POPULATION GENETICS OF *Phalaris arundinacea* L. IN A WESTERN UNITED STATES WETLAND

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of

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POPULATION GENETICS OF *Phalaris arundinacea* L. IN A WESTERN UNITED STATES WETLAND

Abstract

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Turnbull National Wildlife Refuge (henceforth Refuge) is a wetland complex located in east central Washington State. *Phalaris arundinacea* is an invasive grass species on the Refuge. It forms monotypic stands, reducing native plant diversity and can reproduce either sexually or from rhizomes and stem nodes. Due to the extreme density of *P. arundinacea*, we hypothesized that there was only one genetically distinct population of *Phalaris arundinacea* at the Refuge. The entire length and breadth of the refuge was sampled and leaf tissue was taken from each sample plant. Amplified Fragment Length Polymorphism was used to determine the presence or absence of genetic markers for each plant, and analysis using NTSYSpc, STRUCTURE, DISTRUCT and GenAlEx established the presence of 5 genetically distinct populations of *Phalaris arundinacea* at the Refuge. Out of 166 plants sampled only four were of clonal origin, indicating that this species’ reproduction on the Refuge is primarily sexual. Considering likely sources of this genetic variability, we identified the possibility of the species being planted by
farmers relocating to this area prior to establishment of the refuge. At the time, the species was considered to be good forage for livestock. Further, refuge records indicate that the species was planted by refuge staff at least three times between 1945 and 1970, before it was observed to be invasive. Early cultivars available between those dates included both tetraploids and one hexaploid. Multiple introductions are considered to be a source of genetic variability allowing a species to adapt to a site and become invasive. We hypothesized that the *Phalaris arundinacea* plants on the Refuge could either be tetraploid, hexaploid or a mix. Through flow cytometry, we found that only tetraploids were present. The source of the genetic variability among these tetraploids remains unknown. The degree of variability discovered and the prevalence of sexual reproduction imply that this species has the genetic resources to adapt quickly to future changes in its environment and remain a management challenge on the refuge. Control strategies that limit seed production may be useful.
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Dedication

This dissertation is dedicated to my brother James Henry Canwell, who has provided me enormous emotional support through the entire doctoral program. He never missed an opportunity to assist with edits, computer issues and trips to Pullman. At a moment’s notice he would drive 100 miles and take time off work just to assure my well-being. Thank you James Henry Canwell.
CHAPTER 1

EXPLORING THE POPULATION GENETICS
OF REED CANARYGRASS (Phalaris arundinacea)

INTRODUCTION

Although reed canarygrass (Phalaris arundinacea) can be a desirable forage species in some environments, it can also become invasive, competing with preferred native plants species and leading to suppression, displacement or complete elimination of desirable native plant communities. This perennial grass is present on all continents except Antarctica, yet it is invasive only in the northern regions of the United States. P. arundinacea is found in riparian zones, drainage ditches, and moist uplands as well as wetlands. It is able to reproduce both sexually and vegetatively. Decades of control efforts have been largely unsuccessful. The genetic characteristics of Phalaris arundinacea, particularly the results of multiple introductions have been offered as one explanation for the species’ invasiveness (Lavergne and Molofsky 2006). The thinking is that multiple introductions broaden the gene pool and provide more genetic resources for plants to adapt especially well to new sites (Lavergne and Molofsky 2004). The genetic characteristics of entire populations are largely unstudied. We undertook two related studies in an effort to find out more about the population genetics of the species and explore possible reasons for the specie’s invasiveness, all with the intent to develop more effective control strategies in the future.

Both studies were conducted at Turnbull National Wildlife Refuge in southeast Washington State, USA, as conditions on the refuge are typical of problem populations in the interior Pacific Northwest where the conservation mission of the Refuge is threatened by the specie’s
dominance. There are historical reasons why genetic variability is possible in *P. arundinacea* here. The Refuge was established in 1937 and during this first year a series of narrative reports began that provides some history of the species and its management on the refuge. In the late 1800’s, European settlers created as many as 60 small farms on what is now the Refuge (TNWR Narratives 1938-2012). These settlers may have brought the species with them from other regions, possibly contributing to the origin of invasive strains. There is question as to the nativity of this species in the region (Meriglio and Lesica 1998), but we do know that the species was planted on the refuge at least three times between 1945 and 1970 (TNWR Narratives 1938-2012). The origin of the plant material and exact locations were not recorded. This situation matches the multiple introduction theory posited by (Lavergne *et al.* 2009, Ellstrand and Schirenbeck 2000) for the invasiveness of the species in the eastern United States.

No other known introductions occurred on the Refuge after 1970 (TNWR Narratives 1938-2012), potentially allowing the *P. arundinacea* population time to become well adapted to this specific environment. Based on the dense stands and the specie’s strong vegetative reproductive capacity, we hypothesized that only one population of *P. arundinacea* currently existed on the Refuge. This hypothesis was tested by randomly collecting leaf samples of the species from two meadows and two long transects that spanned the entire length and breadth of the Refuge. Amplified Fragment Length Polymorphism (AFLP) and gel electrophoresis were used for DNA analysis. The programs STRUCTURE, NTSys and GenAlEx were used determine the number of populations of the species occurring on the Refuge. We found five distinct populations occurring across the refuge, with no particular geographic pattern associated with their distribution. Further, only two percent of the plants tested were clonal, ninety eight percent originated from seed.
Due to the agronomic importance of *P. arundinacea* in some regions, cultivars have been developed over the years to address perceived shortcomings, including poor reproduction from seed (Carlson *et al.* 1996). At least twelve cultivars existed when *P. arundinacea* was planted on the Refuge including both tetraploids and hexaploids (Carlson *et al.* 1996, USDA ARS GRIN 2015) Tetraploids are the most common form of the species in northern-latitudes while hexaploids are more common at mid-latitudes (Lavergne and Molofsky 2004 Lavergne *et al.* 2009).

