Analysis of the genetic diversity within and between populations of Festuca campestris in the Helena and Lewis and Clark National Forests

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Analysis of the genetic diversity within and between populations of *Festuca campestris* in the Helena and Lewis and Clark National Forests

David Joseph Roach

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Abstract

Rough Fescue is a grass native to the prairies of the American west and southwestern Canada. It is one of the primary plains grasses and has seen a dramatic drop in habitat availability due to encroachment by humans and overgrazing by cattle. The United States Forest Service has begun a reseeding program in areas where it is in danger of being extirpated. The first step of the project was to gather seeds from populations of rough fescue in the Lewis and Clark and Helena National Forests. Although it is an important prairie grass and is the object of a government reseeding project, little is known about the genetics of the populations whose seeds were being collected. In this study, ten samples were taken from ten different sites in the national forests and were analyzed for amounts of genetic diversity within and between the populations. Using the inter-simple sequence repeat (ISSR) technique and gel electrophoresis, eleven different alleles under three primer sets were identified. These were used to analyze the amount of genetic diversity contained within the national forests as a whole as well as to identify patterns of diversity with respect to region and elevation. This information helped to give a guideline for future seed collections in order to ensure that the genetic diversity of the rough fescue in the national forests be maintained in the reseeding zones.

Key words: Rough fescue, genetic diversity, ISSR, PCR amplification, gel electrophoresis, Helena National Forest, Lewis and Clark National Forest.
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Introduction

Rough Fescue (*Festuca campestris* Rydb.) is a native prairie bunchgrass that grows throughout the northwestern United States and southwestern Canada (Lamb, 2008). It is a tetraploid perennial monocot that has been continually threatened due to agricultural expansion over the last 100 years, resulting in isolated patches surrounded by agricultural fields or on mountaintops throughout its range (Qui et al., 2007). Currently, the United States Forest Service is doing a reseeding program to revegetate mid and high elevation areas that have been disturbed and suffer sustained erosion due to mining, road construction, and road rehabilitation. One of the goals of the reseeding program is to establish new populations of rough fescue in these disturbed habitats. I investigated the genetic structure and relationships within and between different isolated populations of *Festuca campestris* in the Helena and Lewis and Clark National Forests where the seeds for the reseeding program were collected.

I chose to use Inter-Simple Sequence Repeat (ISSR) genetic markers as the primers used in Polymerase Chain Reaction (PCR) amplification for the population genetic analysis of the rough fescue populations. ISSR markers are short microsatellites, typically between 10 and 20 base pairs, that consist of a series of either di or tri-nucleotide repeats with a two base repeat that serves as a primer anchor at one end. This anchor prevents slippage of the primers on the DNA and provides a more accurate and consistent amplification. Each primer serves as both the forward and the reverse primer in the PCR. The primers are non-random and their complementary DNA sequences are normally found throughout the genome, which results in multiple sites of amplification.
Due to the small size of the microsatellite loci and the high level of polymorphism in both
the microsatellites themselves and the intervening DNA sequences, this technique is an
efficient way to assess genetic diversity within one species (Wolfe and Liston, 1998;
Reddy et al., 2002).

The use of ISSR genetic markers has been shown to be an effective method for
obtaining phylogenetic relationships and assessing amounts of genetic diversity in a wide
variety of plant species. Culley and Wolfe (2001) used ISSR microsatellites in Viola
pubescens in order to assess the amount of genetic diversity contained within and
between the individual populations that were sampled as well as the total diversity in all
populations. In addition, they used the genetic distances to establish phylogenetic
relationships between the different populations. The ISSR technique showed that a large
amount of diversity existed within the species and allowed the development of a
phylogenetic tree that accurately depicted relationships between populations when
compared to other molecular techniques.

Genetic relationships between species within the same genus have also been
studied using ISSR markers. Ajibade et al. (2000) established the relationships between
different species within the genus Vigna. This genus contains several different plant
species that have agricultural importance throughout tropical areas, most notably the
cowpea of Africa. They were able to create a phylogenetic tree using ISSR markers that
coincided closely to previous studies done on Vigna, which helps to show the accuracy of
the method.
Joshi et al. (1999) did a study of the genus *Oryza* that elucidated both inter and intra-species phylogenetic relationships using ISSR markers. They found a high degree of diversity in the genus as a whole and in each of the different species and were able to create a UPGMA dendrogram that gave phylogenetic relationships with a high degree of certainty, in some cases up to 100%. This study assessed the diversity that exists between cultivated rice and 22 different wild varieties. The goal of the study was to identify species that are better able to tolerate different stresses than cultivated rice, in order to broaden the gene pool of cultivated rice through hybridization and ensure its continued productivity and fitness.

