POPULATION GENETICS OF THE WESTERN PRAIRIE FRINGED ORCHID

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The Population Genetics of the Western Prairie Fringed Orchid

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ABSTRACT

The Western Prairie Fringed Orchid (WPFO) *Platanthera praeclara* is listed as a federally threatened species in the United States. The habitat of the WPFO is highly fragmented, causing the possibility of genetic isolation among populations. I assessed microsatellite polymorphism in eight WPFO populations using six polymorphic microsatellite markers. High levels of within population genetic diversity were found: 12.5 alleles per locus and mean expected heterozygosity (H_E) of 0.622. The genetic structure among the populations was weak; the overall F_{ST} value was 0.0692. A mantel test showed no correlation between genetic and geographic distance r = 0.2793 (P = 0.31). The genetic diversity and genetic structure is comparable to other plant species. Significant inbreeding many be occurring as indicated by a F_{IS} value of 0.1903. More studies with the developed markers are necessary over a larger part of the geographic range of WPFOs to determine if other populations are genetically isolated.

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CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

The western prairie fringed orchid, hereafter WPFO, *Platanthera praeclara* was first described as a distinct species by Sheviak and Bowles (1986). WPFO is native to the tall grass prairie region of the United States and Canada, the majority of which has been converted to agricultural land (Bowles and Duxbury 1986). As a result of habitat destruction WPFO was listed as a threatened species under the Endangered Species Act in 1989 in the United States. The remaining habitat is fragmented, resulting in small populations that are often a great distance from each other, because of this, these populations may be genetically isolated and may be at risk for inbreeding depression and loss of genetic diversity. An understanding of WPFO population genetics can inform management on effective practices to ensure the survival of WPFO.

WPFO has been the subject of numerous studies for decades, which have evaluated several aspects of its biology. My goal is to review the available literature on the biology of WPFO. The review is composed of six sections: the first three sections summarize the biology of WPFO and actions taken to conserve it. The fourth section covers conservation practices specific to the populations I am studying in the ecoregional section 251A which are located in western Minnesota and eastern North Dakota. The fifth section details what is known about the life history of WPFO. The sixth summarizes the conservation genetics approach used for my research project.

1.2 WPFO Ecology and Distribution

WPFO was first described as a separate species from *Platanthera leucophaea* the Eastern Prairie Fringed Orchid (hereafter EPFO) in 1986 (Sheviak and Bowles 1986). WPFO is found

only west of the Mississippi River, while EPFO is found east and west of the Mississippi River. There is no known location where populations of WPFO and EPFO co-occur (US Fish and Wildlife 1996). WPFO and EPFO are very similar but can be distinguished using differences in flower size and flower structure, such as column shape, pollinia spacing, and fragrance (Sheviak and Bowles 1986).

As described in by Sheviak and Bowles (1986), WPFO is an erect, stout, perennial herb that grows to maximum approximately one meter tall. The plant usually has a single straight flowering stalk, with 2 to 5 elongated, lanceolate, sheathing, leaves and bearing from 5 to 25 showy, white flowers on a terminal inflorescence. The flowers are typically 2.5 cm wide and the lower petal is deeply lobed and fringed (Figure 1.1). The plant has several coarse fleshy roots emerging from a fleshy tuber. Blooms open in late June in the southern part of the range and late July in the northern part of their range (US Fish and Wildlife 1996).

Differences in pollinia location and column shape in the flowers most likely limits hybridization between WPFO and EPFO. These morphological differences result in differential placement of pollinia on pollinating insects (Sheviak and Bowles 1986). WPFO pollinia are deposited on the eyes of its pollinators. EPFO pollinia are placed on the proboscis of its pollinators. As a result, pollen placed on pollinators from one orchid species does not touch the stigma of the other orchid species, preventing a pollinator from crossing the two species (Sheviak and Bowles 1986). Thus, it is likely that pollen rarely if ever moves between the two species in the wild (Sheviak & Bowles 1986). Hawkmoths pollinate both species; one hawkmoth species, *Eumorpha achemon* has been identified as a pollinator in both species (Cuthrell 1994). Hybridization between EPFO and WPFO is apparently biologically possible, based on experimental evidence that, when the two species are crossed artificially, seed is produced

Figure 1.1. An example of a WPFO plant in bloom. Photo was taken by J. Challey, downloaded from the USDA Forest Service website.

(Sheviak & Bowles 1986). Populations of EPFO and WPFO both occur in Iowa, and may have occurred near each other historically (US Fish and Wildlife 1996). Currently the nearest WPFO and EPFO populations occur is approximately 26 km apart in Harrison County and Grundy County respectively in Missouri (McKenzie *et al.* 2012). The present amount of gene flow between these species is unknown. However, the two species have probably not diverged extensively based on a population genetics study using allozymes (Pleasants and Klier 1995). There was no discernible difference in allozyme allele frequencies between WPFO and EPFO.

Sheviak & Bowles (1986) stated that EPFO may have diverged from WPFO as its habitat is located in the prairie peninsula region. The prairie peninsula emerged adjacent to the tallgrass region now occupied by WPFO and EPFO approximately 8000 years ago (Nelson *et al*. 2006). EPFO's pollination system is less specific to pollinators than that of WPFO and that may present an advantage (Sheviak & Bowles 1986).

The flowers are typical of plants with a moth-pollinated syndrome: they are fragrant at night, produce relatively large amounts of sugar-rich nectar, and have the longest nectar spur of any orchid in North America (Faegri and van der Pijl 1971, Bowles 1983). Although other insect species may visit the flower of WPFO and even rob nectar, hawk moths are uniquely suited to access the deep nectar spur and effect pollination because of the specialized floral structures that require the removal and deposition of precisely placed pollinia (Sheviak & Bowles 1986, Cuthrell 1994).

The range of WPFO extends from southern Manitoba to northeastern Oklahoma. WPFO was documented in 81 counties in eight U.S. states prior to 1970. It is currently documented in 41 counties in 6 U.S. states (US Fish and Wildlife 1996, Figure 1.2). A single population of WPFO was first documented in Canada in 1984 (Catling and Brownell 1987 cited in Environment Canada 2006).

Prior to the conversion of land to agriculture, WPFO was reported to be widespread and occasionally found in large populations (Sheviak & Bowles 1986). However, as the majority of the WPFO habitat has since been converted to agriculture, populations of plants have been significantly reduced in size and number (Bowles 1983). Two phases occurred in this reduction.