Because of the possibility that hexaploid seed might have been used at the Refuge, we determined the ploidy of 21 *P. arundinacea* plants from across the refuge. Plants were grown in the greenhouse from rhizomes collected in the field and subsequently, the foliage of each plant was harvested for flow cytometry to determine ploidy levels. All of the samples were tetraploid. This does not preclude introduction of hexaploid cultivars, it only indicates that none survived or established. There is still the possibility that tetraploid cultivars may have been introduced at that time.

The next two chapters provide full details of the studies outlined above, while the last chapter integrates their results and implications for understanding the population genetics of *Phalaris arundinacea* under field conditions. Lastly, management recommendations are discussed. Chapters two and three are written as manuscripts for PLOS ONE and Natural Areas Journal, respectively and as such, their formats vary from one to the other.
LITERATURE CITED


Turnbull National Wildlife Refuge (TNWR) (1938-2012) TNWR Narrative Reports. Cheney, WA.

CHAPTER 2

POPULATION GENETICS OF Phalaris arundinacea L. IN A WESTERN UNITED STATES WETLAND

ABSTRACT

Genetic characteristics of Phalaris arundinacea populations are largely unstudied, yet it is an important invader in wetlands and riparian zones in the Northwestern United States. It forms monotypic stands, reducing native plant diversity and can reproduce sexually or vegetatively. One hypothesis explaining invasiveness is the hybrid hypothesis: intraspecific hybrids produce new phenotypes with competitive advantages. Sources of genetic variation in P. arundinacea include introduced European strains and 15 cultivars developed here. We studied the population genetics of an invasive population in an Eastern Washington wetland that had multiple introductions of Phalaris arundinacea and may have had a native population. Amplified Fragment Length Polymorphism was used to determine genetic characteristics of each plant sampled. We used several procedures to estimate the number of groups among the samples, including cluster analysis using NTSYSpc and STRUCTURE to determine likely population and sub-population relationships. Population admixtures were displayed using STRUCTURE’s ancillary program DISTRUCT. The distances among populations hypothesized using STRUCTURE were evaluated using Prevosti’s Distance Coefficient in NTSYSpc, and the extent and significance of divergence was shown using the analysis of molecular variance (AMOVA) Excel add-on in GenAlEx. After analyzing by transect with inconsistent results, data from all transects were combined. Then all procedures identified five groups. Prevosti’s distance demonstrated 85% similarity among the groups and AMOVA showed higher within (89%) than among (11%) group variation (p<0.01). Numerous studies have found that P. arundinacea spreads primarily by
rhizomes, however, our results show that reproduction here is primarily sexual (158 of 166 plants were genetically unique at distances of $\geq 1.5$ m between samples). At this site, there may be sufficient genetic resources to adapt to future manipulations and environmental changes. This genetic diversity supports the hybrid hypothesis of invasiveness and has implications for similar sites across Northwestern North America.
INTRODUCTION

Reed canarygrass (*Phalaris arundinacea* L.) is a widely distributed grass species located on every continent except Antarctica [1]. According to Hitchcock and Cronquist [2], there are seven species of *Phalaris* in the Pacific Northwest. It is believed that *P. arundinacea* was introduced to Eastern North America from Europe in the early 1800’s as a forage plant [1], [3]. The question of whether *P. arundinacea* is native to the northwest United States is still controversial [4]. As *P. arundinacea* has been planted across the United States for forage and riparian stabilization, it has become the most important invasive grass in wetlands and the riparian zones in the United States [5].

The invasive nature of *P. arundinacea* can lead to the formation of monotypic stands that reduce native plant diversity [6], [7]. Perkins and Wilson [8] found that *P. arundinacea* had a significant impact on the diversity of native species in an Oregon Coast Range wetland. For every 7% increase in *P. arundinacea* cover, one native plant species was lost. *Phalaris arundinacea* eliminated most native species within two years in Wisconsin meadows exposed to increased sedimentation, nutrients and flooding [9]. Several characteristics enhance the invasiveness of *Phalaris arundinacea*. These include reproduction by seed as well as vegetative spread by rhizomes and tillers, high stem elongation, wide physiological tolerance and high phenotypic plasticity [5], [10], [11].

Within the Mediterranean region, native *P. arundinacea* appears not to be as invasive as the species is in the United States, particularly in the western states [10]. Lavergne and Molofsky [5] and Kercher et al. [9], have suggested that *P. arundinacea* has become invasive in the eastern United States because of repeated introductions of European plants. *P. arundinacea* clearly is able to out-compete native species wherever it is introduced in appropriate habitats. The impacts
of invasion, both ecological and economic of *P. arundinacea*, have been well-studied, [10], however, the mechanisms underlying its invasiveness are not well understood [5], [12]. Numerous hypotheses have been suggested to explain why some plant species become invasive [13]. Among these is the hybrid hypothesis which states that interspecific or intraspecific hybrids result in new phenotypes with advantages in invaded sites that would not be present in the parent populations [13].

Anderson and Stebbins [14] anticipated the hybrid hypothesis:

“Hybridization between populations having very different genetic systems of adaptation may lead to new adaptive systems, adapted to new ecological niches.”

Ellstrand and Schierenbeck [15] have suggested that hybridization, both interspecific and intraspecific, is an important source of genetic variation leading to invasiveness. They hypothesize that mixing of different genotypes results in individuals that for reasons of evolutionary novelty or genetic variation can start invasive populations. They found 28 examples from the literature of interspecific hybridization that led to invasiveness and argue that hybridization between genetically divergent populations of the same species should have a similar result [15]. Populations from across the geographic range of a species are likely to be more similar than those of closely related species and thus would take multiple introductions and intermating to eventually result in a hybridization favorable to invasiveness in a new environment [15]. A population resulting from such a hybridization event should show higher levels of genetic variation than the parent populations [15].