These studies show the usefulness of the ISSR genetic marker technique in performing population genetics analyses. In addition to being used for the development of dendrograms and assessing amounts of genetic diversity, it can be used for a wide variety of other applications, from finding modes of reproduction (Camacho and Liston, 2001, Wolfe et al., 1998) to elucidating the origin of agricultural crops (Joshi et al., 1999). The ISSR method has been shown to provide consistent and accurate results when used in a variety of different plant species, which is why I chose to use it to look at the genetic structure and relationships of the *F. campestris* populations in the Helena and Lewis and Clark National Forests.

Genetic diversity is important to a species' long term persistence for several reasons: it increases their ability to survive competition between species, it helps populations recover after ecological disturbance, it can increase the productivity of the species, and it can increase the ability of a species to compensate for changes in the
amount or availability of nutrients in the soil (Herrmann et al., 2007; Hughes et al., 2008). Because genetic diversity is important in situations of population recovery, I assessed the amount of diversity contained within the *F. campestris* populations from which the seeds were collected. I hypothesized that there will be diversity both within and between the different populations sampled.

Though *Festuca campestris* is an important native species in the western prairie grasslands (Sheley et al., 2006) little is known about the genetic diversity contained within the species. Through my research project, I tried to fill this gap in scientific knowledge. The main goal of this study was to identify any populations of *F. campestris* within the Helena and Lewis and Clark National Forests that contained unique alleles that should be maintained through the seed collection program. Another goal was to elucidate any allelic patterns that might exist between populations with respect to elevation and region, in order to determine whether these factors should be used as a guideline for future collections to ensure that these patterns are maintained. Determining whether patterns of variation exist among populations, regions, and elevations will help preserve the amount of diversity within the entire *F. campestris* populations in the Lewis and Clark and Helena National Forests, and thereby help ensure the survival of the newly establish populations in this region.
Materials and Methods

Collection

One hundred different samples of *F. campestris* plant tissue were collected at 10 different sites throughout the Helena National Forest and the Lewis and Clark National Forest between August 5th and August 17th, 2008 (Table 1). There were 9 samples collected from the Helena National forest and 1 from the Lewis and Clark National Forest. *Festuca campestris* has a red section of stem that grows beneath the soil just before the roots start. Only the stem and leaf tissue that had this red stem-base attached was collected in order to ensure that no other plant species got mixed with *F. campestris* samples. The samples were then placed in plastic bags containing silica gel and allowed to dry. At each site the elevation, longitude and latitude were recorded. Collection sites were chosen by both region and elevation to ensure samples were taken at both high (above 6,000 ft.) and low elevations (below 6,000 ft.) and across a large regional area, namely on either side of the Continental Divide. This was done to look for any allelic patterns that might follow region and elevation.

Table 1: Collection sites and elevations

<table>
<thead>
<tr>
<th>Site Number</th>
<th>Site Name</th>
<th>Elevation</th>
<th>East/West of C. Divide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MacDonald Pass</td>
<td>6,341 ft.</td>
<td>west</td>
</tr>
<tr>
<td>2</td>
<td>Electric Tower</td>
<td>6,485 ft.</td>
<td>west</td>
</tr>
<tr>
<td>3</td>
<td>Weasel Creek A</td>
<td>6,693 ft.</td>
<td>east</td>
</tr>
<tr>
<td>4</td>
<td>Weasel Creek B</td>
<td>6,158 ft.</td>
<td>east</td>
</tr>
<tr>
<td>5</td>
<td>Granite Butte</td>
<td>7,126 ft.</td>
<td>west</td>
</tr>
<tr>
<td>6</td>
<td>Bald Butte</td>
<td>6,058 ft.</td>
<td>west</td>
</tr>
<tr>
<td>7</td>
<td>Little Blackfoot R.</td>
<td>5,558 ft.</td>
<td>west</td>
</tr>
<tr>
<td>8</td>
<td>Ophir Creek</td>
<td>5,866 ft.</td>
<td>west</td>
</tr>
<tr>
<td>9</td>
<td>Pole Creek</td>
<td>5,713 ft.</td>
<td>east</td>
</tr>
<tr>
<td>10</td>
<td>L &amp; C National Forest</td>
<td>4,868 ft.</td>
<td>east</td>
</tr>
</tbody>
</table>
DNA Extraction

