study is limited to only the extant fescue populations in the northern range of its distribution, offering little resolution on the population demography. Further study on historical population processes with more informative DNA markers may help explain the observation of the longitudinal decline in fescue AFLP variation.

4.4.3 Association with fragmentation and environmental variables

The lack of the associations between the AFLP variation and fragmentation attributes (i.e., population size and geographic distance to the nearest other population; Table 4.4) indicates that habitat fragmentation has not had considerable impact on the fescue populations. Such non-associations were also found in other plant species, such as *Predicularis palustis* in Germany (Schmidt and Jensen, 2000), endangered shrub *Grevillea caleyi* in Australia (Llorens et al., 2004), endangered calcareous grassland plant *Globularia bisnagarica* in Belgium (Honnay et al., 2007), and in three native plant species across Minnesota, U.S.A. (Moncada et al., 2007). The little genetic response to habitat fragmentation may reflect the short period of fragmentation so far

upon the fescue stands. Many more generations of genetic changes after a fragmentation event are needed to display detectable genetic responses (Llorens et al., 2004; Geert et al., 2007; Honnay et al., 2007), particularly for long-lived perennial plant species such as this fescue grass with a high level of genetic variation (Young et al., 1999; Culley et al., 2007; Honnay et al., 2006, 2007; Geert et al., 2007). Also, the assayed populations, although remnant, still were relatively large in size ranging from 104 to 343,527 individuals per sampling site. The founder effects and genetic drift remained weak in these remnant populations. The gene flow may have been active among neighboring sites, given the fact that the geographic distance to the nearest neighbor ranging from 0.5 to 9.0 km was much shorter than 60 km for a significant population differentiation. Moreover, the overlapping generation from seed and rhizome propagation would enhance population resistance to change and may dilute the genetic response to fragmentation.

Historical population processes may also be a contributing factor for the lack of genetic response to habitat destruction (Schmidt and Jensen, 2000; Llorens et al., 2004; Honnay et al., 2007). The relatively short history of the grassland formation in the Northern Great Plains after the last glaciation may indicate a common ancestral history among fescue populations (Still et al., 2005). It is possible that fescue populations have had a long history subdivision, just considering natural habitat discontinuities or favourable aspects of the species biology as mentioned above. In the natural stands assayed, fescue plants were found predominantly on the north-facing slopes and seemed to adapt to an area with certain favourable environmental attributes, particularly related to moisture (Table 4.3). It is known that fescue plants were associated with moist black soil throughout the prairies (Baines, 1973; Hill et al., 2000). Similar results were found in *Echinacea augustifolia* throughout the Great Plains of North America (Still et al., 2005), where climatic variables such as annual mean temperature were related to genetic variation among populations. Recently, climate change with increasing temperature and droughts has been identified as one of the largest potential problems for plant conservation (Thomas et al., 2004).

4.4.4 Implications for fescue conservation

This study has generated the first useful set of genetic and site information to address restoration and conservation concerns for this fescue species. The assayed fescue populations appear to be maintaining large genetic diversity, implying that genetic erosion (or genetic drift and inbreeding) was small (if any) in these isolated fescue stands. The AFLP variation was not significantly

correlated with population size and geographic distance to the nearest neighbor, indicating a minimal impact of the population fragmentation over the last century upon the fescue genetic diversity. Thus, these findings are encouraging for the re-establishment of fragmented fescue network across the Fescue Prairie, as restoration of *F. hallii* where none presently exist should be genetically feasible. Using plant material from the nearby fescue stands should not degrade much the genetic background of the restored stands. However, as the fescue genetic variation was associated with the moisture attributes, the plant materials from the nearby sites with similar moisture conditions should be preferred. The finding of a significant genetic similarity within 60 km implies that the fescue plants should not be translocated between sites more than 60 km apart. A pollen/seed transfer zone within 60 km from current natural populations could be considered. However, these restoration considerations emphasize more on fescue genetics and less on its ecology such as seed germination and seedling establishment. Further studies on seed germination are needed to assess the germination characteristics of seeds collected from natural fescue populations across the geographic range. Also, the finding of the east-west pattern of genetic variation implies that the remnant fescue stands along this gradient should be the target for protection from further fragmentation. Although the protected fescue stands had relatively less genetic variation than the unprotected populations, more emphasis should still be placed on the conservation of remnant fescue plants in the east edge of species distribution.

5. SPATIAL VARIATION IN SEED GERMINATION CHARACTERISTICS OF REMNANT *FESTUCA HALLII* POPULATIONS ACROSS THE CANADIAN PRAIRIE

Abstract

Grassland in North America is expected to expand northward as the climate becomes warmer and drier under many climate change scenarios. Successful adaptation of plant species to climate change depends on regeneration success. This paper aims to identify the spatial variation in seed germination characteristics of *F. hallii* populations and to quantify the effect of genetic, geographic, demographic, or climatic factors on the variation. Seeds were collected from 15 populations in five ecoregions over a wide geographic range in Canada. Seeds were germinated at 7 growth chambers with constant temperatures from 5 to 35^{o}C with 5^{o}C increments. Thermal time models were constructed to generate germination parameters. Correlations between germination parameters and genetic variation, longitude, latitude, population size, geographic distance to the nearest neighbour, mean annual temperature (MAT) and mean annual precipitation (MAP) were assessed. Base temperatures (T_b, minimal or base temperature permitting germination) of the 15 populations fell into a narrow range within 2.2^{o}C with an average of 1.1^{o}C. T_b was positively correlated with latitude and negatively with longitude and the thermal time requirement for 50% germination was negatively correlated with latitude. Seed mass was negatively correlated with MAT and positively correlated with MAP. High final germination percentage was reached at a wide temperature range from 5 to 20^{o}C with the highest germination percentage at 10^{o}C. Germination rate index (GRI) increased with increasing temperature from 5 to 20^{o}C. Final germination percentage at 5^{o}C was negatively correlated with MAT and positively correlated with MAP. GRI at 5 and 10^{o}C were positively correlated with MAP but not with MAT. GRI at 5^{o}C was positively correlated with longitude. Germination was not correlated with any genetic or demographic attributes assayed. Population-wise variation in temperature and precipitation affected seed mass, an important attribute determining seed germinability. Sites with greater precipitation and lower temperature had heavier seeds, which usually had lower thermal time requirements for germination and faster germination. The warmer and drier future climate as predicted for the Canadian prairie may reduce regeneration

success of this species in the current distribution range, particularly after disturbances followed by sexual regeneration from seeds.

5.1 Introduction

Recent years have seen the increasing efforts to address the possible shift of species distribution under predicted climate change (Hogg and Schwarz, 1997; Graae et al., 2008; Levine et al., 2008; Morin et al., 2008; Petru and Tielborger, 2008). Grassland in North America is expected to expand northward as the climate becomes warmer and drier under many climate change scenarios (Sargent, 1988; Rizzo and Wiken, 1992; Hogg, 1994). Whether the grasslands can adapt to these climate changes is uncertain. In this study I report on the germination response of *F. hallii* to simulated variation in environmental conditions.

Successful adaptation of plant species to climate change depends on regeneration success. Even though vegetative regeneration is the main mechanism in long-lived perennial grasses such as *F. hallii*, sexual reproduction plays a major role after disturbances and in restoration (Romo et al., 1991). Seed germination is a critical stage of the life cycle reflecting adaptation to local habitats and affecting seedling establishment and population dynamics (Probert, 2000; Pendleton and Meyer, 2004; Donohue et al., 2005a, b; Zeiter et al., 2006). Understanding seed germination response is important not only for understanding and predicting the ecological adaptation of the species, but also for formulating effective restoration strategies for remnant populations. Remnant populations are at risk of deteriorating genetic variability and, in the long term, these small populations may have reduced ability to adapt to changing environments and potentially become extirpated (Templeton et al., 1990; Ellstrand and Elam, 1993; Young et al., 1996). Seed mass and germination rate are important seed features that have strong adaptive implications for survival (Luzuriaga et al., 2006). However, knowledge of germination thresholds in changing environments is extremely scarce, especially for species targeted for conservation and restoration.

Seed germination is a complex trait influenced by both genetic determinants and environmental factors (Pendleton and Meyer, 2004; Schmuths et al., 2006; Boyd et al., 2007). Seed germination varies with the environmental conditions of the mother plants, such as temperature (Lacey, 1996; Sugiyama, 2003; Murray et al., 2004) and soil moisture conditions (Luzuriaga et al., 2006). A global temperature increase of 3-5°C by the end of this century is predicted and the increasing temperature and drought has been recently identified as one of the

biggest potential problems for conservation (Thomas et al., 2004). The identification of spatial patterns in germination thresholds can help us understand species distribution range, predict possible changes in future distributions as a result of global warming, and develop suitable conservation strategies.

