Characterization of Nuclear and Chloroplast Microsatellite Markers for *Falcaria vulgaris* (Apiaceae)

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**ABSTRACT**

*Falcaria vulgaris* (sickleweed) is native to Eurasia and a potential invasive plant of the United States. No molecular markers have been developed so far for sickleweed. Characterization of molecular markers for this plant would allow investigation into its population structure and biogeography thereby yielding insights into risk analysis and effective management practices of the plant.

In order to characterize the molecular markers, DNA samples were collected from eight populations in Iowa, Nebraska and South Dakota. Nuclear microsatellite markers developed for other Apiaceae taxa were screened and tested for inter-generic transferability to sickleweed. The chloroplast *trnL* intron and *trnL-F* intergenic spacer regions were sequenced and the sequences were used to design primers to amplify the microsatellites present within each region. We characterized eight polymorphic microsatellite markers for sickleweed that included six nuclear and two chloroplast markers. Our result showed inter-generic transferability of six nuclear microsatellite markers from *Daucus carota* to *F. vulgaris*. The markers we characterized are useful for population genetic study of *F. vulgaris*.

**Keywords:** *Falcaria vulgaris*; Invasive Species; Microsatellite; *trnL* Intron; *trnL-trnF* Intergenic Spacer; Sickleweed

1. **Introduction**

Sickleweed (*Falcaria vulgaris* Bernh.; Apiaceae) is native to Europe and Asia [1] and was introduced to the United States in early 1920s [2]. It has been reported from 35 counties across 16 states in the United States and exhibits disjunct distribution in the Midwestern and Eastern USA [3]. Sickleweed exhibits some characteristics of invasive plant species including the production of a large number of seeds, effective seed dispersal mechanism whereby the seeds attached to the stem are carried away by the wind as the stems break at the nodes after the plant senescence, and the ability of the plant to reproduce asexually through root sprouting. These characteristics are perhaps facilitating its emergence as an aggressive weed in the Midwest [4]. Continuous increase in area coverage of sickleweed in Fort Pierre National Grassland (FPNG) and Buffalo Gap national Grassland (BGNG) of South Dakota has attracted attention of ecologists in the Midwest. The plant has also been listed as potential invasive plant by Nebraska Invasive Species Council [5].

Both nuclear and chloroplast DNA markers are commonly used for the genetic analysis of invasive plant populations particularly to predict the invasiveness of the introduced species, identify the source populations and help to design effective control programs for invasive species [6]. Microsatellite markers are one of the most preferred molecular markers because they are co-dominant, hyper-variable and are highly reproducible [7,8]. However, development of novel microsatellite markers is expensive and quite laborious task [9]. Cross-species transferability of microsatellite markers and identification of chloroplast microsatellites using universal chloroplast markers for sequencing chloroplast region are options that can avoid high cost and long time needed for marker development [10,11]. The markers are useful for investigating population structure and phylogeography of the introduced species, and are also useful for the study of comparative studies of different species [10], the process of population divergence and speciation process [12]. No molecular markers have previously been developed for sickleweed that would allow us study the genetic structure of this plant. Here we report on inter-generic transferability of six nuclear microsatellite markers from *Daucus carota* to *F. vulgaris*, and two polymorphic chloroplast microsatellite markers.

2. **Methods**

2.1. **Screening of Microsatellite Markers**

We reconstructed a phylogeny of the family Apiaceae based on nuclear ribosomal Internal Transcribed Spacer
The presence of repeat motifs in the amplicons was evaluated by 1.2% agarose gel to determine the quality of PCR products. The PCR conditions were an initial denaturation at 94 °C for 4 minutes, followed by 40 cycles of 1 minute denaturation at 94 °C, 20 seconds of annealing temperature and 1 minute extension at 72 °C, and final extension of 5 minutes at 72 °C. The PCR products were genotyped using 3730 x1 DNA analyzer (Applied Biosystems) at Iowa State University DNA Facility.

Genemarker V2.4.0 (Softgenetics) was used to visualize the genotyping data and create allele reports. The genotyping data and the presence of repeat motif in the amplicons was verified by re-sequencing the PCR products.