*Echium plantagineum*, was introduced to Australia several times and the invasive hybrid was found to have higher genetic variation than parent populations from Europe [16]. *Bromus tectorum* was introduced to the United States multiple times from different parts of its native range in Europe and Africa. Isozyme analysis found that the within population variation of
Bromus tectorum in the United States was greater than within population variation in its native range [17].

The hybrid hypothesis has been studied in reed canarygrass, in Vermont, North Carolina and in some European populations [10]. It was found that invasive populations of reed canarygrass had relatively high (but unspecified) levels of within population genetic diversity and that each genotype differed in its phenotypic response to environmental conditions. Morrison and Molofsky [18] studied the responses of three *P. arundinacea* genotypes to several environmental conditions. Each responded differently. Genotype 1 produced more root biomass than the other two when there was little competition. Genotype 2 produced more tillers under ideal conditions and genotype 3 produced more roots and shoots under competitive conditions. This study suggests that a number of different genotypes in a population may influence its ability to become invasive. Due to genotypic variation, *P. arundinacea* may vary in biomass allocation, survivorship and reactions to soil moisture, drought and competition [10].

Lavergne and Molofsky [6] found that invasive genotypes of reed canarygrass from Vermont and North Carolina had higher emergence, biomass, tillering and vegetative establishment as compared to native European strains. Similarly, Caño et al., [19] found that invasive populations of *Senecio pterophorus* in Spain had higher growth rates, more seed heads and greater seed production than non-invasive native populations from South Africa when grown in similar conditions.

Until recently most of the invasive characteristics of *P. arundinacea* studied have been anatomical not physiological. *P. arundinacea* genotypes from the Czech Republic (non-invasive) and North Carolina and Vermont (invasive) did not show significant differences in photosynthetic rate, stomatal conductance or water use efficiency [20]. They concluded that
physiological differentiation between native and invasive populations may not be common and may not influence invasiveness.

The hybrid hypothesis requires that there be sources of genetic variation from different populations and that those populations interbreed. Sources of genetic variation in *P. arundinacea* include European strains introduced into the United States and cultivars developed in the United States. It is unknown how many genotypes of *P. arundinacea* were introduced from Europe or where they were planted.

According to Galatowitsch and Anderson [13] the development of cultivars in the United States, as early as 1920 [21] provided even more genetic variation to *P. arundinacea* as an important forage species in the Midwestern United States. Cultivars have been developed in the United States to improve seed production, viability and other characteristics that would also enhance competitiveness. The United States Department of Agriculture (USDA) National Plant Germplasm System collects plant germplasm from around the world and has 115 accessions of *P. arundinacea*. The accessions range in improvement status from wild material to named cultivars [21]. There are 25 North American accessions of *P. arundinacea*, including 15 named cultivars, 2 breeding lines, 6 wild populations, and 2 of unknown status [21]. Some of these cultivars have been widely planted across the United States. Unfortunately, very few records were kept of which cultivars were planted where. Morrison and Molofsky [18] and Gifford, et al. [22], have shown that *P. arundinacea* has high levels of genetic variation in the eastern United States. It is likely that the interbreeding of *P. arundinacea* cultivars in the United States has resulted in the observed genetic variation [15]. Genetic characteristics of *P. arundinacea* populations are largely unstudied as is the question of how these populations distribute themselves across the environment.
To evaluate the hybrid hypothesis as an explanation for the invasiveness of *P. arundinacea* in the Western United States, we studied the population genetics of an established invasive population in an Eastern Washington wetland complex. The research was conducted at Turnbull National Wildlife Refuge in Eastern Washington State. We hypothesized that there is a single genetically uniform population of *Phalaris arundinacea* at the refuge, perpetuated by vegetative reproduction rather than a diverse population resulting from hybridization.

**FIELD PROCEDURES**

Turnbull National Wildlife Refuge (TNWR) is located approximately 47.42N latitude and 117.56W longitude in Eastern Washington State. Annual precipitation averages 419 mm with cool season moisture and dry summers. Long-term summer and winter temperatures are above 27° and -3.8°C, respectively [23]. The landscape is a series of wetland channels and ponds with rocky uplands. Elevation ranges from 670 to 720 m. The emergent plant community includes sedges (*Carex sp.*), bulrush (*Scirpus maritimus*), water buttercup (*Ranunculus aquatilis*) and common cattail (*Typha latifolia*). The riparian areas feature red-osier dogwood (*Cornus stolonifera*), quaking aspen (*Populus tremuloides*) and golden current (*Ribes aureum*). The rocky uplands are dominated by ponderosa pine (*Pinus ponderosa*) with an understory of forbs and grasses.

*Phalaris arundinacea* has invaded the emergent and riparian zones, but the uplands are too dry for the species. Reed canarygrass has been present in the area since at least the late 1800’s. The refuge was established in 1937 on agricultural lands and the species was seeded at least twice since that time [23].

In order to sample as broadly as possible, and because this was a preliminary exploration, transects and sample locations were systematically located. The genetic variation of *Phalaris*
arundinacea was investigated on two scales. Two topographically distinct meadows were selected and 87 samples were taken at 1.5 meter intervals along a transect bisecting each meadow. Two large-scale transects across the entire refuge were set up, the first spanning the greatest length that roughly approximated the prevailing wind direction (SW to NE) which was also the reverse of the general direction of ground water flow (NE to SW), and the other perpendicular to it (Figure 1). These extended 4.9 kilometers from northeast to southwest with 40 samples at 111 meter intervals and northwest to southeast 4.45 kilometers, with 39 samples were taken at 99 meter intervals. All sample locations were recorded by GPS. Transects included all major land types, but sample locations were somewhat irregular due to presence of deep water or uplands where the species was not present. At each sample point, a minimum of 4 cm of live leaf tissue was collected from the closest Phalaris arundinacea specimen. Tissue samples were placed in labeled envelopes and kept on ice.

ETHICS STATEMENT
We were issued a research permit to collect Phalaris arundinacea leaf samples by Michael Rule, the refuge biologist.