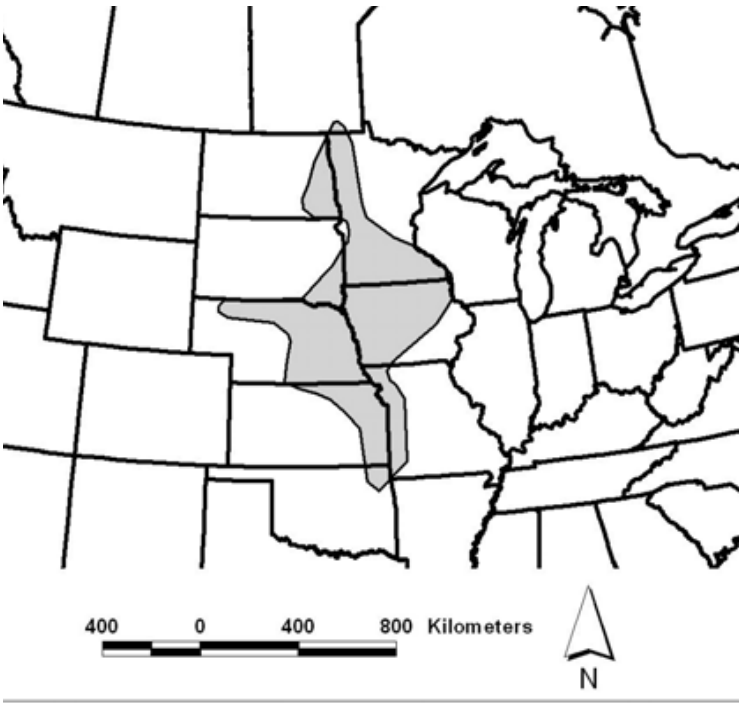

Figure 1.2. The Historical Range of WPFO in the United States and Canada; from the 2006 Canada Western Prairie Fringed Orchid Recovery Plan. This image was derived from Flora of North America Editorial Committee 2003.

The first phase was the initial conversion of prairie to cropland that occurred in the second half of the 19th century. The second continued into the 20th century as hayfields or pastureland were converted to cropland (Bowles and Duxbury 1986).

In the United States, the current WPFO range is located in 3 ecological provinces in 9 ecoregional sections (Figure 1.3). The ecological provinces are based on potential dominant vegetation, the ecoregional sections of each province are based on unique geology and soils (McNab *et al*. 2007). The population in Canada is located north of section 222N on land classified as a mid boreal uplands ecoregion in the boreal plains ecozone (Ecological Stratification Working Group 1995). In 1996 WPFO was recorded at approximately 175 distinct sites in the United States (US Fish and Wildlife 1996). The number of flowering plants at these

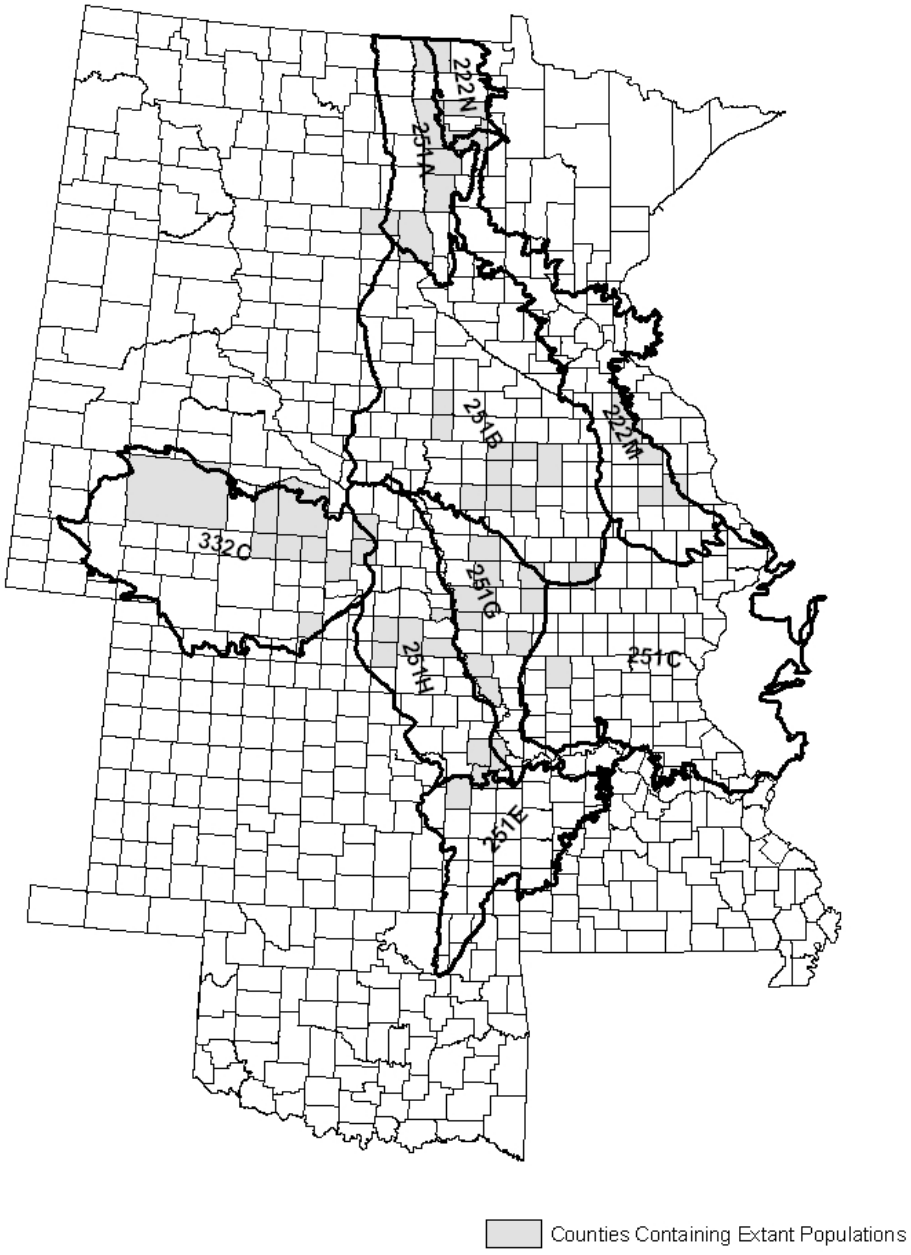

Figure 1.3. The Historical Range of WPFO in the United States separated into ecoregional sections, from the 2009 U.S. Fish and Wildlife 2009 report.

sites varied from one plant to thousands (US Fish and Wildlife 1996). It is possible more small populations exist; it is mentioned specifically by Armstrong *et al.* (1997) that more surveys are necessary in Nebraska. The population in Manitoba Canada is the largest extant population; the number of flowering individuals varied from 1818 in 1995 to 23,530 in 2003. The largest populations in the US occur in the northern part of its range (US Fish and Wildlife 2009). The 2009 US Fish and Wildlife report lists a total of 29,140 orchids in the United States; of these, 21,834 orchids are in the Red River Valley (251A) and Lake Agassiz-Aspen Parklands (222N) ecoregions (Figure 1.2).