For chloroplast microsatellites, we sequenced the trnL intron and trnL-F intergenic spacer (using the primer pair 5’-CGAAATCGGTAGACGCTACG-3’ and 5’-ATTGGAATCGGTAGACGCTACG-3’) of chloroplast gion and found two mononucleotide repeats with more DNA. We searched for nucleotide repeats within the region of interest to amplify these mononucleotide repeats.

### 2.2. Genotyping, Test for Potential Artifacts and Data Analyses

For genotyping, PCR was carried out in a reaction mixture of 15 μl containing 50 ng genomic DNA, 3 μl of 5 X buffer (Promega), 1.2 μl of 10 mM dNTPs (Promega), 2 μl of 25 mM MgCl₂ (Promega), 1 μl each of 10 pM forward and reverse primers and 2 units of Jump Start Taq polymerase (Sigma-Aldrich). The PCR conditions were an initial denaturation of 4 minutes at 94 °C followed by 40 cycles of 1 minute denaturation at 94 °C, 20 seconds of annealing temperature and 1 minute extension at 72 °C, and final extension of 5 minutes at 72 °C. Electrophoresis was carried out in 1.2% agarose gel to evaluate the quality of PCR products and the presence of repeat motif in the amplicons was verified by re-sequencing the PCR products.

### Table 1. Collection sites of the sickleweed population.

<table>
<thead>
<tr>
<th>Collection site</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onawa, Iowa</td>
<td>42°57’4.62&quot;</td>
<td>96°7’34.68&quot;</td>
<td>12</td>
</tr>
<tr>
<td>Wayne, Nebraska</td>
<td>42°14’7.18&quot;</td>
<td>97°8’32.24&quot;</td>
<td>12</td>
</tr>
<tr>
<td>Boyd, Nebraska</td>
<td>42°52’2.99&quot;</td>
<td>98°45’7.22&quot;</td>
<td>12</td>
</tr>
<tr>
<td>Boyd, Nebraska</td>
<td>42°57’9.63&quot;</td>
<td>98°40’9.93&quot;</td>
<td>12</td>
</tr>
<tr>
<td>Alkali West FPNG, South Dakota</td>
<td>44°10’5.85&quot;</td>
<td>100°17’42.87&quot;</td>
<td>12</td>
</tr>
<tr>
<td>Grass Creek FPNG, South Dakota</td>
<td>44°11’45.35&quot;</td>
<td>100°18’26.49&quot;</td>
<td>12</td>
</tr>
<tr>
<td>BGNG, South Dakota</td>
<td>43°55’56.12&quot;</td>
<td>101°24’8.54&quot;</td>
<td>12</td>
</tr>
<tr>
<td>Brookings, South Dakota</td>
<td>44°18’53&quot;</td>
<td>96°47’38&quot;</td>
<td>12</td>
</tr>
</tbody>
</table>

### 3. Results and Discussions

The probability of microsatellite marker transferability reduces with increased phylogenetic relationship [20]. There are several evidences of microsatellite marker transferability within genus but few evidences at higher taxonomic level than genus. For example, microsatellite transferability success rate within the genera in eudicots are approximately 60% compared to 10% across genera [10]. Therefore, it is always beneficial to identify phylogenetically close relatives before screening the microsatellite markers for cross species transferability when microsatellites markers are not available for sister species within the genus. In our phylogenetic analysis, we found Apium graveolens, D. carota, Eryngium alpinum and H. mantegazzianum were the only taxa with microsatellite markers developed within the clade and they were distantly related to F. vulgaris. Falcaria is a monotypic genus. Previous studies have shown transferability of Daucus and Heracleum microsatellite markers to other species [13,15]; therefore, we chose microsatellite markers developed for these two species to test their transferability to sickleweed.

None of the six H. mantegazzianum microsatellites
markers amplified the *F. vulgaris* DNA; however, all six markers did amplify the DNA of *H. maximum* which was used as a positive control in this test. The transferability of these microsatellite markers (see [15] for primer sequences) from *H. mantegazzianum* to *H. maximum* has not been previously reported. With *Daucus* microsatellite primers, 26% of the primers amplified *Falcaria* DNA. Based on the quality of electrophoretic bands, *Daucus* primers tested for the amplification of *Falcaria* were classified: six primers producing a clean band with nearly expected amplicon size (7%), 16 primers producing multiple bands (19%) and 63 primers did not produce any band (74%). The six markers that produced clean bands are presented in Table 2. The identified six nuclear microsatellites and two chloroplast microsatellite primers were used for genotyping sickleweed samples collected from eight populations.