LABORATORY PROCEDURES
At the USDA Western Regional Plant Introduction Station genetics lab, at Washington State University, samples were frozen at -80 C for a minimum of 4 hours, followed by freeze-drying for 72 hours at -48 degrees C. Molecular markers revealed by amplified fragment length polymorphism (AFLP) were used to characterize genetic differences. We used AFLP because it is reproducible, no prior DNA sequence is necessary, and only a small amount of DNA is required. The AFLP procedure is particularly useful in differentiating the taxonomy of highly related species, cultivars, accessions and strains, allowing researchers to look at genome
evolution and genetic diversity, as well as conduct genetic mapping [24]. AFLP markers are biallelic and dominant, and although less informative at a locus, they allow for efficient sampling of many loci [25, 26]. Thus, AFLP’s lend themselves to studies in which more loci are needed to estimate genetic diversity because genomic heterogeneity is high [27]. Despite being dominant markers, AFLPs have shown themselves effective in discriminating among populations and correctly assigning individuals to populations [26, 28, 29].

Two cm of freeze-dried plant tissue were placed in a labeled 1.5 ml Eppendorf tube containing 5-6 glass beads 3 mm in diameter. Eppendorf tubes were secured in a plastic container surrounded by Styrofoam blocks and plant tissue was pulverized using a Tornado Two ® paint shaker. DNA was extracted from pulverized plant tissue using the MagnaSil Kit Methodology ®, quantified using fluorometry, and adjusted to a concentration of 25 ng/µl. The resulting DNA was digested using restriction enzymes EcoR1 and Mse1, followed by ligation of restriction-specific adapters [24].

Pre-amplification using primer sequences complementary to the adapter sequences but with a selective nucleotide at the 3’ end was used to produce template DNA (Biolase DNA Polymerase from Bioline USA, INC. methodology) [24]. A second selective amplification using 3 selective nucleotides produced markers visible on the Licor® sequencer for separation using polyacrylamide gel electrophoresis (Li-Cor Gene ReadIR 4200 \(^1\) Lincoln, NE). Gel images were printed using GeneImagIR® software from Scanalytics (Beckton-Dickenson, Rockville, MD) and each molecular weight was scored as either present (1) or absent (0) for each sample.

**ANALYTICAL PROCEDURES**

A simple matching coefficient cluster analysis was run using the 0/1 matrices to graphically represent relationships among the sampled individuals using NTSYSpc [30]. This was performed
using the un-weighted pair group mean algorithm (UPGMA) from the Sequential Agglomerative Hierarchical and Nested clustering method (SAHN) function of NTSYSpc. Distance matrices were used to generate dendograms [30]. Principle Coordinate Analysis (PCoA) graphically displayed major differences among the plants in the first 2 and 3 dimensions of variance.

STRUCTURE [31], a heuristic analysis of genetic population structure was run as employed by Evanno et al. [32], to determine the most likely sub-population relationships. STRUCTURE estimates the number of populations or clusters present (K), using a Bayesian algorithm [33], [34]. The Bayesian algorithm used in STRUCTURE employs knowledge of prior events to predict the likelihood of future events [33], [34], and [35].

The admixture model was chosen assuming no prior population information, burn in length period set at 10,000 repetitions and the number of Markov chain Monte Carlo repetitions after burn in was set at 50,000 repetitions with five iterations per assumed population (K) [31], [35]. Falush et al [33] determined that increasing the amount of burn in time or run length beyond these values did not affect the final estimates of K. DISTRUCT, an ancillary program of STRUCTURE graphically displays populations [36]. The allele frequencies and population destinations are placed in individual Q-Data files to show admixtures and define groups that should correspond with Prevosti’s Distance Coefficients. NTSYSpc generates Prevosti’s Distance Coefficients in order to measure the distance between populations by observing the variance of marker frequencies characterizing the genetic distance. Prevosti’s Distance Coefficient is considered the most precise measurement for population distance [29].

GenAlEx (an MS Office 2003 Excel add-in), was run using the output from Structure to obtain an analysis of molecular variance (AMOVA). The AMOVA measures genetic structure directly from scored data (1’s and 0’s), and differences within and between populations [37, 38].
RESULTS

The analyses described above were run to identify separate populations among plants on each transect (Table 1). Data from the two short transects indicated three or four populations on transect A (p = 0.02), while transect B consisted of a single population. We then combined data from both transects to effectively double the sample size and analyzed these data as one sample. This resulted in identification of five or six populations (p = 0.01). This procedure was repeated for the two long transects, suggesting three or four populations on transect C and four on transect D (p = 0.64, and 0.16 respectively). When data from the two long transects were combined and analyzed, four or five populations were identified (p = 0.10). Lastly, data from all four transects were combined.

When plants from all transects were pooled, cluster analysis with NTSYSpc [30] for the 166 plants together showed five populations (Figure 2) of 37, 27, 45, 36 and 18 individuals, including 4 pairs of identical plants. Identical plants were found only on the short (1.5 m between samples) transects but only one of the four pairs were adjacent plants. The 3-dimensional PCoA (Figure 3) clearly shows five populations consistent with the probability LnP(X/K) and dK as described by Evanno et al. (Figure 4) [31]. The Q distribution at K=5 displays five populations consisting of 38, 50, 23, 23, 32, respectively, of the 166 plants (Figure 5).

Analysis of molecular variance showed higher within (89%) than among (11%) group variation. The p value of 0.01 supports the hypothesis of five groups (Table 2). Prevosti’s distance (not shown), demonstrated approximately 85% similarity for the five groups. Comparing the results from each transect or group of transects (Table 1) reveals that larger samples generate more consistent results between tests. We interpret our results to mean that our sample size was sufficient to generate consistent results from all analyses when data from all transects were
combined. Because analysis of individual transects yielded from one to six possible groups, we believe the results from all transects combined are the most reliable at a consistent five groups (Table 3) a value bracketed by both pairs of transects (A + B, and C + D) (Table 1). The five populations were mapped together (Figure 6) and separately (Figures 7 – 11), but no pattern was evident in the distribution of plants from any one population. Nor was there any correlation between the plant community type and population. The plant did not occur on the upland or deep water habitats.