The population sizes are based on surveys of flowering individuals (US Fish and Wildlife 2009, Environment Canada 2006). The population sizes in both the 1996 WPFO recovery plan and the 2009 WPFO 5 year update are based on the largest number of observed flowering individuals counted in a season (US Fish and Wildlife 1996, US Fish and Wildlife 2009). The absence of data on non-flowering plants is due to the difficulty of finding non-flowering plants thus annual censuses may not accurately reflect the true number of individuals. A survey of 5 sites in the Sheyenne National Grasslands used 16 belt transects, each 30–80 meters long and 10 meters wide. Over four seasons every visible plant was counted; 73% of present orchids were found to be non-flowering vegetative plants (Sieg and King 1995). In contrast Alexander (2006) found 3.4% of the 5,518 orchids observed were vegetative in her work in the Sheyenne Grasslands.

The amount of attention given to the orchid populations varies over its range, as several organizations are involved in surveying the populations. Populations in Minnesota for example, are surveyed annually whereas populations in Iowa are surveyed irregularly (Phil Delphey personal communication 2012). There may be some difference between the actual number of

WPFO populations listed in the 1996 Western Prairie Fringed Orchid recovery plan and the number of orchids listed 2009 Western Prairie Fringed Orchid Recovery Plan 5 year update.

Three metapopulations have been suggested in the northern part of the WPFOs range: the Sheyenne River Delta in North Dakota, the Vita Prairies in Manitoba, and the Pembina Trail Prairie complex in Minnesota (U.S. Fish and Wildlife 1996). These metapopulations are composed of dynamic groups of subpopulations totaling to at least 3000 individuals. Three other population complexes of subpopulations have also been suggested that are each composed of 100 to 1000 plants located on a 5 to 6 square mile area. The population complexes are located in Kittson County and Clay County Minnesota, and in Cherry County Nebraska (U.S. Fish and Wildlife 1996). The purported metapopulations may not reflect biological reality (Alexander 2006). Gene flow may be possible among them, via pollen movement or seed dispersal, but the level and relatedness among the populations is unknown.

Outside of the metapopulations and population complexes and over the vast majority the range of WPFOs' range, the remaining orchid populations all are either scattered and/or are composed of a small number of individuals (US Fish and Wildlife 1996). No single population in Kansas and Missouri, in the southern part of the range is composed of more than 50 individuals (US Fish and Wildlife 1996). Moreover, two sections, 251C and 251E, have only one known population each (U.S. Fish and Wildlife 1996). The southernmost populations in Oklahoma were first observed in 1979; these were documented until 1996 and may have represented a temporary colonization (Environment Canada 2006, Phil Delphey, personal communication 2010).

1.3 WPFO Life History

The growing season of WPFO starts in mid April in the southern part of its range and

late May in the northern part of its range. As a result, it blooms in mid June in the southern portion of the range and late July in the northern portion. A flowering individual will produce a 4.8 to 11.6 cm inflorescence with 5 to 25 flowers (Sheviak and Bowles 1986, US Fish and Wildlife Service 1996). Flower buds open first at the base of the inflorescence and then sequentially upward over a period of one to three weeks. Each flower remains open for approximately seven to ten days Over a 24 hour cycle, starting at dusk, individual flowers produce a sweet scent to attract pollinators (Bowles 1983, Sheviak and Bowles 1986). Because the flowers are both hermaphroditic and self-compatible they are capable of producing seed from self pollinations (Sheviak and Bowles 1986). However, in the absence of pollinator visitation they will not set seed (US Fish and Wildlife 1996).

The orchid is known to be visited by seven species of hawkmoth (US Fish and Wildlife Service 2009). WPFO appears to be highly co-evolved with its pollinators. To successfully pollinate WPFO a visitors' proboscis must be long enough to reach the nectar but not so long that it can reach the nectar without contacting the pollinia (Sheviak and Bowles 1986). The necessary length of proboscis is estimated to be 34-43 mm long by Sheviak and Bowles (1986). Westwood *et al.* (2011) estimated the proboscis must be 20-30 mm long to reach the top of the nectar. Pollinators must have a distance of 5.8-6.4 mm between the outer edges of their eyes to contact the pollinia in WPFO. Moths with longer proboscis and a smaller distance between their eyes may function as nectar thieves (Sheviak and Bowles 1986).

The pollination success rate can be highly variable. Fruit set can be used as a proxy to pollinator visitation (Fox 2008). In the Sheyenne Grassland the percentage of flowers that set fruit ranged from 9% to 20% in 1993 (Cuthrell 1994). In a study from 2004-2007 Fox (2008) found overall the number of plants with one or more seed capsules ranged from 47.6% (2007) to

80.9% (2005). This contrasts with Vita Prairie population in Canada where only 2.1% of stems produced at least a single seed capsule (Westwood and Borkowsky 2004). Low fruit set may be caused by asynchrony with pollinator activity and orchid flowering or a lack of pollinator species (Westwood and Borkowsky 2004).

Once flowers are pollinated they will produce an approximately 2.5 cm long seed capsule (US Fish and Wildlife Service 1996). These pods have been classified into various types based on shape and size such as plump, inflated, partially inflated, twisted and atrophied (Alexander et al 2010a). Alexander (2006) described a more specific classification of using pod diameter: 3.7 mm to 7.4 mm for "inflated," 2 mm to 3.7 mm for "partially inflated," and less than 2 mm as "atrophied". There is no correlation with seed production and pod size unless the seed pod is atrophied; atrophied capsules have no seeds. The average number of seeds reported per capsule has varied among research projects. Richardson *et al*. (1997), cited by Alexander (2006), reported a mean of 15,722 seeds per capsule, while Ericson *et al.* (2006) reported an average of 15,000 seeds per capsule, and Alexander (2006) reported an average 9,825 ± 4294 seeds per capsule. The percent of viable seed has been evaluated by Alexander (2006) on the basis that the seeds contain a distinct round hyaline embryo as 80%. Richardson *et al*. (1997); cited by Alexander 2006, placed the number at 53% and Ericson *et al.* (2006) at 13%.