The signals of the genotyping results were clean and did not show stuttering bands. Micro-Checker showed no evidence of scoring error due to stuttering and large allele drop-out. The program did not identify any genotyping error due to null alleles either. Our test of genotyping artifacts suggest that any deviation from Hardy-Weinberg equilibrium in our analysis is the result of change in allele frequency but not due to genotyping artifacts.

All six nuclear microsatellite loci were polymorphic with mean number of allele per locus of 8.8 (range 3 to 19) and most of these loci except ESSR80 have high polymorphism content value (Figure 1). Three out of six nuclear microsatellite loci were monomorphic for population from Iowa. This could be because of small sample size of the population. Two, three and five loci showed significant deviation from Hardy-Weinberg equilibrium for the populations from Iowa, Nebraska and South Dakota, respectively (Table 3).

Among six nuclear microsatellite loci, three loci (ESSR9, GSSR24 and GSSR25) produced three to four alleles for some samples (Figure 2). These multiple alleles per sample indicates that sickleweed in the novel range might have undergone gene duplications perhaps through polyploidization [21].

Chloroplast DNA markers are often used for the study at higher taxonomic level as they have very slow evolutionary rate and do not reveal much variation within species [22]. However, chloroplast microsatellites have been effectively used for the study of intra-specific variation of the plant [11]. The two chloroplast microsatellites markers used in our study were also polymorphic and detected two (CSSR1) and three alleles (CSSR2). The average mean haploid diversity for these two loci was 0.23 and these two loci detected three chloroplast haplotypes. The number of alleles and the haploid diversity of these two loci in different populations are presented in Table 4.

These polymorphic microsatellite markers can be used for the study of population structure and gene flow in the introduced as well as native range and will ultimately be useful in the identification of source population(s). Use of non-recombinant chloroplast microsatellite markers and nuclear microsatellite markers will exactly determine if the gene flow is the result of seed or pollen flow [11]. The nuclear and chloroplast microsatellite markers together can provide important insights about the genetic

### Table 2. Characteristics of microsatellite markers for *F. vulgaris*.

<table>
<thead>
<tr>
<th>Locusa</th>
<th>Primer sequences (5'-3')</th>
<th>Repeat motif</th>
<th>Tm (°C)</th>
<th>Size range (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear microsatellites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| ESSR9 (FAM) | F: ATCTGGGGAACCTTGCTGTTG  
R: AGCATCAGCAGCAGTACAA | (TGC)6 | 58 | 290 - 311 |
| ESSR80 (FAM) | F: ACAGCCAGTGGAGCAAGACT  
R: GAGATTGGCAATGTGGGAT | (CA)9 | 53 | 235 - 239 |
| GSSR24 (FAM) | F: GCCAACCATCAAAATCACTTCT  
R: GAATAAATGCCTGCAATACCG | (TC)12 | 51 | 281 - 321 |
| GSSR25 (FAM) | F: CCAGAAAATGTATTTTTATGTCGGCC  
R: CGTTTTTCAATTTAATACCTCAATC | (CATA)21 | 53 | 166 - 222 |
| GSSR154 (FAM) | F: CTTATATGATGCGGTCGAAA  
R: GACTGACCGCTCTTACTCTCAC | (TCT)11 | 53 | 302 - 316 |
| BSSR53 (FAM) | F: GCTTATAGACCTTCTTCTAGTCGTCCA  
R: CTACATGAGCTTACCTCATTACCT | (AT)8 | 53 | 193 - 211 |

Chloroplast microsatellites

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequences (5'-3')</th>
<th>Repeat motif</th>
<th>Tm (°C)</th>
<th>Size range (bp)</th>
</tr>
</thead>
</table>
| CSSR1 (FAM) | F: GTTCAATGGGGAGCTCGCTT  
R: TAT CCCCAA AAA GCC CAT T | (A)11 | 53 | 376 - 377 |
| CSSR2 (FAM) | F: CGG AAG TTT CAA TGG AAG GA  
R: TAA TTC CGG GGT TTT TCT GAA | (T)11 | 53 | 177 - 179 |