Fescue prairie, bordering the boreal forest to the south and centred in Saskatchewan, Alberta, Manitoba, Montana, and North Dakota (Aiken et al., 1996, 1997; Anderson, 2006), is one of the most threatened ecosystems in the Canadian prairie (World Wildlife Fund, 1988). Since the time of European settlement, widespread conversion of Fescue prairie has created a landscape with small, isolated patches of remnant fescue embedded in a dissimilar matrix of cultivated fields, grazed pastures, roads, and human settlements (Archibold and Wilson, 1980; Romo, 2003). *Festuca hallii* [(Vasey) Piper] (plains rough fescue) is the distinctive characteristic species in the Fescue prairie (Aiken et al., 1996, 1997; Anderson, 2006). Few ecological studies have been conducted on this species (Coupland and Brayshaw, 1953; Pavlick and Looman, 1984; Romo et al., 1991) and little information is available on the ecological impacts of habitat fragmentation on this species. The objectives of this study were 1) to assess the germination characteristics of seeds collected from 15 populations across the Canadian prairie and 2) to evaluate the correlations of germination thresholds with genetic, geographic, demographic and climatic variables.

5.2 Materials and methods

5.2.1 Site characteristics, demographic and genetic properties and seed collection

We collected seed from remnant fescue populations. Twenty four *F. hallii* populations in Saskatchewan and Manitoba were visited in summer 2005 of which only 15 had seed sets (Table 5.1; Fig. 5.1). The 15 sampled populations were distributed in five Ecoregions (Ecological Stratification Working Group, 1996): Mixed Grassland, Moist Mixed Grassland, Aspen Parkland, Boreal Transition, and Mid-Boreal Upland, belonging to two ecozones: Prairie and Boreal Plain representing a diversity of land forms across a longitudinal and latitudinal gradient.

To characterize site environmental conditions, mean annual temperature (MAT) and mean annual precipitation (MAP) corresponding to the ecodistrict (climate normals from 1961 to 1990) where each population was collected were extracted from the attribute data of the National Ecological Framework for Canada (Marshall et al., 1999). Mean annual temperature was from

0.5 to 3.2^{o}C and annual mean precipitation was from 322.4 mm to 503.5 mm among the study sites (Table 5.1).

Table 5.1 Major site characteristics and three estimates of genetic variation of the 15 *F. hallii* populations.

Site (label)	Eco-zone	Eco-region	Latitude (N)	Longitude (W)	MAT (°C)	MAP (mm)	Size	GDN (km)	PPB	MBF	WPV
Batoche (Ba)	B	BT	52°45'05"	106°08'25"	0.6	412.4	2,118	0.8	0.902	0.412	74.3
Hague (Hag)	P	AP	52°32'22"	106°33'17"	1.1	397.5	12,426	2.3	0.927	0.436	80.8
Kernen (Ker)	P	MM	52°09'55"	106°31'41"	2.2	362.2	16,754	3.7	0.931	0.398	74.0
Macrorie (Mac)	P	MG	51°13'48"	107°13'48"	3.2	322.4	7,669	0.6	0.935	0.414	80.1
Monet (Mon)	P	MM	51°08'35"	107°56'11"	3.2	324.5	2,260	0.7	0.902	0.430	79.4
PASouthend (PAS)	B	BT	53°35'16"	106°02'26"	0.5	441.0	11,117	2.5	0.915	0.430	77.7
Riding Mountains (RM)	B	MB	50°48'35"	100°14'42"	1.2	503.5	118,598	6.2	0.870	0.364	69.4
Hearts Hill (S31)	P	MM	51°55'20"	109°59'43"	2.4	367.3	1,606	0.5	0.919	0.394	77.9
Scott (S47)	P	MM	52°18'42"	108°47'15"	1.7	367.5	2,156	1.7	0.947	0.445	80.3
Unity (S52)	P	MM	52°20'56"	109°11'44"	1.7	367.5	4,795	3.0	0.947	0.424	82.8
Macklin (S54)	P	AP	52°20'56"	109°53'02"	2.1	407.6	420	1.2	0.931	0.423	79.8
Cando (S57)	P	MM	52°22'28"	108°25'47"	1.7	367.5	47,260	0.5	0.894	0.417	73.5
Denzil (S59)	P	MM	52°27'17"	109°33'24"	1.9	398.3	1,977	2.4	0.911	0.426	80.8
Hafford65 (S65)	P	AP	52°50'35"	107°39'37"	0.8	392.6	9,098	1.0	0.963	0.424	81.9
Turtleford (S77)	B	BT	53°27'38"	109°03'12"	1.2	421.6	8,690	1.3	0.890	0.435	75.3

Note: Population: Monet = Monet PFRA community pasture; PASouthend = Prince Albert National Park Southend meadows; Hearts Hill = Hearts Hill PFRA community pasture.
Ecozone: B = Boreal Plain; P = Prairie.
Ecoregion: AP = Aspen Parkland; BT = Boreal Transition; MB = Mid-Boreal Upland; MG = Mixed Grassland; and MM = Moist Mixed Grassland.
MAT = mean annual temperature; MAP = mean annual precipitation; Size = estimated population size; GDN = geographic distance to nearest neighbour.
Genetic diversity estimates: PPB = proportion of polymorphic bands; MBF = mean band frequency; WPV = within population variation.

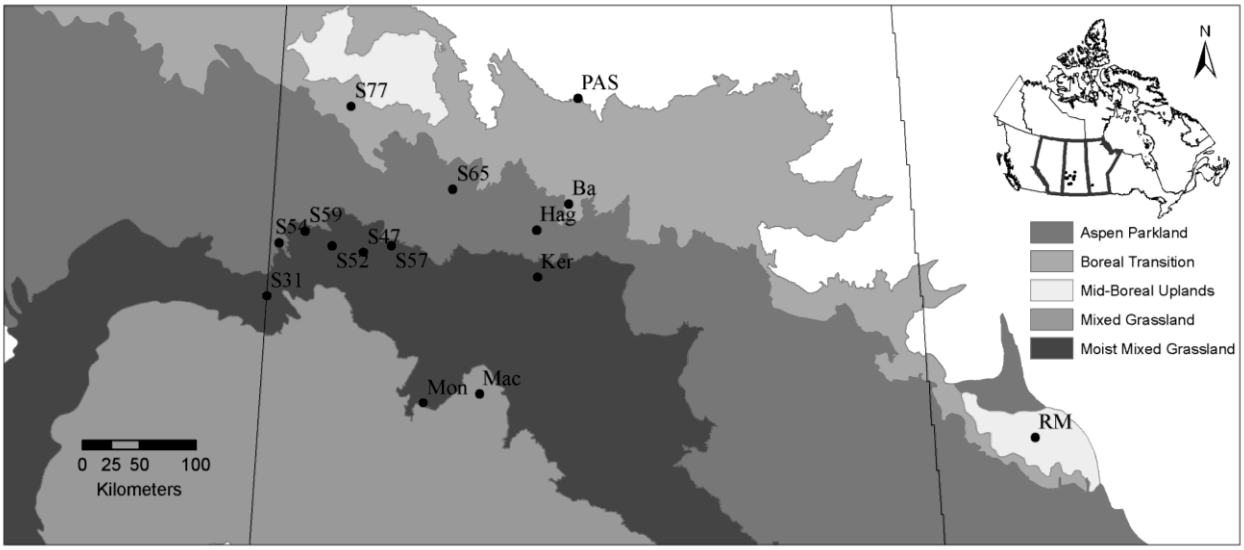


Fig. 5.1 Geographic locations of 15 *F. hallii* populations across the Canadian Prairie. Population abbreviation follows those in the text and Table 5.1.

Two measures of population fragmentation were estimated: population size and population isolation. GPS positions of site boundaries were recorded using a Trimble GeoXT™ GPS receiver (submeter accuracy) (Trimble, Sunnyvale, California, USA) and used to calculate site area. The coverage of *F. hallii* at each site was estimated using frequency frames (0.5 × 0.5 m) (Vogel and Masters, 2001). Each frame contained 25 squares (10 x 10 cm each). The number of squares in which at least one plant occurred was recorded. The process was repeated 20 times at each site. The relative population size at each site was estimated by multiplying the coverage of *F. hallii* with the site area. Geographic distance to the nearest neighbour with *F. hallii* was used as the measure of population isolation that we measured by surveying the surrounding area with the GPS receiver. The estimated population size ranged from 420 to 118,598 individuals per site. The geographic distance to the nearest neighbour ranged from 0.5 to 6.2 km among sites (Table 5.1).