The average number of capsules per plant has been evaluated by different projects. Pleasants and Moe (1993), as cited by Armstrong *et al.* (1997) estimated 7 flowers per plant with 20% setting seed. Another research project in that report found 1.14 capsules per plant in a group of control plants that were not manipulated (Armstrong *et al*. 1997). Alexander (2006) found an average of 11 seed capsules per plant. Herbivory by deer, cattle, and insects have all been shown to reduce the number of seed capsules per plant (Cuthrell 1994, Alexander 2006).

As in all orchids, the seeds are small and can be dispersed by wind or water (Arditti and Ghani 2000). They are composed of an embryo and seed coat (Sharma 2002). Alexander *et al*. (2010a) classified a large embryo size as 1.9 μm in length and 1.1 μm in width.

Seed germination and development into seedlings is likely another major limiting stage in the orchid life cycle. This was demonstrated further in a study of *in situ* germination. After one year *in situ*, Alexander *et al.* (2010b) found that only 0.4% of seeds developed into seedlings, which then have a approximately 25% survival rate (Armstrong *et al.* 1997). Based on an estimate of 9,825 seeds per capsule Alexander (2006) estimated each plant could theoretically produce 132 new plants each year. There has been no study of the average amount of seed production over a large portion of WPFO's range. It is possible that over subsequent years more seeds in Alexander's (2006) experiment could develop to seedling stage.

One limitation at the germination stage is the obligate relationship with mycorrhizal fungi (Bowles 1983). To develop, the seeds must be inoculated with a symbiotic fungus. Two genera of fungi, *Ceratorhiza* and *Epulorhiza* have been found to be associated with WPFO (Sharma 2002). Stratification and inoculation with fungus is important to germination in WPFO. The seeds can be germinated without the symbiotic fungus but the germination rate is far lower. In a laboratory setting, 31% of seeds germinated within 60 days when inoculated with fungus and stratified at 41^0 C (Sharma 2002).

After inoculation with fungus the seed forms a protocorm of undifferentiated tissue. After the protocorm phase a seedling is formed that is the first above ground stage with a single visible leaf (Sharma 2002). Sharma found that up to 10.6% of seeds would form protocorms if inoculated with two fungus strains after being vernalized for four months of 41^0 C. The lengths

of the seed, protocorm and seedling phases are not well understood. In Alexander's 2006 study, when seeds were buried for one year, 13,290 viable seeds resulted in 94 protocorms and 51 seedlings after one year.

After the seedling stage the plant will most likely be vegetative, no documented plants have been shown to flower in their first year (Alexander 2006). A long term study by Sather (2004) cited by Alexander (2006) recorded plants flowering only after their sixth year of growth.

After flowering the plant can be dormant, vegetative, or can flower again (Sieg and King 1995, Sieg and Wolken 1999). Approximately two months of growth in a season is necessary before WPFO can flower (Bowles 1983). The flowering can be affected by several factors such as precipitation and burning (US Fish and Wildlife Service 2009).

The primary reproduction of WPFO is claimed to be from seed; e.g. Armstrong *et al.* (1997) estimated that 99% or orchid reproduction is from seed. This is based on studies by Bowles (1983) and Bowles and Duxbury (1986). Vegetative reproduction is possible through the production of a new perennating bud and tuber that can form a new plant (Bowles 1983). When compared to other orchids that are mainly seed dependent or mainly dependent on vegetative reproduction WPFOs' life history appears to be similar to the mainly seed dependent orchids (Roberts 2003).

The lifespan of the orchid is difficult to determine due to its erratic flowering pattern and dormancy periods. Bowles (1983) determined WPFO has a long lifespan based on observations of orchids persisting under periods of intense haying or grazing that would have prevented seed set. Sather (2005) cited by Alexander (2006) determined the orchid can have a long life span of possibly 15 years based on surveys of visible orchids returning above ground. Research in the

Sheyenne Grassland has shown that a much shorter lifespan may be common. A project undertaken there from 1987 to 1994 used 16 belt transects in five study sites representing five management regimes. Ten flowering orchids were marked on each transect initially in 1987; in 1990 all individuals in the original transects were marked and recorded. Overall, 4 percent of the original orchids marked in 1987 were present in 1994 (Sieg and King 1995). This study determined that once an aboveground orchid was observed absent there was an 82-100% it would remain absent. This may indicate a short average lifespan of approximately three years in the Sheyenne Grassland.

In a similar study evaluating flooding impacts on WPFO, Sieg and Wolken (1999) monitored 66 orchids in 15 flooded swales in the 1993 growing season. The orchids selected were growing in 5 cm of water. Of twenty-three plants that survived the growing season, three plants, 13% reemerged in 1994. Ten sites where an orchid was found in 1993 but not in 1994 were excavated, and no evidence was found of an underground tuber at any of the ten sites. This indicates flooding most likely causes mortality rather than dormancy. This suggests that the conclusion of Sieg and King (1995) may be accurate that once an orchid is not visible it will most likely not reappear. The methods of surveying flowering individuals may be an accurate estimate of the number of individual orchids in the Sheyenne National Grassland as dormant plants appear to be uncommon. Both Sieg and King (1995) and Sieg and Woken (1999) indicated a short lifespan, however their work was limited to the Sheyenne National Grasslands. The orchids in both studies were affected by drought and flooding. More long term monitoring in other populations is necessary to determine if WPFO on average can have a longer lifespan.

1.4 WPFO Habitat

Western Prairie Fringed Orchid generally is found in late successional remnant tallgrass

prairie and is associated with wetlands. However, it can also colonize disturbed areas such as road ditches and abandoned fields and is found in several soil types (US Fish and Wildlife 1996).

There is difficulty in classifying WPFO habitat and its associated vegetation due to the seasonal variation in its habitat. Several researchers have attempted to describe and quantify its habitat. When associated with wetlands WPFO may be limited to a narrow band nearby that is variable, due to the necessity of high soil moisture content and lack of tolerance for flooding (Alexander 2006).