*The fluorescent dye used to label forward primer is given in parentheses.*

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Table 3. Number of samples (n), Number of alleles (A), observed heterozygosity (H_o) and expected heterozygosity (H_e) of different nuclear microsatellite markers for the samples from Iowa, Nebraska and South Dakota.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Iowa n</th>
<th>A</th>
<th>H_o</th>
<th>H_e</th>
<th>Nebraska n</th>
<th>A</th>
<th>H_o</th>
<th>H_e</th>
<th>South Dakota n</th>
<th>A</th>
<th>H_o</th>
<th>H_e</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESSR9</td>
<td>12</td>
<td>3</td>
<td>na</td>
<td>na</td>
<td>36</td>
<td>7</td>
<td>0.52*</td>
<td>0.80</td>
<td>48</td>
<td>5</td>
<td>0.73*</td>
<td>0.71</td>
</tr>
<tr>
<td>ESSR80</td>
<td>12</td>
<td>1</td>
<td>na</td>
<td>na</td>
<td>36</td>
<td>2</td>
<td>0.02</td>
<td>0.03</td>
<td>48</td>
<td>3</td>
<td>0.02*</td>
<td>0.06</td>
</tr>
<tr>
<td>GSSR24</td>
<td>12</td>
<td>3</td>
<td>1.00*</td>
<td>0.52</td>
<td>36</td>
<td>12</td>
<td>0.80*</td>
<td>0.84</td>
<td>48</td>
<td>11</td>
<td>0.91*</td>
<td>0.92</td>
</tr>
<tr>
<td>GSSR25</td>
<td>12</td>
<td>4</td>
<td>0.33</td>
<td>0.42</td>
<td>36</td>
<td>3</td>
<td>0.27</td>
<td>0.33</td>
<td>48</td>
<td>9</td>
<td>0.48</td>
<td>0.43</td>
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<tr>
<td>GSSR154</td>
<td>12</td>
<td>3</td>
<td>1.00*</td>
<td>0.65</td>
<td>36</td>
<td>7</td>
<td>0.69*</td>
<td>0.80</td>
<td>48</td>
<td>5</td>
<td>0.43*</td>
<td>0.68</td>
</tr>
<tr>
<td>BSSR53</td>
<td>12</td>
<td>1</td>
<td>na</td>
<td>na</td>
<td>36</td>
<td>3</td>
<td>0.06</td>
<td>0.05</td>
<td>48</td>
<td>6</td>
<td>0.16*</td>
<td>0.39</td>
</tr>
</tbody>
</table>

*Departure from Hardy-Weinberg equilibrium at p < 0.05; na—Locus is monomorphic and test for H-W equilibrium was not done.

Table 4. Number of alleles and haploid diversity of two chloroplast microsatellite markers.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Iowa n</th>
<th>h</th>
<th>Nebraska n</th>
<th>h</th>
<th>South Dakota n</th>
<th>h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSSR1</td>
<td>2</td>
<td>0.32</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CSSR2</td>
<td>2</td>
<td>0.32</td>
<td>2</td>
<td>2</td>
<td>0.27</td>
<td>2</td>
</tr>
<tr>
<td>CSSR2</td>
<td>2</td>
<td>0.32</td>
<td>0.47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Polymorphism information content values displayed by the microsatellite loci.

Figure 2. Electropherograms of a sample at GSSR 25 locus. Similar multiple peaks were present at GSSR24 and ESSR9 loci. Multiple peaks displayed by several loci suggest that sickleweed in the United States might have undergone polyploidization.

4. Conclusion
Our results demonstrated successful inter-generic transferability of microsatellite markers from *Daucus carota* to *Falcaria vulgaris*. Since these two species belong to two distantly related genera, transferability of microsatellite markers between these species indicates that these microsatellite markers may work for other genera within the Apiaceae family. We are also reporting two chloroplast microsatellite markers for sickleweed. Sequencing other chloroplast region using universal chloroplast markers may reveal more chloroplast microsatellite markers. These nuclear and chloroplast microsatellite markers are polymorphic and are useful for population genetics and phylogeographic study of sickleweed.

5. Acknowledgements
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