DISCUSSION

Genetic characteristics of *Phalaris arundinacea* populations are largely unstudied. Morrison and Molofsky [18] and Gifford, *et al.* [22], have shown that *P. arundinacea* has high levels of genetic variation in Eastern North America. It is likely that interbreeding of *P. arundinacea* cultivars in North America has resulted in observed genetic variations [15]. The species has been introduced to North America numerous times since the early 19th century. The strains introduced are of European origin, although *Phalaris arundinacea* is considered native to North America [6]. Turnbull National Wildlife Refuge experienced multiple introductions of *Phalaris arundinacea* on top of what may have been a native population [4]. In the late 1800’s, 60 homesteads occupied what is now the refuge. The settlers cleared timber and attempted to farm the dry rocky uplands. When this failed they drained the lowlands and planted livestock forage species, including *P. arundinacea* [39].

*Phalaris arundinacea* was still a desirable species after the refuge was established in 1937. Three acres were planted with the species in 1945; however, the seed source was not noted [24]. Again in 1970, *P. arundinacea* was planted on several acres, but neither the exact location, nor the seed sources were recorded. By the 1990’s, views of the species had changed, and its aggressive
behavior was considered a threat to biodiversity. In 1991, *P. arundinacea* was targeted for control. Between 1991 and 1998, a series of control practices were implemented. Another site was flooded repeatedly along with use of an aquatic formulation of glyphosate, mowing and tilling. This treatment combination proved to be successful for several years. In 2001, after mowing and disk ing, a third site was seeded with meadow foxtail (*Alopecurus pratensis*) and redtop (*Agrostis gigantea*), however neither species took hold and *P. arundinacea* regained dominance the following spring.

The species’ ability to reproduce sexually or vegetatively makes control difficult. Numerous studies throughout the United States, but primarily in the east found that *P. arundinacea* spreads primarily through rhizomes [10] [22]. The relatively dense continuous stands at Turnbull National Wildlife Refuge suggest a clonal origin, however, our results show that on a population scale, reproduction is primarily sexual (162 of 166 plants sampled were genetically unique). Five distinct population groups are evident and the genetic variation within populations (89%) greatly exceeds variation between populations (11%), suggesting that the species may have significant genetic resources to adapt to future control efforts and environmental changes and retain dominance of these plant communities. This supports the hybrid hypothesis as a possible explanation for the invasiveness of *P. arundinacea* at the refuge and perhaps numerous sites with similar history across Northwestern North America. A comparison of current genotypes at the refuge with known cultivars might determine which cultivars had been seeded and provide insight into their effect on producing invasive populations through hybridization. Such a comparison might also help clarify questions regarding the nativity of the species in this area.
LITERATURE CITED


Table 1. Summary of sample size (N), range of population (K) estimates and p value from AMOVA using GenAIEx [34] for data from plants on each transect, transect pair, and all transects combined from Turnbull National Wildlife Refuge, WA.

<table>
<thead>
<tr>
<th>Transect</th>
<th>N</th>
<th>K</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>43</td>
<td>3 or 4</td>
<td>0.02</td>
</tr>
<tr>
<td>B</td>
<td>44</td>
<td>1</td>
<td>Na</td>
</tr>
<tr>
<td>A&amp;B</td>
<td>87</td>
<td>5 or 6</td>
<td>0.01</td>
</tr>
<tr>
<td>C</td>
<td>40</td>
<td>3 or 4</td>
<td>0.64</td>
</tr>
<tr>
<td>D</td>
<td>39</td>
<td>4</td>
<td>0.16</td>
</tr>
<tr>
<td>C&amp;D</td>
<td>79</td>
<td>4 or 5</td>
<td>0.10</td>
</tr>
<tr>
<td>A, B, C and D</td>
<td>166</td>
<td>5</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 2. Analysis of molecular variance output from GenAIEx [34] showing both among and within group variance for Phalaris arundinacea plants on transects A, B, C and D combined from Turnbull National Wildlife Refuge, WA.

<table>
<thead>
<tr>
<th>Source</th>
<th>N</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>Est. Var.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSTOT</td>
<td>32.066</td>
<td>1038.946</td>
<td>165</td>
<td>6.450</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>Pop</td>
<td>124.960</td>
<td>94.960</td>
<td>311.719</td>
<td>176.846</td>
<td>219.030</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>25</td>
<td>25</td>
<td>57</td>
<td>26</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>SSWP</td>
<td>124.960</td>
<td>94.960</td>
<td>311.719</td>
<td>176.846</td>
<td>219.030</td>
<td></td>
</tr>
</tbody>
</table>