Several classifications of WPFO and associated vegetation exist (Alexander 2006). Differences in classifications may be due the seasonal and transitional nature of WPFO habitat and lack of classifying plants in close association with WPFO. A wide diversity of plants have been found in association with WPFO (Alexander 2006).

The 1996 Western Prairie Fringed Orchid recovery plan classified two general WPFO habitats. The first is lowland in wet mesic grasslands often classified as sedge meadows, usually dominated by wooly sedge (*Carex pellita*), northern reedgrass (*Calamagrostis inexpansa*), and baltic rush (*Juncus balticus*). Shrubs are also common in the northern part of the range. The second habitat type is in the wetter portions of tallgrass prairie, dominated by species such as big bluestem (*Andropogon gerardii*) and little bluestem (*Schizachyrium scoparium*).

Research by Woken *et al.* (2001) evaluated factors that determine the presence or absence of WPFO. Four factors were significantly correlated with the presence of WPFO: 1) the percent cover of Baltic Rush (*Jucus baliticus*), 2) the percent cover of hedge-nettle (*Stachys palustris*), 3) the soil surface moisture level in August and, 4) the level of soluble soil magnesium. Using a logistic regression model incorporating these factors, 84% of swales were correctly classified as

containing or not containing WPFO. This research, was limited to the Sheyenne National Grasslands and may not be representative of the entire range of WPFO.

The soil moisture level in the top 10 centimeters of soil is crucial as WPFO has shallow roots and the maximum rooting distance is approximately 16 centimeters (Woken *et al.* 2001, Woken 1995 cited by Alexander 2006). During droughts there are far fewer flowering individuals than during wet years either due to mortality or decreased propensity for flowering under relatively dry conditions (U.S. Fish and Wildlife 2009, Sieg and King 1995). Flowering individuals are more common in deeper swales during droughts and found at higher elevations during wet years (Woken 1995 cited by Alexander 2006, Sieg and King 1995).

Flooding is common in WPFO habitat and has also been shown to decrease the number of flowering orchids. Sieg and Woken (1999) concluded that flowering plants are better able to tolerate flooding than vegetative plants due to their height. Sieg and Woken (1999) also excavated ten flooded plant sites, where plants were observed in 1993, but not in 1994. No evidence of roots was found; suggesting that flooding may cause mortality.

Willson *et al.* (2006) concluded that the timing of precipitation has a significant effect on the number of flowering individuals. Greater precipitation in August is associated with a greater number of flowering orchids the following year, while October to March precipitation decreases flowering orchid numbers the following growing season

Hawk moth pollinators are necessary for WPFO reproduction, in the absence of pollinators WPFO will not set seed (US Fish and Wildlife 1996). Pollinators feed on other host plants during their larval stage (Tuttle 2007). For example the as wild cherry sphinx (*Sphinx drupiferarum*) requires *Prunus* species, such as chokecherry as host plants (Environment Canada

2006). In their adult stage, hawk moths use nectar from other plants as food sources (Fox 2008). Many plants may be necessary to maintain the pollinators of WPFO.

Periodic disturbances are necessary to maintain WPFO habitat. In tallgrass prairie the main historical disturbance regimes were seasonal burning, grazing by bison and flooding. Fire prevents encroachment by woody species and releases nutrients from litter accumulation (Bragg and Hulbert 1976, Seastedt and Ramundo 1990). Fire and grazing may create regeneration niches by removing competition for the orchids and stimulation of mycorrhizal fungi (Bowles 1983).

Current and future threats to orchid habitat include invasive species such as leafy spurge and reed canary grass, lack of management of prairie remnants, overgrazing, and indirect effects of adjacent land use such as water table reductions and pesticide drift (US Fish and Wildlife 2009, Environment Canada 2006).

1.5 WPFO Management

WPFO was listed as threatened in the US in 1989 and endangered in Canada in 2003 (US Fish and Wildlife 1996, Environment Canada 2006). In the US, state regulations specify that WPFO is endangered in Missouri and threatened in Iowa, Minnesota and Nebraska. North Dakota and Kansas have no additional protections. The individual populations are protected to varying degrees explained below (US Fish and Wildlife 2009).

The two current WPFO recovery plans identify key areas needing more effort in order to maintain this species (U.S. Fish and Wildlife Service 1996, Environment Canada 2006). The main areas are: 1) habitat maintenance, 2) public knowledge, 3) location of new potential habitat, and 4) the monitoring and research related to existing populations. Both recovery plans state research in population demographics and pollinators is necessary. The US report states the

importance of determining the rate of vegetative reproduction (US Fish and Wildlife Service 1996).

As stated in the WPFO recovery plan, the major management goal is the recovery of the species to a level at which it can be delisted. There are two major approaches to managing the remaining WPFO populations. The first is to protect current habitat from anthropogenic factors; specifically to place 90% of habitat with present orchid populations in each ecoregion at a 4-9 level of protection. The lowest levels of protection, 0-3 are either unprotected or involve voluntary agreements with land owners or managers. Level 4 protection is the habitat being a part of a lease license or management agreement. Protection Levels 5-9 involve further protection such as being part of a conservation easement, or public land designation. To this end, the number of orchids under level 4 or higher level of protection varies by ecoregion. Overall 83% of all orchids in the US are at a minimum level of level 4 protection (US Fish and Wildlife Service 2009). The second approach is to effectively manage the populations and their habitat to ensure persistence of the orchids. Management practices must maintain the spatial, successional, and hydrologic ranges that allow persistence of WPFO (U.S Fish and Wildlife 1996).

A second major goal is managing WPFO populations to ensure their persistence once they are protected from anthropogenic factors (US Fish and Wildlife 2009). It is often difficult to determine which management practices are the most effective in maintaining orchid populations. WPFO has many characteristics that make it difficult to manage and study, such as the erratic nature of its flowering and different life stages (Sieg 1997, Bowles 1983). Several variables affect the WPFO that have management implications. An understanding of all of the variables and their effects is necessary to effectively manage the remaining WPFO populations.

Managing a disturbance regime that maintains tall grass prairie is necessary. The three main management tools in native prairie are grazing, fire, and mowing. These practices can possibly kill or negatively impact WPFO populations depending on how they are carried out (US Fish and Wildlife 1996, US Fish and Wildlife 2009, Environment Canada 2006).