Total genomic DNA was extracted from approximately 25-30 mg of dried leaf material per sample by initially grinding the tissue with the FastDNA® Kit (Q-Biogene, Carlsbad, CA) followed by extraction using a Qiagen DNeasy® kit (Valencia, CA) and accompanying protocol. Extracted DNA was stored in a -20° C freezer. DNA concentration was fluorometrically assayed using a 100 μg/mL solution of calf thymus DNA as a standard. The concentration of the *F. campestris* DNA was recorded and DI water was then added to put the concentration of each DNA sample at 1 μg/ml.

Polymerase Chain Reaction DNA Amplification

The PCR amplification was performed using an Eppendorf® thermocycling machine (Westbury, NY). The three primers used are listed in Table 2.

Table 2: ISSR primers used in PCR amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB 8</td>
<td>5'-GAGAGAGAGAGAGAGGG</td>
</tr>
<tr>
<td>HB 11</td>
<td>5'-GTGTGTGTGTGTGCC</td>
</tr>
<tr>
<td>HB 12</td>
<td>5'-CACCACCACCACGC</td>
</tr>
</tbody>
</table>

These primers and two others were chosen from a list of ISSR primers developed by Ballard in 2001. The three primers listed in Table 2 resulted in consistent amplification within rough fescue. For each PCR amplification, GoTaq® Colorless Reaction Buffer was used which maintains the pH at 8.5 and contains 7.5mM MgCl₂ (Promega Product Information). GoTaq® Colorless Master Mix was used, which contains, 400μM dATP, 400μM dGTP, 400μM dCTP, 400μM dTTP, and 3mM MgCl₂ (Promega Product Information). Each reaction tube was then filled with 0.1 μL of Taq
bacterially derived DNA polymerase, 4 μL of PCR Buffer, 2 μL of the master mix, 0.7 μL of primer, 0.5μL BSA, 8.7 μL of dH2O, and 1 μL of sample DNA to give a total volume of 16μL per reaction tube. In addition, a negative control tube was made that contained all the elements of the master mix but no DNA. The PCR cycling conditions are listed in Table 3.

Table 3: PCR cycling temperatures and times

<table>
<thead>
<tr>
<th>Step Number</th>
<th>Cycling Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94° C for 1 min</td>
</tr>
<tr>
<td>2</td>
<td>94° C for 45 sec</td>
</tr>
<tr>
<td>3</td>
<td>45° C for 30 sec</td>
</tr>
<tr>
<td>4</td>
<td>72° C for 45 sec</td>
</tr>
<tr>
<td>5</td>
<td>repeat steps 2-4 35 times</td>
</tr>
<tr>
<td>6</td>
<td>72° C for 5 min</td>
</tr>
<tr>
<td>7</td>
<td>4° C hold</td>
</tr>
</tbody>
</table>

**Gel Electrophoresis**

The PCR amplified DNA was then run out on 1% agarose gels with 1μL of ethidium bromide added. Sixteen μL of DNA and 2 μL of loading dye were added to each well. Ten samples were added per gel, as well as the negative control and a lane that contained a standardized ladder for band comparison. The gels were then run for approximately 45 minutes and were photographed using an ultraviolet camera.

**Analysis of Bands**

The photographs were studied and all the different bands (alleles) within each primer set were identified. Individual samples were recorded as having either the
presence or absence of bands. Any bands present in the negative control due to the presence of primer dimers were not considered alleles and were not documented in the results and were not used for subsequent genetic analysis. The raw data was then put into the Popgene (Yeh et al., 1997) population genetics analysis program and analyzed for any diversity that might exist within and between populations.

For each population the number of alleles per locus and percent polymorphism were calculated. Nei’s genetic distance (Nei, 1972) between pairs of populations was also calculated and a UPGMA dendrogram was constructed based on these pairwise genetic distances. The populations were subsequently subdivided into different groups for analysis.

I first analyzed the data based on elevation to determine if there were differences between the populations that occur at high elevations (above 6,000 ft) or lower elevations (below 6,000 ft). Populations 1-3, 5, and 6 were included in the high elevation category while populations 4 and 7-10 were in the low elevation category (Table 1).