Summary AMOVA Table

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>SS</th>
<th>MS</th>
<th>Est. Var.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among Pops</td>
<td>4</td>
<td>111.430</td>
<td>27.858</td>
<td>0.689</td>
<td>11%</td>
</tr>
<tr>
<td>Within Pops</td>
<td>161</td>
<td>927.516</td>
<td>5.761</td>
<td>5.761</td>
<td>89%</td>
</tr>
<tr>
<td>Total</td>
<td>165</td>
<td>1038.946</td>
<td>6.450</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>Stat</td>
<td>Value</td>
<td>P(rand &gt;= data)</td>
<td>0.107</td>
<td>0.010</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Summary of analyses, programs used, estimated populations (K) and size of each population for *Phalaris arundinacea* plants from transects A, B, C and D combined, from Turnbull National Wildlife Refuge, WA. Totals may vary slightly depending on how program treats genetically identical plants. * = Program does not designate.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>K</th>
<th>Size of each group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster Analysis (NTSYSpc, STRUCTURE)</td>
<td>5</td>
<td>37, 27, 45, 36, 18</td>
</tr>
<tr>
<td>PCoA-2-D (NTSYSpc)</td>
<td>5</td>
<td>*</td>
</tr>
<tr>
<td>PCoA-3-D (NTSYSpc)</td>
<td>5</td>
<td>*</td>
</tr>
<tr>
<td>Posterior Probability (STRUCTURE)</td>
<td>5</td>
<td>*</td>
</tr>
<tr>
<td>dK (Structure)</td>
<td>5</td>
<td>*</td>
</tr>
<tr>
<td>Q-Distribution (STRUCTURE)</td>
<td>5</td>
<td>38, 50, 23, 23,32</td>
</tr>
<tr>
<td>Q-Data (STRUCTURE)</td>
<td>5</td>
<td>38, 50, 23, 23,32</td>
</tr>
<tr>
<td>Prevosti’s Distance (NTSYSpc)</td>
<td>5</td>
<td>*</td>
</tr>
<tr>
<td>AMOVA (GenAlEx)</td>
<td>5</td>
<td>25, 25, 57, 26, 33</td>
</tr>
</tbody>
</table>
Figure 1. Turnbull National Wildlife Refuge showing sampling scheme (transects A, B, C and D).
Figure 2. Cluster diagram adjoining admixture of five populations of *P. arundinacea* at Turnbull National Wildlife Refuge.
Figure 3. Three Dimensional PCoA for all transects (A, B, C and D) combined.
Figure 4. Probability of populations (A) and change in populations (B) (Evanno et al. [31]).
Figure 5. The Q distribution of populations one through five.
Figure 6. All plants on long transects (C and D) combined mapped by population.
Figure 7. Map of plants in population one.
Figure 8. Map of plants in population two.
Figure 9. Map of plants in population three.
Figure 10. Map of plants in population four.
Figure 11. Map of plants in population five.
CHAPTER 3

USING PLOIDY IN SEARCH OF CULTIVARS IN A NATURALIZED POPULATION OF REED CANARYGRASS (*Phalaris arundinacea*)

ABSTRACT

*Phalaris arundinacea* invasions are a significant threat to native biodiversity in wetlands and riparian areas in the interior Pacific Northwestern United States. One recognized source of invasive characteristics is multiple introductions of a species. We recently found five distinct populations of this species at Turnbull National Wildlife Refuge in Eastern Washington State and hypothesized that this could reflect past introductions of cultivars whose selection for positive forage characteristics could contribute to invasiveness in natural settings. Because some popular cultivars are hexaploid, but plants in more northern environments are usually tetraploid, the presence of hexaploid plants could confirm this hypothesis. In July 2013, 24 random samples of *P. arundinacea* rhizomes were collected from three areas of the Refuge including one known to have been seeded with *P. arundinacea* in 1945 and 1946. Rhizomes were grown into plants whose leaves were sampled and analyzed by flow cytometry. All the plants were tetraploid. This does not rule out past introduction of tetraploid cultivars, but no evidence of introduction or survival of hexaploid cultivars was found. According to many researchers, tetraploid plants are most common at northern latitudes and hexaploids more common at warmer mid-latitudes. Our results are consistent with this assertion.
INTRODUCTION

*Phalaris arundinacea* is a rhizomatous perennial grass that can reach three to six feet in height. It spreads by seeds and creeping rhizomes, and also produces roots and shoots from the nodes of severed culms (Marten and Heath, 1973). In the Pacific Northwest, reed canarygrass is invasive, outcompeting many native plant species and reducing biodiversity in critical wetland and riparian habitats Lavergne and Molofsky 2004; Ruchert 2004).

We recently completed a landscape level analysis of the genetic variability of reed canarygrass at Turnbull National Wildlife Refuge in southeastern Washington State (Canwell, Hardesty and Kisha, Unpublished Data). This area is typical of many inland Northwest sites where the grass is invasive. We found five genetically distinct populations within the 7372 Ha refuge. Despite the appearance of dense clonal stands, only 5% of plants tested were of clonal origin. All of the rest arose from seed. This result led to questions about the source of this genetic variability.

There are historical reasons why we might expect to find genetic variability in reed canarygrass (*Phalaris arundinacea*) populations at Turnbull. The Refuge was created in 1937 from many small farms dating from European settlement. *Phalaris arundinacea* may have been native to the site and/or may have been introduced by settlers (Merigliano and Lesica 1998). The Refuge has kept a series of narrative records since 1937. The first mentions of reed canarygrass in the narratives were May, June and July of 1940-1942, noting the location of many duck species in ponds surrounded by the species (TNWR Narratives, 1938-2012). In 1945 and 1946, three acres were planted with reed canarygrass each year in the area of Lower Pine Creek. No record was made of the method of planting or source of plant material. For almost two decades, there is no further record of the species except mention of it being harvested from the mid-1950’s through the early 1960’s.
In 1969 and 1970, 90 and 76 tons, respectively, of reed canarygrass hay were harvested from several areas of the Refuge. It is presumed that cattle-grazing was also occurring on the refuge during this period, as was common on refuges at the time. In 1969 a burning slash pile ignited a reed canarygrass meadow, burning 12 acres before being contained. The following year refuge staff planted 10 acres with reed canarygrass. The exact location and source material were not recorded (TNWR Narratives, 1938-2012). In 1972, a cattle grazing study revealed that grazed areas had 40% more sedges and rushes than the area where reed canarygrass was fenced off and left ungrazed. William Bennington (1972) noted in his thesis that cattle prefer reed canarygrass and that it is excellent for erosion control and the reclamation of low lying poorly drained soils.

It was not until 1988 that reed canarygrass was mentioned as a concern as it was encroaching into drying wetland basins during a drought. From that point, the species came to be seen as a major constraint to reaching refuge goals, and was selected as a target species for control for the 1991 management plan. In line with revised USDI Fish and Wildlife Service policy, cattle grazing on the refuge was terminated in 1993. This change in wetland and moist soil management likely exacerbated problems with reed canarygrass dominance. In 2004, a technical report on invasive plant species affecting the Refuge reported that over 75% of the refuge is now “infested” with invasive species and reed canarygrass was listed as the most problematic of the invasive species (Ruchert 2004).