Population genetic variation was estimated using amplified fragment length polymorphism markers in a companion genetic diversity study. The estimation procedure is described in Qiu et al. (2007). Three major genetic variation estimates were used in this study: proportion of polymorphic bands (PPB), mean band frequency (MBF), and within population variation (WPV). PPB of the 15 populations ranged from 0.870-0.963, MBF ranged from 0.364-

0.436, and WPV ranged from 69.4-82.8 (Table 5.1).

Seeds from the 15 *F. hallii* populations were collected in late July within one week. Seeds were collected from widely spaced plants representing the whole population. Seeds were stored in paper bags at room temperature for two months and cleaned using a seed blower. Fifty seeds, randomly selected from each population, were weighed and an average seed mass was calculated. This procedure was repeated five times. The weighed seeds were used later for germination. Cleaned and weighed seeds were stored at –18^{o}C until use. The 0.05% benomyl powder was applied to sterilize seeds at the beginning of germination tests.

5.2.2 Seed germination test

Germination tests were conducted in seven growth chambers (Sanyo Versatile Environment Chamber MLR-350H, Sanyo Scientific, USA) with constant temperatures between 5 and 35^{o}C with 5^{o}C increment. Temperatures inside the growth chambers were monitored continuously using dataloggers (21X Campbell Scientific Inc., USA) and recorded at hourly intervals using three temperature probes per chamber. A randomized complete block design with five replicates was used and replicates were put into growth chambers at one-week intervals. Designated temperatures were randomly allocated to each growth chamber. For each replicate, a unit of 50 seeds was imbibed on top of two layers of filter paper (Whatman No.1) in 9 cm plastic Petri dishes. Seventy-five Petri dishes (15 populations x 5 replicates) were randomized within each chamber in darkness. Seeds were sprayed with 0.05% benomyl solution whenever there was a sign of microorganism contamination during germination tests. Seeds with coleoptiles greater than 3 mm were considered germinated. Seeds were counted and removed daily under room temperature, and germination tests were conducted for 40 days. Non-germinated seeds were then tested for viability by finger pressing. Viable seeds were firm when pressed with finger tips. The number of viable and dead seeds was recorded. Results showed that un-germinated seeds were mostly dead. Final germination percentage was calculated based on total seeds incubated and seed viability, which was obtained on 250 seeds stained with 0.1% Tetrazolium Chloride (TZ) solution.

5.2.3 Data analysis

Germination rate for sub-population g ($GR_{(g)}$) was calculated with the reciprocal of germination time ($1/t_g$). Base temperature (T_b) and thermal time for 50% germination ($\theta_{T(50)}$) were estimated

using extrapolation (graphical) methods (Garcia-Huidobro et al., 1982; Qiu et al., 2006). Temperature was treated as the dependent variable and plotted against $GR_{(g)}$. The intercept of the linear regression line was T_b and the linear relationship between temperature T and $GR_{(g)}$ varies among sub-populations as indicated by the slope of the regression line, which equals the thermal time ($\theta_{T(g)}$).

$$T = T_b + \theta_{(g)} GR_{(g)} = T_b + \theta_{(g)} \frac{1}{t_{(g)}}$$

The rate of seed germination was calculated using Germination Rate Index (GRI):

$$GRI = (G_{tot}/p)*(\sum g_i/t_i)$$

where G_{tot} = the total number of germinated seeds at the end of the germination test, p = the total number of seeds, and g_i = the number of seeds germinated between time t_{i-1} and t_i (h) (Steinmaus et al., 2000).

One-way ANOVA was used for seed mass as affected by seed origin and a two-way ANOVA was conducted for germination percentage and GRI as affected by seed origin and germination temperature (SAS/STAT Software V.9.1 for Windows, 2002). Percentage data were arcsine square root transformed before analysis. Data were transformed back to percentage for presentation. Correlations between seed germination characteristics (seed mass, final germination percentage, GRI, T_b and thermal time for 50% germination $\theta_{T(50)}$), and genetic variation (PPB, MBF, and WPV), fragmentation measures (population size and GDN), geographic variables (latitude and longitude), and climatic variables (MAT and MAP) were explored using linear regressions (SAS/STAT Software V.9.1 for Windows, 2002). Population size was log- transformed and mean percent germination was arcsine square-root transformed for regression analysis.

5.3 Results

5.3.1 Differences in germination characteristics among seed collections in response to temperature

Final germination percentages for most populations were high over a wide range of temperatures from 5°C to 20°C and decreased sharply at 25 and 30°C (Fig. 5.2). There was no significant interaction between population and temperature for germination percentage. Seeds collected from Denzil which is located at the western part of the Moist Mixed Grassland Ecoregion had the highest germination percentage ($\overline{X}$ = 99.8%), and those from Macrorie which is located at the Mixed Grassland Ecoregion had the lowest germination percentage ($\overline{X}$ = 72.7%) over the tested temperatures at 5-25°C. Overall, seeds from the Boreal Transition Ecoregion had the highest germination percentage ($\overline{X}$ = 86.5%) and those from the Mixed Grassland Ecoregion had the lowest germination percentage ($\overline{X}$ = 67.2%).

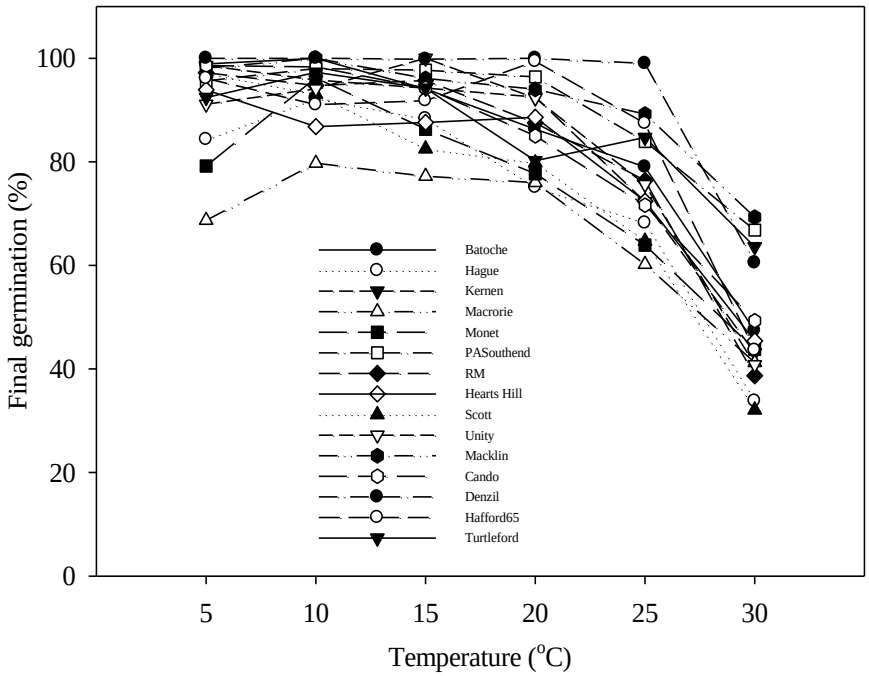


Fig. 5.2 Germination percentage of 15 *F. hallii* populations in six different temperature regimes from 5-30°C.

Germination rate measured as GRI was significantly affected by the origin of the seeds and the germination temperature. The interaction between site and temperature was significant

(P <.0001). GRI increased with increasing temperature from 5 to 20°C and then decreased when the temperature was above 25°C (Fig. 5.3). Seeds from the Mid-Boreal Upland Ecoregion had the highest GRI. Seeds from the Boreal Transition and Aspen Parkland Ecoregions had higher GRI than those from the Moist Mixed Grassland and Mixed Grassland Ecoregions.