In addition to the analysis based on elevation, another was performed with the populations divided by forest. Populations 1-9, which are from the Helena National Forest, were in one group while population 10, the Lewis and Clark National Forest sample, was in the other.

When the data were subdivided this way, Nei’s analysis of Gene Diversity in Subdivided populations was performed and Gst (coefficient of genetic differentiation (Nei, 1972)) values were calculated. Gst provides a measure of the differences in allele frequencies between populations. Gst values near 1.0 indicate that subgroups are
completely different in their allelic composition; values near 0 indicate that subgroups are nearly identical in their allelic composition and allele frequencies.
Results

Table 4: Alleles identified in different primer sets

<table>
<thead>
<tr>
<th>HB 11 alleles</th>
<th>HB 8 alleles</th>
<th>HB 12 alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB 11-1</td>
<td>HB 8-1</td>
<td>HB 12-1</td>
</tr>
<tr>
<td>HB 11-2</td>
<td>HB 8-2</td>
<td>HB 12-2</td>
</tr>
<tr>
<td>HB 11-3</td>
<td>HB 8-3</td>
<td>HB 12-3</td>
</tr>
<tr>
<td>HB 11-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB 11-5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In all, 11 different alleles were identified under the three primer sets (Table 4). Genetic polymorphism was present for all alleles in the entire sample set, although some sampling sites had no polymorphism for certain alleles. The population located in the Lewis and Clark National Forest had an entirely unique allele under the primer set HB 12 which was at an allelic frequency of 60% (Fig. 1). The UPGMA tree for all populations and alleles (Fig. 2) shows that the Lewis and Clark population is an outlying group compared to the populations within the Helena National Forest. Even when the unique allele was removed from the analysis, the Lewis and Clark population remained distantly related to the other populations, with only the Granite Butte population being somewhat related (tree not shown). The Gst value was 0.205 for all populations and alleles. When the analysis was run with the populations grouped according to high or low elevations, the value dropped to 0.032. The value between the two different national forests was 0.11. When the populations within the Helena National Forest were grouped by elevation, the Gst value was 0.041.
Fig. 1: Site of HB 12 unique allele bands from Lewis and Clark population
Fig. 2: UPGMA tree showing phylogenetic relationships between populations based on Nei's genetic distance
Discussion

The use of ISSR genetic markers has been shown to be effective in the analysis of population genetics in a variety of studies. Liston et al. (2003) used ISSR markers to study the number of genetic individuals vs. clonal individuals in a population of Idaho fescue (*Festuca idahoensis*) that had made a recovery in an area of southeastern Oregon where cattle overgrazing had taken place. They also assessed the amount of diversity that existed over all by analyzing different allele frequencies and different haplotypes and found high levels of diversity within the population being studied.

A study done by Tsumura et al. (1996) used a method similar to that which was used in the current study to identify different ISSR genetic marker alleles and their relative frequencies. Their study was done using DNA from Douglas fir (*Pseudotsuga menziesii*) and sugi (*Cryptomeria japonica*) trees. This study supported the use of ISSR markers and PCR analysis in plant population genetics analysis because of the high degree of polymorphism revealed with these markers and the relative ease with which these types of studies can be performed.

Genetic diversity is important for maintaining healthy populations of individuals in a variety of ways, from helping populations manage fluctuations in nutrient availability to providing a greater edge in competition with other species for space, nutrients, or disease resistance (Herrmann et al., 2007; Hughes et al., 2008). In the case of the *F. campestris* populations in the Helena and Lewis and Clark National Forests, however, perhaps the most important advantage of having high levels of genetic diversity is the increase in the ability of a species to recover after habitat disturbance (Hughes et al., 2008).
The data collection and subsequent analysis showed that there is indeed genetic diversity in the different populations of rough fescue within the Helena and Lewis and Clark National Forests. The analysis for all populations and all alleles showed a Gst value of 0.205, which suggests that approximately 20% of the total genetic variation for these populations exists between the different populations while 80% exists within the individual populations. This level of diversity between populations must be taken into account in future seed collections, because it suggests that there is a need to collect seeds from as many populations as possible in order to capture and maintain the current genetic structure of the *F. campestris* populations.