We know that several introductions of *P. arundinacea* occurred on the refuge. There were at least three cultivars of the species available in 1945-6, and another seven or eight by 1970, the time of the last known planting (Table 1). Cultivars have been developed to improve perceived agronomic weaknesses of the species: seed production and viability have been improved in
cultivars, potentially contributing characteristics that could make the species more competitive in a natural environment.

Grasses are capable of accumulating genetic material, reflected as increased numbers of chromosomes, referred to as ploidy. *Phalaris arundinacea* is considered diploid (2n) (Østrem, 2008); however, it can occur as a polyploid: 4n or 6n (Anderson, 1961, and Beest *et al.*, 2012).

Ploidy can assist in tracing the history of the species across landscapes. Elberson and Johnson (USDA-ARS 2013) analyzed the ploidy of all of the accessions of *P. arundinacea* in the USDA ARS Western Plant Introduction Center collection. They found that while most were tetraploids, the cultivars Superior, Auburn and Arkansas Upland were hexaploid. Of these hexaploids, Superior was available as early as 1930, while the latter two were available by 1970 (Table 1). Hexaploids crossed with tetraploids produce sterile seed, so the presence of hexaploids should still be detectable. This fact, coupled with our analysis of the genetic characteristics of the Turnbull population suggested that we might be able to determine if hexaploid cultivars have contributed to the invasiveness of the species at Turnbull.

**OBJECTIVES**

1. Determine the ploidy level of a representative sample of reed canarygrass plants from Turnbull National Wildlife Refuge.

2. Evaluate ploidy results for indications of previous introduction of hexaploid cultivars to the Refuge.

**METHODS AND MATERIALS**

In July 2013, random samples of rhizomes from *Phalaris arundinacea* were obtained from three
areas in the refuge. Lower Pine Creek, the only known site of prior planting was sampled along with two long transects spanning the length and breadth of the refuge. As each rhizome was collected, it was placed in a wet paper towel in an envelope labeled by location, then in a cooler for transport to Washington State University in Pullman, Washington. Individual rhizomes were placed in potting soil and grown for approximately 2 weeks in a greenhouse.

Two centimeters of leaf tissue was collected from each plant for ploidy analysis. Leaf tissue was chopped with a razor blade for 0.5 to 1 minute in extraction buffer, incubated 1.5 to 2 minutes, filtered through 30 um mesh, stained and incubated in the dark for 1 minute before analysis. Ploidy was measured with a Partec CyFlow Ploidy Analyzer 'DAPI' using Partec CyStain UV P extraction buffer and staining buffer.

**RESULTS AND DISCUSSION**

All of the samples were tetraploid (Tables 2 and 3). If hexaploid seed had been planted at TNWR, there is no evidence that it survived or established itself on the site. This does not preclude use of one of the tetraploid cultivars available when Refuge staff planted the species.

Reed canarygrass is found worldwide, and climate seems to correlate with ploidy level. McWilliam and Neal-Smith (1962) reported that the tetraploid version of reed canarygrass was more abundant in Europe and Asia with moderate temperature regimes. In 2004 Lavergne and Molofsky reported that all the plants they sampled from Europe and the United States were tetraploid. In further studies by Lavergne and Molofsky (2004), they concluded that tetraploids are found in cooler environments as they do go through a dormancy phase. Johnson and Evans (2014) also concluded that the tetraploid form of *Phalaris arundinacea* is widespread in northern locations with cooler seasons while the hexaploid form was more prevalent in
temperate mid-latitude locations. Our results are consistent with this conclusion.

The next step in evaluating the possible role of cultivars in the invasiveness of this population would be to compare the genetic characteristics of each of the five populations found at the Refuge with those of tetraploid cultivars available when planting occurred there.

ACKNOWLEDGEMENTS

Thanks to Lisa Taylor for assistance in the field, taking samples to the ARS-USDA lab in Pullman and running the Cyflow Partek unit. Gratitude also to Dr. Jinguo Hu for use of the cytometry equipment and Mike Rule for use of the Refuge as well as knowledge of the manuscripts dating back to 1937.
LITERATURE CITED


Table 4. *Phalaris arundinacea* cultivars that may have been available for planting at Turnbull National Wildlife Refuge, Cheney, WA from 1945 to 1970. Shaded lines represent hexaploid cultivars. Ploidy from USDA ARS GRIN database, or otherwise unavailable (*). Year of release from accession notes in USDA ARS GRIN database unless otherwise noted.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Release year</th>
<th>Source</th>
<th>Ploidy</th>
<th>Reference for year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superior</td>
<td>@ 1930</td>
<td>Oregon</td>
<td>hexaploid</td>
<td>Carlson et al. 1996</td>
</tr>
<tr>
<td>Iowa Phalaris</td>
<td>1930</td>
<td>Iowa</td>
<td>*</td>
<td>Carlson et al. 1996</td>
</tr>
<tr>
<td>Ioreed</td>
<td>1946</td>
<td>Iowa</td>
<td>tetraploid</td>
<td>Carlson et al. 1996</td>
</tr>
<tr>
<td>Auburn</td>
<td>1952</td>
<td>Alabama</td>
<td>hexaploid</td>
<td>Carlson et al. 1996</td>
</tr>
<tr>
<td>Flare</td>
<td>1959</td>
<td>Iowa</td>
<td>*</td>
<td>Carlson et al. 1996</td>
</tr>
<tr>
<td>Arkansas Upland</td>
<td>1962</td>
<td>Wisconsin</td>
<td>hexaploid</td>
<td>Carlson et al. 1996</td>
</tr>
<tr>
<td>syn 4 Ioreed</td>
<td>1962</td>
<td>Wisconsin</td>
<td>tetraploid</td>
<td></td>
</tr>
<tr>
<td>Ames 85</td>
<td>1962</td>
<td>California</td>
<td>tetraploid</td>
<td></td>
</tr>
<tr>
<td>Rise</td>
<td>1965</td>
<td>Iowa</td>
<td>tetraploid</td>
<td>Carlson et al. 1996</td>
</tr>
<tr>
<td>ML 4694 Ioreed</td>
<td>1968</td>
<td>Missouri</td>
<td>tetraploid</td>
<td></td>
</tr>
<tr>
<td>Grove</td>
<td>1970</td>
<td>Ontario</td>
<td>tetraploid</td>
<td></td>
</tr>
<tr>
<td>NCRC1</td>
<td>1973</td>
<td>Minnesota</td>
<td>tetraploid</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Ploidy as determined by line numbers from flow cytometry. * no triploid is known.