Fire has a unique relationship to WPFO. Fire maintains their prairie habitat by killing other plant types, thus favoring prairie grass species that regrow quickly after fire (Steuter and McPherson 1995). Fire also frees up nutrients and possibly stimulates mycorrhizal growth (Bowles 1983). WPFO is not specifically adapted to fire, and fire can kill the orchids (US Fish and Wildlife 2009). Often WPFO would have been found in areas that were periodically flooded; areas that may not have burned regularly (USDA Forest Service 2001). The timing of the burning is important, if it occurs in early spring it has little possibility of destroying WPFO. The most important time span for the orchids is May through September, from when the orchids emerge to when they set seed. If the burning takes place in this time, it will reduce the number or orchids and their reproduction by killing the orchids prior to producing seed (USDA Forest Service 2001). Historically, burning took place at all times of the year.

Grazing also maintains tallgrass prairie habitat and impacts the orchids as well. The grazing may create microhabitats suitable for the orchid, however intensive grazing has been shown to reduce the number of WPFO individuals and their reproductive output (Alexander 2006, Alexander *et al*. 2010c). Grazing has to be managed for timing and intensity to allow the orchids to complete their life cycle. Mowing has effects similar to grazing; it has to be practiced in a way that will not kill flowering WPFOs. Mowing may also spread the seeds of exotic cool season grasses (US Fish and Wildlife Service 2009).

There are many other variables that have to be managed for the WPFO specifically. Hydrology is important, and protecting the area from development may prevent wetlands from being drained, but adjacent activities, such as agriculture or road building can affect the water table and the soil moisture levels necessary for orchids (USDA Forest Service 2001). Pesticide drift from nearby agricultural, or weed control activities can affect WPFOs and their habitat (Cuthrell 1994, U.S. Fish and Wildlife Service 1996). For example, in 2010 in the Sheyenne Grasslands, 197 WPFO plants were killed by a 2,4-D Amine herbicide sprayed at roadside ditches by a private company (United States Attorney's Office, District of North Dakota, 2011).

Invasive species are a unique issue. Several invasive plants are present in WPFO habitat, such as leafy spurge (*Euphorbia esula*), musk thistle (*Carduus nutans*) and creeping foxtail (*Alopecurus arundinaceus*) (U.S. Fish and Wildlife Service 1996, Environment Canada 2006). These plants could impact WPFO populations by out competing them for resources. The practices used to control these species can also impact WPFOs. For example, the herbicide Imazapic has been shown to cause a decrease in flowering and seed production in orchids the season following treatment (Erickson *et al.* 2006, US Fish and Wildlife Service 2009)

Reproduction of the orchid is limited by low seed germination rate and low vegetative production (Alexander *et al*. 2010b, Armstrong *et al.* 1997). Populations may also be limited by low seed production (Westwood and Borkowsky 2004).

1.6 WPFO Management in Ecoregion 251A

The Northern section of the WPFO range in the US is region 251A. This is where the populations in my study are located. Region 251A contains two metapopulations, The Pembina Trail population and the Sheyenne Grassland population. It also contains two population

complexes composed of 100-1000 plants. There are several additional small populations in this ecoregion as well. This ecoregion has the largest number of remaining WPFOs in the US (US Fish And Wildlife Service 1996).

Due to its importance as a multi-use area and metapopulation status the Sheyenne Grassland is covered first. The management of the areas where the other six populations are located in will be covered as well; the data are not as complete or detailed when compared to Sheyenne Grassland.

The Sheyenne Grassland is managed by the US Forest Service and cover an area of 27,244 hectares. There are several vegetation types and habitats present (Sieg and Bjugstad 1992) in a patchwork of different land use types of private and public land. A variety of management practices have been used since the Sheyenne Grassland were established. The management practices were and continue to be, cattle grazing, mowing, noxious weed treatment, blowout stabilization, and burning (USDA Forest Service 2001).

To maintain the WPFO populations the management practices that maintain its habitat must be practiced in a way that minimizes damage to WPFO populations. The US Forest Service has developed a management plan with the stated goal of maintaining and expanding orchid populations as much as possible (USDA Forest Service 2001). The focus of the current management plan is the strategy and practices regarding the metapopulation concept of WPFO management. The Sheyenne Grassland management plan addresses the multiple use area and attempts to balance multiple uses.

The management plan designates core, satellite, and other WPFO populations. Core populations are designated by the number of individuals, their location, and distribution.

Satellite populations are more transitory, the orchids can disappear and be reestablished; they are located in areas peripheral to core populations. Additional populations that are not core or satellite are not managed in the same manner as core or satellite populations though efforts are made to protect them.

An example of management is: in core areas where 1/3 of the area where orchids are have been documented cannot be grazed from June 1st to September 15th. In satellite areas 1/10 of areas where orchids are found cannot be grazed. Research and monitoring are an important part of management and are given high priority. All of the management rules have exceptions for research projects and WPFO sites are monitored to determine appropriate management practices (USDA Forest Service 2001).

The Bluestem population is located in the Bluestem Prairie Scientific and Natural Area in Clay County Minnesota. The land is managed by the Nature Conservancy Bluestem Prairie Office. The site the orchids are located on was an intensely grazed hayfield up to 1985; it has been burned on an approximately four year rotation since. Invasive weed control has taken place using four types of herbicides: 2,4-D, Plateau, Milestone, and Roundup. The application is done with backpack sprayers and the workers try to avoid the orchids as much as possible. The nearby Hartke population is located on private land that is a hay field and is hayed annually (Rhett Johnson personal communication 2011).

The Syre population is located in Norman County Minnesota on easement land located adjacent to the Syre wildlife management area. It is managed by the Fergus Falls Minnesota DNR office. The easement was burned once since being established, the only other management

action has been the girdling of several aspen growing near the area where the orchids are found (Shelley Becklund personal communication 2011).

The Dalby population is located in a wildlife management area in Norman County Minnesota. The wildlife management area is managed by the Detroit Lakes Minnesota DNR office. The management of this area is less well documented than others. The last controlled burn was approximately 15 years ago. Brush cutting has occurred since this time (Tom Kucera personal communication 2011).

The Bicentennial population is located on the Bicentennial Scientific and Natural Area in Clay County Minnesota. It is managed by the Fergus Falls Minnesota DNR office. It was burned approximately every 5 years from 1987 to 2009. The burns are undertaken primarily in spring prior to May 15th to limit damage to orchids. Leafy spurge has been treated in the area of the orchid population with Tordon and Plateau herbicides. Other herbicides were used in the early 1980s (Shelley Becklund personal communication 2011).