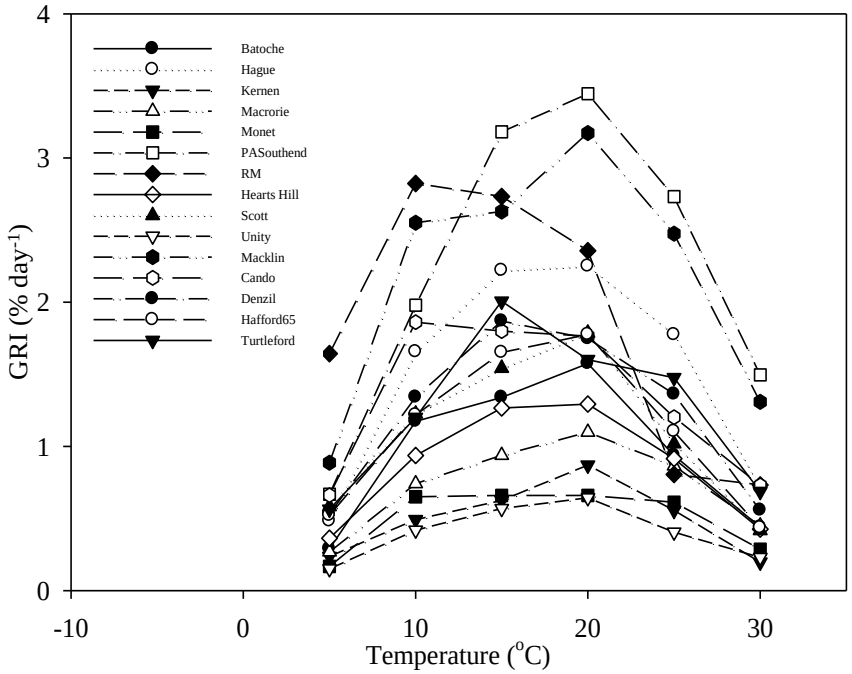


Fig. 5.3 Germination rate index (GRI) of 15 *F. hallii* populations in six different temperature regimes from 5-30°C.

The average dry weight per seed was significantly different among the 15 populations, ranging from 0.8 to 1.4 mg (Table 5.2). Seeds from Macrorie located in Mixed Grassland Ecoregion were the lightest and those from RM located in Mid-Boreal Upland Ecoregion were the heaviest. The lowest temperature for germination to start was close to the freezing point in most populations. Seeds from Riding Mountain, the most eastern population, had a subzero T_b at -0.3°C and those from Hafford had the highest T_b at 1.9°C (Table 5.2). The amount of thermal time required to reach 50% seed germination ($\theta_{T(50)}$) also varied among populations, ranging from 190 (Denzil) to 295 (Macrorie) °C days. Populations with a lower T_b had a higher $\theta_{T(50)}$, indicating a trade-off between T_b and ($\theta_{T(50)}$) to ensure successful germination at their local environments. Seeds from the Mixed Grassland Ecoregion had a higher $\theta_{T(50)}$ but a lower T_b.

Table 5.2 Seed mass (mean ± SD), base temperature (T_b) and thermal time requirement for germination of the 50% subpopulation($\theta_{T(50)}$) of 15 populations of *F. hallii* with 95% confidence intervals.

		T_b (°C)		$\theta\tau_{(50)}$ (°C d)	
Population (label)	Seed mass (mg seed^{-1})	T_b	Confidence intervals	$\theta\tau_{(50)}$	Confidence intervals
Batoche (Ba)	1.29 ± 0.18	1.0	0.47 ~ 1.47	218	211.6 ~ 223.6
Hague (Hag)	1.18 ± 0.20	1.6	0.43 ~ 2.77	215	208.2 ~ 222.7
Kernen (Ker)	0.85 ± 0.06	1.0	-0.15 ~ 2.11	241	233.4 ~ 249.2
Macrorie (Mac)	0.80 ± 0.16	0.4	-1.98 ~ 2.81	295	282.3 ~ 313.7
Monet (Mon)	0.78 ± 0.22	0.8	-1.23 ~ 2.77	278	267.5 ~ 290.1
PASouthend (PAS)	1.29 ± 0.16	1.2	0.31 ~ 2.10	200	194.7 ~ 205.3
Riding Mountains (RM)	1.42 ± 0.14	-0.3	-0.68 ~ 0.14	239	233.1 ~ 245.1
Hearts Hill (S31)	0.92 ± 0.10	1.6	1.08 ~ 2.19	193	187.9 ~ 197.6
Scott (S47)	1.05 ± 0.14	1.0	0.37 ~ 1.69	262	252.3 ~ 272.9
Unity (S52)	0.80 ± 0.08	1.0	-0.51 ~ 2.50	245	237.1 ~ 253.6
Macklin (S54)	1.17 ± 0.12	1.2	0.23 ~ 2.19	205	200.4 ~ 210.9
Cando (S57)	0.84 ± 0.16	0.3	-1.29 ~ 1.97	260	252.6 ~ 268.5
Denzil (S59)	1.06 ± 0.20	1.7	1.19 ~ 2.29	190	184.9 ~ 194.3
Hafford (S65)	1.20 ± 0.16	1.9	0.84 ~ 2.93	209	203.0 ~ 215.8
Turtleford (S77)	1.24 ± 0.24	1.5	0.51 ~ 2.41	210	204.1 ~ 215.5

Population: Monet = Monet PFRA community pasture; PASouthend = Prince Albert National Park Southend meadows; Hearts Hill = Hearts Hill PFRA community pasture.

There was a significant correlation between GRI and seed mass (Table 5.3). At 5 and 10°C, the temperatures with high germination percentage, 40-50% of the variance in GRI can be explained by seed mass. The correction between seed mass and the amount of thermal time required to reach 50% seed germination ($\theta_{T(50)}$) was negative, indicating less thermal time requirements for populations with heavier seeds.

5.3.2 Geographical variation in seed characteristics and germination attributes among populations

Seed mass was positively correlated with longitude but its correlation with latitude was not significant (Table 5.3), indicating eastern seeds were heavier. It was negatively correlated with mean annual temperature and positively correlated with mean annual precipitation, indicating that as site temperature decreases and precipitation increases, heavier seeds were produced. Final germination percentage at 5°C was not correlated with seed mass, but was negatively correlated with mean annual temperature and positively correlated with annual mean

precipitation. Final germination percentage at 10°C was not correlated with any variable tested (data not shown). GRI at 5 and 10°C were positively correlated with annual mean precipitation and longitude, but their correlations with latitude and annual mean temperature were not significant. Seed mass and germination characteristics were not significantly associated with any of the three genetic parameters (PPB, MBF, and MPV; data not shown), nor with population size or geographic distance to the nearest neighbour (data not shown).

Base temperature was positively correlated with latitude and negatively correlated with longitude, but its correlation with mean annual temperature and precipitation was not significant. The thermal time requirement to reach 50% germination $\theta_{T(50)}$ was negatively correlated with latitude and positively correlated with mean annual temperature; it was negatively correlated with mean annual precipitation but not correlated with longitude.

Table 5.3 Correlation coefficients between seed attributes such as seed mass, germination percentage (G %) at 5°C, germination rate index (GRI) at 5 and 10°C, base temperature (T_b), and thermal time requirement for germination of the 50% subpopulation($\theta_{T(50)}$) and spatial and demographic variables among 15 populations of *F. hallii*.

Spatial and demographic variables	Seed mass (mg)	G % at 5°C	*GRI* at 5°C	*GRI* at10°C	T_b (°C)	$\theta\tau_{(50)}$ (°C d)
Seed mass		ns	0.67*	0.70*	ns	-0.62*
Latitude	ns	ns	ns	ns	0.61*	-0.54*
Longitude	0.51*	ns	0.61*	ns	-0.62*	ns
MAT	-0.79***	-0.58*	ns	ns	ns	0.51*
MAP	0.90***	0.54*	0.81**	0.76*	ns	-0.53*

*, **, and ***, significant at P = 0.05, 0.01, and 0.001, respectively.
MAT = mean annual temperature; MAP = mean annual precipitation.

5.4 Discussion

Festuca hallii seeds germinated over a wide range of temperatures, therefore, temperature does not appear to be a major limitation for germination of this species, suggesting that it would be unlikely for climate warming to affect seed germination in wild populations of this species. The wide temperature range for high germination is consistent with the findings of Romo et al. (1991). Seeds from Denzil, located in Moist Mixed Grassland Ecoregion, showed the highest final germination percentage under high temperatures (20-25°C) among all collections. The regeneration success of this collection may be enhanced under climate change if moisture is not

limiting. Seeds from Macrorie located in the Mixed Grassland Ecoregion have the lowest germination at high temperature. The regeneration success of this collection may not be favored under climate change.