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>n</th>
<th>lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>2n</td>
<td>50</td>
</tr>
<tr>
<td>Triploid</td>
<td>3n</td>
<td>*</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>4n</td>
<td>100</td>
</tr>
<tr>
<td>Pentaploid</td>
<td>5n</td>
<td>*</td>
</tr>
<tr>
<td>Hexaploid</td>
<td>6n</td>
<td>150</td>
</tr>
<tr>
<td>Octoploid</td>
<td>8n</td>
<td>200</td>
</tr>
<tr>
<td>Decaploid</td>
<td>10n</td>
<td>250</td>
</tr>
</tbody>
</table>
Table 6. Results of ploidy analysis of *Phalaris arundinacea* from Turnbull National Wildlife Refuge, Cheney, WA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean Lines</th>
<th>CV</th>
<th>Particles</th>
<th>Ploidy</th>
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</thead>
<tbody>
<tr>
<td>D1</td>
<td>93.25</td>
<td>4.8</td>
<td>2832</td>
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</tr>
<tr>
<td>D2</td>
<td>92.36</td>
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<td>D5</td>
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<tr>
<td>D6</td>
<td>81.95</td>
<td>10.2</td>
<td>9708</td>
<td>tetraploid</td>
</tr>
<tr>
<td>D7</td>
<td>93.54</td>
<td>11.2</td>
<td>4931</td>
<td>tetraploid</td>
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<tr>
<td>C1</td>
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<tr>
<td>C2</td>
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<td>8.7</td>
<td>1565</td>
<td>tetraploid</td>
</tr>
<tr>
<td>C3</td>
<td>95.14</td>
<td>9.3</td>
<td>3810</td>
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<td>C4</td>
<td>94.17</td>
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<td>tetraploid</td>
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<td>85.58</td>
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<td>C7</td>
<td>88.09</td>
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<td>4134</td>
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<td>11</td>
<td>4865</td>
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<td>PC2</td>
<td>81.32</td>
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<td>4463</td>
<td>tetraploid</td>
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<tr>
<td>PC3</td>
<td>83.26</td>
<td>11.3</td>
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<tr>
<td>PC4</td>
<td>91.01</td>
<td>9.3</td>
<td>3806</td>
<td>tetraploid</td>
</tr>
<tr>
<td>PC5</td>
<td>81.00</td>
<td>9.8</td>
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<td>PC6</td>
<td>80.49</td>
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</tr>
<tr>
<td>PC7</td>
<td>79.41</td>
<td>14.4</td>
<td>15995</td>
<td>tetraploid</td>
</tr>
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</table>
CHAPTER 4

SYNTHESIS AND MANAGEMENT IMPLICATIONS

Given the extreme density of the *Phalaris arundinacea* stands located on the Refuge, we had hypothesized that there was a single population, perpetuated by vegetative means. We found however that all four transects sampled represented five distinct populations. Further, 162 of the 166 plants analyzed were genetically distinct from each other: only four plants arose vegetatively. Sexual reproduction is the dominant form of reproduction for the species at the Refuge. This offers maximal opportunities for adaptation to particular microsites and other ecological challenges. But how did this variability arise? Among the possibilities were historical introductions by early European settlers who farmed the area before the refuge was created. Historical records from the refuge also report several times when the species was planted when it was considered a desirable forage plant for cattle that grazed on the Refuge. There have been cultivars of the species available since the 1930s and seed from cultivars could have been planted. Those cultivars included both tetraploids and hexaploids. It was possible that some of the genetic variability could be from hexaploid plants. Because the more common tetraploid plants do not interbreed with hexaploids, hexaploids might still be present if they were planted and survived in the area. However, we found no hexaploid plants. The origin of the current genetic variability among the reed canarygrass populations on the Refuge remains unknown. The earlier hypotheses remain untested. Comparing the genetic characteristics of cultivars available at the time the species was planted on the refuge might identify tetraploid cultivars as predecessors of some of the existing populations. But decades of sexual reproduction may have developed plants that have adapted sufficiently to the site that they no longer closely resemble their predecessors.
Perhaps the more important question is what are the management implications of knowing that multiple populations exist and mix sexually? First, this provides genetic material for continuing adaptation to both current and future conditions on the Refuge, which is not good news from a species control perspective. This also implies that we need to be as concerned about seed distribution as we have been about vegetative propagules being transported to uninvaded sites. We need to know what the soil seed bank of the species looks like at the Refuge to determine if efforts to reduce seed production could limit future genetic adaptation of the species. If seed is short-lived in the soil, then practices such as mowing that reduce seed production might be useful over the long term. It is difficult to imagine how a *P. arundinacea* (or any other species) seedling could become established in the typically dense existing stands of the species. It may be that only disturbance allows this opportunity, thus it would be interesting to know how old existing plants are. Perhaps the current five populations do not represent the full genetic diversity of the seed bank, but rather are elderly relics of earlier disturbances. A last significant implication is that introduction of further genetic diversity should be avoided. For example, movement of seed or rhizome contaminated heavy equipment, water or soil could introduce genetic material from other sites that might differ from existing populations and generate further genetic diversity.