The Ulen population is located on a wildlife management area in Clay County Minnesota. It is currently managed by Fergus Falls Minnesota DNR office. It was established in 1958 and was hayed prior to this. It is currently managed using fire; a total of 5 burns have taken place from 1984 to 2011 on intervals from 2 to 12 years. It was also recorded that large cottonwoods were cut in 2004. A management plan has been developed for the Detroit Lakes Wildlife Management District that has several similarities to the Sheyenne Grasslands management plan. For example, all burns must take place prior to May 15th or after September 25th (Doug Hedtke personal communication 2011).

The populations of WPFO in this study are all managed in some way. Seven of the eight populations are managed specifically for the orchid to persist.

1.7 Conservation Genetics

The modern field of conservation genetics was initiated in the 1970s. Frankel (1970, 1974) proposed that mitigating the loss of genetic diversity and evolutionary potential is a priority, and in particular the conservation of primitive crop varieties and wild species. Scientists in this field were concerned with better understanding how population and species viability was related to inbreeding depression (Frankel and Soulé 1981), minimum population size (Shaffer 1981) and mutation accumulation (Lynch *et al.* 1995, Frankham 2005).

One focus area within conservation genetics is determining what measures can sustain, the often small, remaining populations of organisms that have been impacted by human activity. In small populations reduced genetic variability and inbreeding are highly likely (Frankham *et al*. 2009). There are two major types of threats for species: deterministic and stochastic. Deterministic threats are over-exploitation, habitat destruction, and pollution. Stochastic threats are random events that can be environmental, demographic or genetic. A small population is more vulnerable to stochastic events (Shaffer 1981).

Controversy emerged over the relevance of genetics in the conservation of species. Lande (1988) argued that deterministic factors, such as habitat destruction, have a large effect on demographics and may drive populations to extinction before genetic factors will have much of an effect. This has become known as the Lande scenario (Frankham 2005). Another factor in the debate is the effectiveness of natural selection in purging deleterious alleles from reduced populations. It was argued that a restriction in population size can cause the purging of

deleterious alleles from populations (Lande 1988). While purging may eliminate lethal alleles, detrimental alleles that are not lethal can accumulate and result in a loss of fitness and increased extinction probability (Lynch *et al.* 1995). It has also been shown that mildly deleterious alleles can accumulate and become fixed due to genetic drift, thereby causing a reduction in fitness (Frankham 2005).

Significant research has been done on the effects of genetics in small populations since Lande's 1988 publication. Three major approaches have shown the effects of inbreeding and lack of genetic diversity in populations: computer models, lab experiments, and field observations (Frankham *et al*. 2009). It has been demonstrated that inbreeding significantly lowers fitness in wild populations. In 90% of species across 34 taxa, inbreeding depression was found to lower reproductive success regardless of phyletic group (Crnokrak and Roff 1999). The loss of fitness due to inbreeding depression significantly increases extinction risk. A study by Frankham (2005) demonstrated that estimated extinction times are significantly reduced in 20 modeled species. Another study has shown a significant drop in genetic diversity in most endangered species, indicating a correlation between loss of genetic diversity and threatened status (Spielman *et al*. 2004). The importance of genetic "health" for the existence of populations and species has been emphasized by studies of effective population size, inbreeding depression, and interactions of inbreeding depression with other stochastic factors (Frankham 2005).

The processes of inbreeding depression and loss of genetic diversity are closely related. Overall, inbreeding depression has been shown to have an immediate short term impact on viability and extinction risk of populations whereas loss of genetic diversity and evolutionary potential has a long term impact on populations that emerges during environmental change (Frankham 2005). These factors, combined with other stochastic events can result in an

extinction vortex in which a population declines reducing genetic diversity and further increasing inbreeding, leading to further decline in population (Gilpin and Soulé 1986).

The importance of understanding other factors such as, deterministic or stochastic threats, habitat requirements, and demographics are highly relevant to maintaining threatened species. Conservation of rare or endangered species requires a balanced approach to genetic and other factors ensure the persistence of a species **(**Allendorph and Luikart 2007).

There are several important reasons to understand the conservation genetics of wild and captive populations. Three roles of conservation genetics that apply to the current project as defined by Frankham *et al.* (2009) are detection of inbreeding, determining genetic diversity, and determining population structure. These roles of are central to conservation genetics as they apply to historically large populations which have been reduced to small populations and/or fragmented populations.

1.8 Conservation Genetics and WPFO

WPFO often occurs in small populations that are likely populated by close relatives and are often separated by tens to hundreds of kilometers. Moreover, WPFO is susceptible to inbreeding via geitonogamy do to multiple flowers being open on the same plant at one time (Bowles 1983). Despite being described as outcrossing by Bowles (1983) the flowers are self compatible (Sheviak and Bowles 1986). Therefore the possibility of inbreeding must be considered. A previous study in EPFO demonstrated a significant decrease in viable seed production from 77% to 16% when the plants were selfed (Wallace 2003). Inbreeding depression of this sort may also be important in WPFO; selfing and other types of inbreeding are more common in small isolated populations.

An important determinant of gene flow in WPFO is undoubtedly pollinator behavior. Specifics of pollinator behavior that affect inbreeding and geneflow, such as the rate at which specific pollinators engage in geitonogamy and the rate at which pollinators to cross closely spaced plants or travel among populations in not understood. A study of the orchid *Satyrium longicauda* found that pollinators are more likely to self-pollinate plants in small populations which increases the possibility of inbreeding (Johnson *et al.* 2009).

A limited number of orchid species have been evaluated for genetic measures of diversity, inbreeding, and genetic divergence. A review by Forrest *et al.* (2004) listed 76 studies in orchid 63 species. Of these studies 70 used allozyme markers. In a literature search I was able to find 6 studies that evaluated 9 orchid species using microsatellite loci (Gustafson 2000, Gustafson and Sjögren-Gulve 2002, Cozzolino *et al* 2003, Solvia and Widmer 2003, Mant *et al*. 2005, Swarts *et al*. 2009).

Two studies designed to examine genetic diversity within populations and genetic divergence among populations of WPFO used allozyme markers. The first study by Pleasants and Klier (1995) studied 14 populations of WPFO over the majority of its range and 7 populations of EPFO on a limited section of its range. The second study by Sharma (2002) examined eight populations in Minnesota. Both studies found no significant genetic structure or divergence among populations in WPFO. Nor did they find evidence of extensive inbreeding. The overall genetic diversity was similar to other orchid species (Pleasants and Klier 1995, Sharma 2002).