The low base temperatures (< 2^{o}C) indicates that *F. hallii*, as a cool season grass, can accumulate thermal time and germinate in early spring, enhancing the competitiveness of seedlings in the grasslands. The average monthly temperature is around 3 and 10^{o}C in April and May, respectively, over the western prairie (Marshall et al., 1999). Therefore, seeds start to accumulate thermal time in April and germinate in May when the temperature is favorable for rapid growth. With a 3-5°C increase in temperature predicted by the end of the century (Thomas et al., 2004), germination time may be as early as April at these locations.

We found that temperature and precipitation are two climatic variables closely involved in seed mass. Maternal environmental conditions such as soil moisture content and temperature have been found to affect seed size/seed mass in many plant species (Ouborg and Van Treuren, 1995; Murray et al., 2004; Luzuriaga et al., 2006; Boyd et al., 2007). In tropical areas, such as Australia, large seed mass is required in area with high annual mean temperature for larger seedlings, due to increased metabolic requirement at high temperatures (Lord et al., 1997; Murray et al., 2004). In temperate areas, such as western Canada, where the climate is cold, larger seeds were produced in areas with low annual mean temperature, allowing those individuals to germinate appropriately for their climate. The relatively dry climate in our area makes more precipitation the favorable factor for larger seed mass.

Seed mass as a component of seed quality affects germination rate (Ouborg and Van Treuren, 1995; Sugiyama, 2003; Wang et al., 2004; Boyd et al., 2007). Among the 15 seed collections of *F. hallii*, those with heavier seeds germinated faster and required less thermal time to germinate. Wang et al. (2004) found a positive relationship between seed size and germination in winterfat (*Eurotia lanata* (Pursh) Moq.) seeds due to lower T_b in large seeds than that in small ones, which also allows large seeds to accumulate more thermal time units than small seeds. Seed mass did not significantly affect final germination percentage in *F. hallii*. Similar results were reported in *Sinapis arvensis* L. (Luzuriaga et al., 2006), while a positive correlation between seed mass and germination percentage was found in *Arabidopsis thaliana* (L.) Heynh. (Boyd et al., 2007).

We found a longitudinal pattern in seed germination that correlated with precipitation. In general, germination was faster in our eastern populations that were in areas of higher precipitation. Rapid germination was the result of eastern populations having larger seed mass with lower T_b and/or shorter thermal time requirements, emerging faster under similar spring temperatures as our western sites. Therefore, we speculate that seed germination of *F. hallii* has a strong adaptation along the west-east precipitation gradient. Relative to precipitation, temperature had less influence on *F. hallii* seed germination. The effect of MAT was only reflected in the ability of seeds to germinate at 5^{o}C but not at 10^{o}C. Schmuths et al. (2006) studied 73 accessions of *A. thaliana* and found a correlation between percentage germination and temperature of origin at 10^{o}C germination temperature but not at higher germination temperatures. Boyd et al. (2007) found that for *A. thaliana*, germination percentage was higher for seeds from lower latitude in the spring environment but was higher for seeds from higher latitudes in the fall environment.

Thermal time for 50% germination was positively correlated with MAT in *F. hallii*, suggesting that seeds of populations at sites with lower temperatures require less $\theta_{T(50)}$ than those with higher temperatures, a possible adaptation to low temperature environment. This adaptation strategy is further supported by the positive correlation between latitude and T_b and the negative correlation between latitude and $\theta_{T(50)}$. Northern populations with lower temperature produce seeds with higher T_b, which has adaptive value for survival. This prevents seeds in cold habitats from germinating too early in the season to ensure seedling survival. On the other hand, seeds from those regions had lower thermal time requirements, thus germinate faster.

The geographical variation in seed characteristics and germination attributes was reflected in Ecoregions where seeds were collected. Seeds from the Boreal Transition and Aspen Parkland Ecoregions were heavier and had higher germination percentage and germination rate than those from the Mixed Grassland Ecoregion. Although ecological conditions in each of these ecoregions, such as soil conditions, landscape patterns, and associated plant species, etc., may be involved in these spatial patterns, the five Ecoregions from where seeds were collected are largely determined by climate (Ecological Stratification Working Group, 1996). The present study has revealed the relative importance of climatic features including temperature and precipitation for the spatial patterns of seed mass and seed germination thresholds along geographical gradients across Canadian prairie.

Correlations among climate variables and seed attributes reflect strong local adaptation at the reproductive stage for *F. hallii*. Both precipitation and temperature related to evaporation can affect soil moisture, which is generally recognized as a critical factor governing the vegetation distribution in temperate prairies (Hogg, 1994; Hogg and Schwarz, 1997). *F. hallii* is generally limited to habitats with cool and moist conditions (Coupland and Brayshaw, 1953; Baines, 1973; Hill et al., 2000). Thus, it is not surprising that climate was the major factor influencing the distribution of *F. hallii* populations. The larger seed mass and better seed germination with cool and moist conditions as detected in this study implies the importance of seed germination on the geographical distribution of *F. hallii* populations. The current distribution and adaptation of *F. hallii* in the Canadian prairie may be traced back to the last Glaciation. Genetic variability of several species in North America has been related to Pleistocene glaciation and post-glacial colonization (Schmidtling and Hipkins, 1998; Beck et al., 2008). Our results suggest that future climate with increasing temperature and drought may result in shifting of this species further north and east where the conditions are more suitable for the germination.

Genetic variation or fragmentation was not correlated with seed characteristics or germination responses. Genetic variation and population size were found to affect fitness traits of rare, endangered species and species suffering from fragmentation and habitat deterioration (Menges, 1991; Soons and Heil, 2002; Vergeer et al., 2003). However, cases have also been reported in which population size did not affect seed germination (Ouborg and Van Treuren, 1995; Lammi et al., 1999; Morgan, 1999; Eisto et al., 2000; Hooftman et al., 2003; Krauss et al., 2007). Several reasons are relevant to the lack of correlation. First, the genetic variation was high within populations and did not differentiate much among populations. Secondly, the detected AFLP variation was presumably selectively neutral and may not be directly linked to the genes controlling seed germination. High AFLP variation does not necessarily mean high germinability. Thirdly, the population sizes assayed were relatively large (> 400 individuals) with a nearby neighbor (< 6.5km away), so fragmentation may not have had detrimental effects on seed germinability. On the other hand, the lack of correlation between population size and germinability also suggests the species abundance has not been affected by sexual regeneration in historical climate conditions and disturbance regimes by grazing and fire.

In conclusion, germination thresholds of *F. hallii* are affected by seed locations differing in environmental conditions. Moisture in the sites from where seeds are collected is one of the

most important factors affecting seed quality and germination. Under climate change scenarios, many ecotypes may become more restricted to or shift towards moister areas, which are predicted to shift northwards. The drier future climate as predicted for the Canadian prairie may reduce regeneration success of this species, particularly after disturbances which rely on seeds for regeneration. Restoration efforts may fail in the south and west, and protection will be most successful in the north and east. If moisture change scenarios are drier than predicted, then this species could suffer across its range.

6. PATTERNS OF AMPLIFIED RESTRICTION FRAGMENT POLYMORPHISM IN DIFFERENTIAL GERMINATION OF *FESTUCA HALLII* SEEDS

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 this differential seed germination response remains poorly understood. Amplified restriction fragment polymorphism (AFLP) technique was applied to characterize the genetic variation of differentially germinated seeds collected from three *Fastuca hallii* populations in the Canadian prairie. Three groups of seeds (early, intermediate, and late germinators) were identified from each population based on germination test under 10, 15 and 20^{o}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 differential 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 were present among seeds of the three populations, 0.3% among seeds grouped for germination timing, 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. Early and intermediate germinators shared similar genetic backgrounds and were genetically differentiated from late germinators. These results indicate seed genotypes respond slightly differently to environmental variation, resulting in significant but weak genetic differentiation in differential germination of *F. hallii* seeds. Implications for plant establishment and fescue restoration are discussed.

6.1 Introduction

Seeds within a population germinate over a period of time even under optimal germination conditions without dormancy. Early germinators in a population may suppress late ones due to the advantage in competition for light, water, and nutrients. In contrast, late germinators may

stay longer in the soil to persist the population (Cabin et al., 1997; Marshall et al., 2000; Mandak et al., 2006). Thus, such an adaptive germination is critical to the lifetime performance of the resulting plant (Donohue, 2002; Donohue et al., 2005a, b; Mandak et al., 2006). Differential seed germination can also affect 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 differential 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 (Cabin et al., 1998, Squire et al., 1997; Marshall et al., 2000). However, the extent to which germination timing is genetically or environmentally controlled remains poorly understood (Donohue, 2005). For example, Squire et al. (1997) showed that early and late germinators 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 favored particular genotypes and significant genetic differentiation existed between early and late-germinating seeds (Cabin et al., 1997; Mandak et al., 2006). These studies, however, focused on differential germination in a single seed population of either annual or short-lived perennial plants, not long-lived perennials. Environmental conditions may impose more influence on genotypes for seed germination in long-lived perennials, especially in natural populations.