The presence of the unique allele and the different allelic frequencies of the Lewis and Clark population suggest a high variability of genotype between the two national forests. The phylogenetic analysis shows that these groups are clearly distinct from one another. The Gst value of 0.11 suggests that 11% or the total genetic variation exists between the different forests, while 89% exists within the different national forest populations. These results show that a considerable amount of diversity exists between the two forests, and as a result it is important to do collections from both in order to ensure that the diversity that currently exists is included in the reseeding program.

When the different populations were grouped according to elevation, the Gst value was rather small at 0.032 overall and 0.04 within the Helena National Forest. This suggests that only 3-4% of the variation exists between the populations, while 96-97% exists within. Although there is not a large amount of difference between the populations according to the parameters of this study, there is enough to suggest that there could be more diversity if a larger and more varied sampling were done. This data warrants
further, more in depth research in order to accurately determine the differences that might exist between the *F. campestris* populations growing at the high and low elevations.

When taken as a whole, all of the populations exhibited at least some differences from one another. This supports the first part of my hypothesis, which stated that genetic diversity would exist between the different populations.

Although the results do show that genetic diversity exists between the populations, there are limitations to the study that must be discussed. The first major limitation is the sampling size from the different populations. The small size does not allow a high level of confidence with respect to the relationships that were established between the different populations. The high vs. low elevation distinction that was chosen, 6000 ft., was an arbitrary number and may not reflect true patterns that may exist according to altitude. Finally, there was only one population sampled in the Lewis in Clark Forest, and it was about 700 ft. below the lowest sample taken in the Helena National Forest. This could mean that the unique allele and allelic patterns found at this site are due to an altitude factor that manifests itself somewhere below 5,558 ft., which is where the lowest sample was taken in the Helena National Forest. Due to only having one sampling site in the Lewis and Clark Forest, it is difficult to know the reason for the unique population with any degree of certainty.

In addition to the diversity between the populations, there is also a considerable amount of diversity within each of the populations sampled. Although certain alleles are fixed within some of the populations, no populations had individuals with identical haplotypes. The diversity contained within populations could be due to a variety of factors. Soil and light microhabitats, interspecific competition, and different amounts of
moisture contained within the soil could all play a role in driving the diversity within a population. In addition, because the ISSR markers are located in non-coding regions of the DNA, random mutations could play a role in driving differences in banding patterns.

No matter what drives it, the presence of diversity within the populations is an encouraging sign. It may help the *F. campestris* populations recover on their own from the habitat disturbances which are currently threatening its presence in the national forests (Hughes et al., 2008, Herrmann et al., 2007) and it may assist in population establishment and longevity after reintroduction. In addition, this data supported the second part of my hypothesis, which stated that there would be diversity within populations.

The high levels of diversity found in *F. campestris* are consistent with the results of similar studies using ISSR genetic markers. Wolfe et al. (1998) found that up to 95% of the alleles identified in their study of *Penstemon* populations were polymorphic. In their study of *Viola* using ISSR markers, Culley and Wolfe (2001) found that 100% of the alleles were polymorphic at the species level. In addition, they found a Gst value of 0.29 when all populations were taken into account. This is an even greater amount of total diversity than I found in the *F. campestris* populations.

Over all, it is important that the diversity of these Montana rough fescue populations be maintained throughout the course of the reintroduction program. Because of this, I would advise that the reseeding program involve collecting seeds from both the Helena and Lewis and Clark National Forests in addition to simply collecting from high and low elevations. This collection pattern will ensure that the maximum amount of
diversity is obtained and preserved in future generations, which will give rough fescue the best chance of reestablishing its presence as a dominant prairie grass species.

Although my project showed that there is indeed genetic diversity within and between the Lewis and Clark and Helena National Forests populations of *F. campestris* and has begun to elucidate some of the geographical patterns that it follows, further research will greatly aid in understanding the different *F. campestris* haplotypes and their distribution with respect to altitude and region. The unique allele found in the samples taken from Lewis and Clark National Forest shows particular promise for further research. A study of greater breadth and depth of the differences between the populations of the two national forests would greatly aid in developing a better reseeding program for the Forest Service.

The goal of this research project was to elucidate the presence and patterns of diversity within the *F. campestris* populations in the Helena and Lewis and Clark National Forests. In doing so, I hope to provide a guideline for future collections that will help to reestablish the presence of this important species to the level that it once had. Although this goal has been partially realized, further study and planning must be done in order to give the complete genetic picture of *F. campestris* in the reseeding zones and to ensure its long term survival in the Helena and Lewis and Clark National Forests.
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