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, 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 (Romo et al., 1991; Johnston and MacDonald, 1967). 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 differential 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 differentially germinated seeds collected from three *F. hallii* populations in the Canadian prairie using amplified restriction fragment polymorphism (AFLP) technique. The AFLP technique (Vos et al., 1995) is a robust, highly effective molecular tool and 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 germinator groups of seeds (early, intermediate, and late germinators) 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* differentially affected by germination temperature? Does the same germinator group of seeds display genetic difference under variable germination temperatures? Do different germinator groups 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?

6.2 Materials and methods

6.2.1 Seed collection, seed germination, and sub-population allocation

Three *F. hallii* populations were selected for this study: Prince Albert National Park (53^{o}35'16"N, 106^{o}02'26"W, located in Boreal Transition Ecoregion), Turtleford (53^{o}27'38"N, 109^{o}03'12"W, in Boreal Transition Ecoregion) in Saskatchewan, and Riding Mountain National Park (50^{o}48'35"N, 100^{o}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 in late July within one week, as described in Chapter 3. Collected seeds were kept in paper bags under room temperature for two months, then cleaned using a seed blower, and stored at –18^{o}C until use. The 0.05% benomyl powder was applied to sterilize seeds at the beginning of germination test.

Seed germination was investigated under 10, 15, and 20^{o}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 one-week interval. 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 PCR plates. 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 with proper labels for population, temperature, replicate, and testing time. 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 embryo. The number of viable, dead and empty seeds were recorded. Results showed that un-germinated 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 was not significantly different among replicates, the transplanted seedlings were pooled among replicates and ranked by the days to germination. The first 0-10% of the seeds germinated were classified as early germinators, the 45-55% of the seeds germinated as intermediate germinators, and the last 90-100% as late germinators (Fig. 6.1).

6.2.2 AFLP analysis

Twenty resulting seedlings were randomly selected from each germinator group and a total of 540 seedling samples were obtained from 27 groups representing germinator group, temperature regime, and seed sources. Young leaf tissue of selected seedlings was individually harvested after 45 days in the greenhouse from transplanting, freeze-dried and stored at –80^{o}C until use. Genomic DNA was extracted from the leaf samples using the DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's instruction. Extracted DNA was quantified by fluorometry using Hoechst 33258 stain (Sigma Chemical Co., St. Louis, MO, USA), and diluted to 25 ng μL^{-1} for AFLP analysis.

The AFLP™ Analysis System 1 (Life Technologies, Burlington, ON, Canada) was applied following the protocol described by Vos et al. (1995) with a modification of using γ^{33}P to label *Eco*RI 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 *Eco*RI: *Mse*I 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.

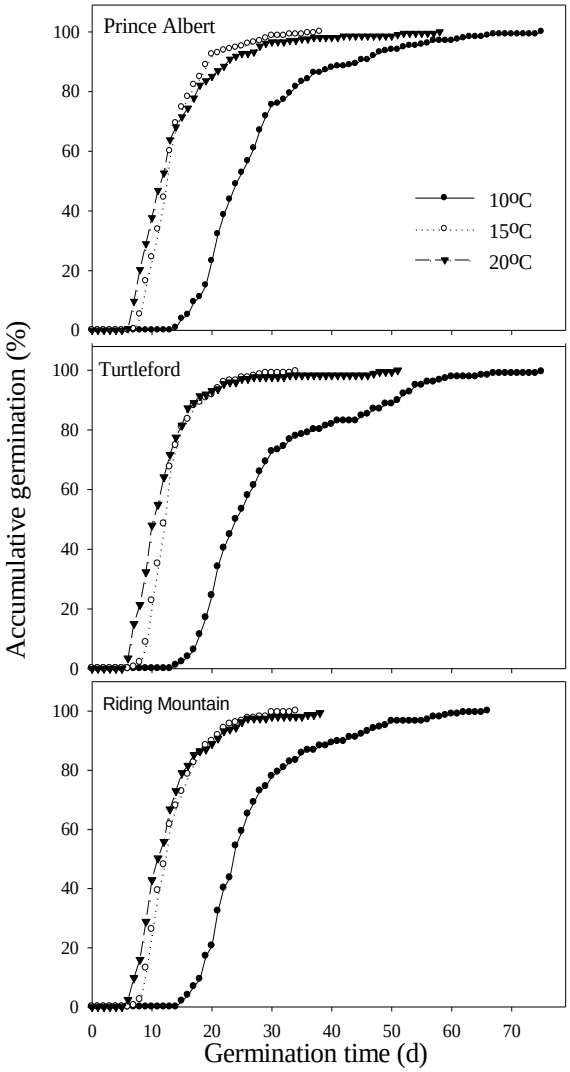


Fig. 6.1 The time-course of seed germination at 10, 15, and 20°C for three *F. hallii* populations. Germination percentages based on the total seeds incubated minus dead and empty seeds were used to identify germinator groups. The first 0-10% of the seeds germinated were classified as early germinators, the 45-55% of the seeds germinated as intermediate germinators, and the last 90-100% as late germinators.

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 pairs were output for data analysis.

6.2.3 Data analysis

A three-way ANOVA was conduced for the days to germinate (in log scale) with respect to population, germinator group and temperature. Since population displayed significant interactions with other two factors, a two-way ANOVA was then conducted for germinator group and temperature. Significance of group means was assessed with *t*-test.

The presence/absence data from the AFLP samples were analyzed 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 population, germinator group and temperature. To identify the chromosome regions or segments associated with differential germination, a χ^2-test was performed following Fu et al. (2005) to determine the significance of the AFLP differences at each AFLP band between early and late germinators. 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 population, germinator group and temperature group. This analysis not only allows partitioning of the total AFLP variation into within- and among- group components, 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 10098 random permutations. Three models of genetic structuring for differentially germinated seeds were assessed: source population, germinator group, and temperature group.

A group specific AMOVA analysis was also performed to assess the differences in AFLP variation among 27 groups representing source population, germinator group and temperature regime. The resulting group-distance matrix was analyzed 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.

6.3 Results

The interactions between germination timing and temperatures were significant for the days to germinate (Table 6.1). Less differences in germination time between early and intermediate germinators were found than those between intermediate and late germinators. Much more days to germinate at 10°C were observed than those at 15 and 20°C. No significant difference in the days to germinate was found between 15 and 20°C, except for those of late germinators from Riding Mountain and Turtleford populations.

Table 6.1 Days to germinate with respect to germinator group 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 germinator			Intermediate germinator			Late germinator		
	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

Three germinator groups in each population displayed variation in mean band frequency under three temperature regimes (Fig. 6.2). Germinating seeds in all three populations had higher estimates of mean band frequency at 20°C than at 10°C. Early germinators had consistently lower estimates of mean band frequency than late germinators at 10°C but had consistently higher estimates of mean band frequency than late germinators at 15°C. At 20°C, the estimate of mean band frequency was higher in early germinators than the others from the Prince Albert population, but it was lower than the others from the Riding Mountain and Turtleford populations. None of 188 polymorphic AFLP bands displayed a significant AFLP difference between early and late germinators under any temperature in any population assayed, after Bonferroni correction.

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 6.2). 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.

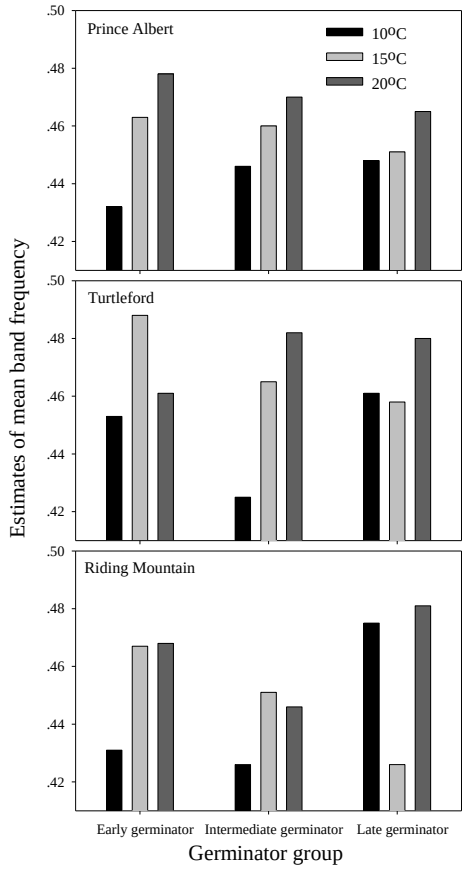


Fig. 6.2 Patterns of estimates in mean AFLP band frequency with respect to germinator group, test temperature and source population.

Assessment of group-wise genetic differences with the proportional AFLP variations showed significant genetic differences among three germinator groups (Table 6.3). The largest difference was present between intermediate and late germinators and the smallest between early and intermediate germinators. For specific populations and germination temperatures, the AFLP differences among germinator groups could be up to 3.7%. The differences between early and late germinators were found in all populations and temperatures, and those between early and intermediate germinators were found only in the Riding Mountain population under the germination temperature of 20^{o}C.

Table 6.2 Analysis of molecular variance (AMOVA) within and among groups of germinating seeds of the three *F. hallii* populations under variable test temperature. Three models of genetic structuring were assessed with respect to source population, germinator group and test temperature.

Source of variation	df	Variance components	% variation
Overall:			
Among populations	2	1.48	5.72
Among germinator and temperatures within populations	24	0.49	1.88
Within germinator and temperatures	483	23.99	92.40
Group for germination timing:			
Among germinator groups	2	0.07	0.26
Within germinator groups	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

Note: all the variance components were statistically significant at $P < 0.001$ level, as calculated from 10098 random permutations.

Table 6.3 Genetic differentiations between germinator groups of *F. hallii* seeds with respect to source population and test temperature, as measured by the proportion of the total AFLP variation.

Population / temperature	Sub-population pairs		
	Early vs. Intermediate	Early vs. Late	Intermediate vs. 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.

Early germinators from both Prince Albert and Riding Mountain populations did not display significant AFLP differences under variable germination temperatures (Table 6.4).

Intermediate germinators from the Prince Albert population and late germinators 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.

Table 6.4 Genetic differentiations within the same group of *F. hallii* seeds under variable test temperatures with respect to source population and germinator group, as measured by the proportion of the total AFLP variation.

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

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

Assessment of the genetic differentiation among 27 groups of germinating seeds revealed three distinct clusters with each population grouped together (Fig. 6.3). The most genetically distinct cluster consisted of various groups of germinating seeds from the Riding Mountain population. Within each distinct group, early and intermediate germinators tended to be clustered together.

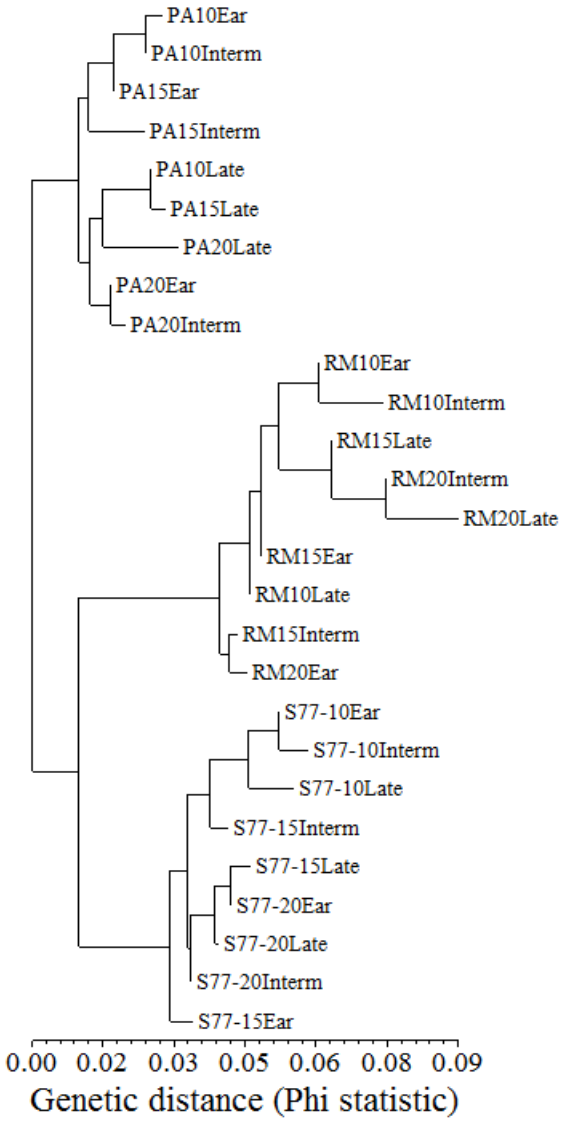


Fig. 6.3 A neighbour-joining tree of the 27 groups of germinating seeds representing source population, test temperature, and germinator group, which was based on pairwise genetic distances (Phi statistics) derived from the analysis of AFLP variation. Each group is labeled with source population (PA=Prince Albert National Park, RM=Riding Mountain National Park, and S77=Turtleford) and test temperature (10=10^{o}C; 15=15^{o}C; 20=20^{o}C) and germinator group (Ear, Interm, and Late for early, intermediate, and late germinators, respectively).

6.4 Discussion

This AFLP analysis of germinating fescue seeds revealed several interesting patterns of genetic variation with respect to differential germination. First, nonrandom patterns of mean band frequency was observed with variable germination temperature. Second, no chromosomal

regions or segments were identified to be significantly associated with differential 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 germinators shared similar genetic backgrounds and were genetically differentiated from late germinators. These findings clearly indicate a weak genetic basis of adaptive seed germination and provide the first molecular evidence for the genetic differentiation in differential germination of *F. hallii* seeds.

Significant changes detected in mean band frequency between early and late germinators under increased temperature indicate that *F. hallii* seed genotypes respond differently to environmental variation during 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). Assessing genetic differentiations among *F. hallii* seeds grouped for germination timing and test temperature further confirms that early germinators are genetically differentiated from late germinators. Thus, the differential germination of *F. hallii* seeds was under weak genetic control.

A weak genetic differentiation of the differential 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 breakage, and germination after dormancy is broken (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, no detection of significant chromosomal regions associated with differential germination may be due to the use of limited sample size (i.e., 20) per germinator group 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 due to test temperature signaled that environmental factors or their interactions with genetic factors may play 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 germinators (see Fig. 6.2).

The results of this study appear to imply that the germinating 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. Variation among individual seeds in hormone sensitivity (Liptay and Davidson, 1971) and enzyme activity such as endo-β-mannanase (Still et al., 1997a. b) 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. 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% un-germinated canola seeds imbibed in water, GA, saline and ABA solutions. These changes may be due to genetic sorting during imbibition. Assessing the patterns of change in mean band frequency (Fig. 6.2) appears to suggest variation existed in genetic sorting of seed genotypes among various germinator groups within a population.

Adaptive seed germination 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 favor early germinators, as these groups of seeds could germinate early in early spring. Different habitats with different environmental conditions could favor different alleles and select different seed genotypes during a certain favorable 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 differential germination revealed in the three populations should provide rough guide to secure 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. halliii* populations were more correlated with mean annual precipitation than mean annual temperature of sites (Chapter 4 and 5). 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 genetic control for differential germination and establishment of seed genotypes in response to spatial and temporal environmental variation. This information should be helpful for explaining how fescue seeds germinate, how fescue plants

adapt to changing environment, and how variable patterns of genetic variation are formed among fescue stands. However, this study provides only a preliminary glance at the genetics of differential seed germination in a native grass. Further research is needed to determine the number, effect, and location of genes controlling germination timing. As differential germination is a complex trait, proper experimental designs with better control of environmental factors are needed (Lynch and Bruce, 1998). With the development of informative molecular markers, such research will offer many insights into the behaviors 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.

7. GENERAL DISCUSSION AND CONCLUSIONS

7.1 General discussion

The comparison of AFLP variation among three tissue types, i.e., reproductive tiller, vegetative tiller and seed (Chapter 3) indicated that these sampling methods yielded significant, but small, AFLP differences for all the six *F. hallii* populations we examined, which suggests that different sampling methods could differ in the effectiveness of capturing genetic variation from natural stands. Small sample differences reflected in AFLP markers also imply that both seed and tiller samples are adequate for genetic diversity research of this species. The tiller samples revealed slightly larger among-population variation than the seed samples. The tiller sampling also appears to be more efficient because we do not have to conduct seed germination.

This study shows *F. hallii* maintains a high level of genetic diversity despite the reduction and fragmentation in its geographic distribution (Chapter 4). The relative high genetic diversity of *F. hallii* might be explained by the life history traits of this species as an outcrossing, wind-pollinated perennial, which are factors associated with high levels of within-population diversity (Larson et al., 2000; Larson et al., 2001a; Nybom and Bartish, 2000; Ferdinandez and Coulman, 2002; Fu et al., 2004a; Fu et al., 2005a, b). Only 6.5% of genetic variation occurs among populations, suggesting weak population differentiation. This weak population differentiation may reflect extensive gene flow through seed and pollen dispersal among populations indicating that habitat fragmentation may not have substantially hindered genetic exchange among fescue remnants (Culley et al., 2007). Comparison of AFLP variation in natural populations and corresponding seed collections of *F. hallii* indicated that seed samples had higher within-population variation and lower among-population variation than tiller samples (Chapter 3), suggesting gene flow is not restricted in recent generations (seedlings from seed collections). However, the pattern of isolation-by-distance was identified and when the geographic distance was beyond 60 km, the genetic similarity declined gradually. Nevertheless, the time since isolation may have been too short relative to the generation time to permit detectable genetic drift and, consequently, low population differentiation.

We found genetic variation and germination rate at low temperatures was significantly correlated with longitude, with western populations having higher genetic variation but lower

germination rate than eastern populations (Chapter 4, 5). This east-west pattern is partially related to moisture, which is one of the causal factors affecting the distribution of *F. hallii* populations. *F. hallii* is generally limited to habitats with cool and moist conditions (Coupland and Brayshaw, 1953; Baines, 1973; Hill et al., 2000). Germination rate was positively correlated with MAP and longitude of sites, both of which were also significantly correlated. This east-west pattern may be also related to Pleistocene glaciation and post-glacial colonization, as many species became restricted or retreated into glacial refugia (Schmidtling and Hipkins, 1998; Elderkin et al., 2007; Beck et al., 2008). As glaciers retreated, areas of suitable habitat to the east with more moisture were colonized by individuals from adjacent western refugia. The post-glacial colonization resulted in reduced genetic diversity in newly-founded populations (Beck et al., 2008).

Temperature, as one of the most important environmental factors, affects seed quality, which in turn affects the ability of seeds to germinate at low temperature (Chapter 5). The influence of seed germination by temperature reflects a possible adaptation to low temperature environment. This adaptation strategy is supported by the positive correlation between latitude and T_b and the negative correlation between latitude and $\theta_{T(50)}$ and positive correlation between MAT and $\theta_{T(50)}$. Northern populations with lower temperature produce seeds with higher T_b, which has adaptive value for survival. This prevents seeds in cold habitats from germinating too early in the season to ensure seedling survival. On the other hand, seeds from those regions had lower thermal time requirements, thus germinate faster.

Germination timing shows genotype-by-environment interaction (Chapter 6). The significant, although small, non-random AFLP differences for early, intermediate and late germinators yielded from different germination temperatures suggest that seed genotypes respond differently to environmental variation during germination process. This genetic sorting under different temperatures significantly affected the genetic structure of plant subpopulations. The affect of genotype-by-environment interaction on germination time further suggests germination timing is an adaptive trait under natural selection (Donohue et al., 2005c). When and where a seed with a particular genotype can germinate depend on the germination conditions.

No significant correlation between fragmentation measures (population size and geographic distance to the nearest neighbor) and genetic variation within *F. hallii* populations was found (Chapter 4). Nor were genetic variation and fragmentation correlated with seed

characteristics or germination responses (Chapter 5). This suggests that habitat fragmentation has not had consequences upon the population genetics and fitness-related life history of *F. hallii*, which is common in plant populations, especially in long-lived perennial plant species (Ouborg and Van Treuren, 1995; Lammi et al., 1999; Morgan, 1999; Young et al., 1999; Eisto et al., 2000; Hooftman et al., 2003; Culley et al., 2007; Honnay et al., 2006, 2007; Geert et al., 2007). The lack of correlation between population size and genetic variation and seed germinability also suggests the species abundance has not been affected by genetic variation and sexual regeneration in historical climate conditions and disturbance regimes.

We examined only the effect of germination temperature on genetic variation of seeds germinated at different times within a population (Chapter 6), which suggests genetic sorting under different temperatures (Cabin et al., 1997; Mandak et al., 2006). We conducted seed germination only using seeds collected from the natural populations and found that environmental attributes, such as moisture, has shown significant effect on seed germination and genetic structure of plant population (Chapter 4, 5). Maternal environmental effects on seed germination have been also widely detected in many species (Roach and Wulff, 1987; Lacey, 1996; Pendleton and Meyer, 2004; Luzuriaga et al., 2006; Boyd et al., 2007). Further studies are needed to examine if there is difference in genetic constitution of the emerged populations using reciprocal seeding or by manipulating moisture conditions. These future efforts would help to understand the genetic variation among populations. For reciprocal seeding, clones of different ecotypes should be isolated in common gardens for seed production to remove the maternal environmental effect on seed germination.

We analyzed only AFLP variation in this tetraploid organism. AFLP markers are scored without reference to dominance (i.e., without distinction between homozygotes and heterozygotes), and co-migrating AFLP bands are not always homologous (Koopman 2005; Mechanda et al. 2004a, b). Research of this type is difficult to do on the basis of AFLP alone and measures of AFLP diversity are difficult to compare among plants. Further studies are needed to examine genetic variation using an additional marker system, such as microsatellite markers (Saha et al., 2004; Luttikhuizen et al., 2007). These future efforts would help to examine the genetic diversity among plants using measures of the allelic richness and observed heterozygosity of individual genotypes.

This study is helpful for formulating effective strategies for conservation and restoration. The finding of the east-west pattern of genetic variation implies that the remnant fescue stands along this gradient should be the target for protection. Moisture is one of the most important factors affecting plant species distribution across Canadian Prairie. Under climate change scenarios, many ecotypes may become more restricted to or shift towards moister areas. Conservation efforts should be concentrated on populations in the Mixed Grassland Ecoregion which is the driest region in the Canadian Prairie. The finding of a significant genetic similarity within 60 km implies that the fescue plants should not be translocated between sites more than 60 km apart. A pollen/seed transfer zone within 60 km from current natural populations could be considered. Because of the high genetic diversity identified in this species, using plant material from the nearby fescue stands should not degrade much the genetic background of the restored stands. However, plants in colder and wetter areas produced heavier seeds. Therefore, seeds from colder areas with better moisture conditions are desirable for restoration. The base temperature of *F. hallii* is lower than 2^{o}C, which allows seeds to utilize the moisture after snow melts in early spring. This supports the suggestion that *F. hallii* should be planted in the spring when temperatures are rising and soil moisture is highest.

7.2 Conclusions

Using AFLP technique, we revealed interesting patterns of AFLP variation and germination responses in the remnant populations of this widespread, native grass species. These variation in genetic diversity and germination were correlated with geographic variation and climatic attributes. These findings are significant for the understanding of the evolution of the remnant populations and for the development of the effective strategies for conserving fragmented plant populations. Major conclusions from this research are summarized below:

1. Different sampling methods could differ in the effectiveness of capturing genetic variation from natural stands. The tiller samples revealed slightly larger among-population variation than the seed samples, and captured significant associations of AFLP variation with population geographic distances.
2. *F. hallii* has high genetic diversity and weak genetic differentiation. The AFLP variation displayed a longitudinal decline and was significantly associated with environmental attributes related to moisture, indicating local adaptation. However, the AFLP variation

was not significantly associated with the estimated population size and geographic distance to the nearest neighbor, suggesting that fragmentation has not generated considerable genetic impact on the fescue populations.

3. Population-wise variation in temperature and precipitation affects seed mass, an important attribute determining seed germinability. Sites with greater precipitation and lower temperature had heavier seeds, which usually had lower thermal time requirements for germination and faster germination. The warmer and drier future climate as predicted for the Canadian Prairie may reduce regeneration success of this species, particularly after disturbances followed by sexual regeneration from seeds.
4. Marked differences in estimates of mean band frequency were observed for various groups of germinating seeds under different test temperatures. Comparisons of AFLP variation among 27 groups of seeds representing population, germination timing and test temperature indicates seed genotypes respond slightly differently to environmental variation, early and intermediate germinators shared similar genetic backgrounds and were genetically differentiated from late germinators. These results indicate weak genetic control of the germination timing and provide molecular evidence for the genetic differentiation in differential germination of *F. hallii* seeds.

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