New DNA based molecular markers have allowed these population parameters to be measured more accurately (Swarts and Dixon 2009). Microsatellites are now a commonly used

marker in population genetics Microsatellites are useful because of their high polymorphism rate often allowing small populations to be evaluated (Allendorf and Luikart 2007). Microsatellite markers have been shown to uncover subtle population genetic patterns not evident from using allozymes (Hughes and Queller 1993).

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CHAPTER 2. ANALYSIS OF THE POPULATION GENETICS OF THE WESTERN PRAIRIE FRINGED ORCHID

2.1 Introduction

Effective management of endangered species to ensure their long term survival requires an understanding of the basic biology of the species (Soulé 1986). Endangered species are often at increased risk of extinction relative to common species due to inbreeding depression, loss of genetic diversity, and mutation accumulation (Frankham 2005). Thus, to better understand the biology of endangered species, and manage long term survival, it is necessary to assess the genetics and genetic health of individual populations. In plants, the genetic health of populations can depend on seed dispersal, pollen dispersal, pollination patterns, plant growth, lifespan, and genetic diversity (Avise 2004). The goal of my research is to explore the population genetic structure and genetic diversity characteristics of wild populations of a rare orchid species native to the Great Plains of North America: the Western Prairie Fringed Orchid (*Platanthera praeclara*) (Sheviak and Bowles 1986).

The Western Prairie Fringed Orchid (hereafter, WPFO) is a perennial herb native to tall grass prairie. The orchid has been classified under the Endangered Species Act as threatened in the United States and endangered in Canada (U.S. Fish and Wildlife Service 1996, Environment Canada 2006). The overwhelming majority of the pre-1900's WPFO habitat has been converted to agriculture and as a result populations have been significantly reduced; WPFO has disappeared from 75% of the counties where it was historically found (U.S. Fish and Wildlife Service 1996, Environment Canada 2006). Two phases occurred in this reduction. The first phase was the initial conversion of prairie to cropland. The second continues as hay or pastureland is converted to cropland (U.S. Fish and Wildlife Service 1996). Disturbances such as fire, grazing,

or mowing may be important in maintaining the habitat of WPFO; however, depending on how these are practiced they can negatively impact orchid populations (U.S. Fish and Wildlife Service 1996, Alexander *et al.*2010b). Other current threats to the remaining populations are invasive species, lack of management, and effects of pesticides used to kill other plants (U.S. Fish and Wildlife Service 2009, Environment Canada 2006).

Inbreeding due to either selfing or mating with close relatives is a possible in the WPFO. First, over the majority of its range WPFO occurs in small populations which are often a great distance from each other; for example, ecoregions 251C and 251E each have a single WPFO population (Figure 1) (US Fish and Wildlife 2009). This may limit gene flow among populations. As a consequence mating is probably more likely within than between populations. Individuals close to one another are likely to be from the same maternal plant. Second, the flowers are self compatible, and multiple flowers on an inflorescence are open simultaneously increasing the likelihood of geitonogamy (pollen transfer between flowers within a plant). A study of the orchid *Satyrium longicauda* found that pollinators are more likely to self pollinate plants in small populations which increases the possibility of inbreeding (Johnson *et al.* 2009). Therefore the possibility of inbreeding depression must be considered (Zimmerman 1988). A previous research project on Eastern Prairie Fringed Orchid (EPFO) demonstrated a decrease in viable seed production when the plants' flowers were selfed (Wallace 2003). Inbreeding depression of this sort may also be important in WPFO. The combination of small population sizes, reduced gene flow, and inbreeding represent a possible threat to this unique orchid species.

Two prior studies characterized the genetic diversity within populations of WPFO and examined divergence among populations using allozyme genetic markers. Pleasants and Klier (1995) examined 14 WPFO, and 7 EPFO populations over their entire range in the U.S. and

quantified genetic variation within and among populations using allozyme markers. In their study the majority of genetic variation (80%) was within, rather and among, populations suggesting a high level of gene flow between populations. Sharma's (2002) study of eight Minnesota populations using allozyme markers found no significant evidence for inbreeding and a positive relationship between population size and allelic richness. There was no evidence of genetic divergence among populations (Sharma 2002). Both of these studies suffer from the use of allozyme markers which provide only a coarse-grained estimate of genetic diversity. The type of molecular marker used has been shown to influence the amount of genetic diversity found in a species (Avise 2004).

In order to assess the genetic health and history of divergence among WPFO populations; I developed and used microsatellite markers to answer the following study questions:

1. What is the genetic structure and evidence for gene flow among eight local Western Prairie Fringed Orchid populations in Ecoregion 251A?
2. What is the genetic diversity of eight local Western Prairie Fringed Orchid populations in Ecoregion 251A?

2.2 Materials and Methods

2.2.1 The Study Species

WPFO generally occurs in poorly drained, mesic soils and often near wetlands. The flowering stalks of this plant grow from a basal rosette of leaves and can reach 12 to 34 inches in height. A single raceme can produce up to 24 white showy flowers (U.S. Fish and Wildlife Service 1996). Individual plants flower for approximately three weeks from late June to early July and are capable of flowering multiple years (Sieg and Wolken 1999, Bowles 1983). The flowers are typical of plants with a moth-pollinated syndrome: they are fragrant at night, produce

relatively large amounts of sugar rich nectar, and have the longest nectar spur of any orchid in North America (Bowles 1983, Fagri and van der Pijl 1971). Previous studies have established that WPFO is primarily pollinated by 7 species of nocturnal hawkmoth (US Fish and Wildlife 2009). Although other insect species may visit the flower of WPFO, the specialized floral structures require the removal and deposition of precisely placed pollinia which favors the morphology of hawkmoths that are uniquely suited to access the deep nectar spur and effect pollination (Sheviak and Bowles1986). The behavior of pollinators within and among WPFO populations is not well understood.

After pollination, seed pods fully mature in mid-September and release small dust-like seeds (Alexander *et al.* 2010a). In a growing season plants can grow from seed or from a rhizome. Previous investigators have determined that reproduction of WPFO is most likely, mainly from seed (Bowles 1983, Bowles and Duxbury 1986). It is believed that: 1) not all underground rhizomes give rise to shoots every year, 2) plants do not necessarily produce flowering stalks in a given year even if they produce leaves, and 3) the average lifespan of an individual plant is no more than eight years (Alexander 2006, Sieg 1997). However, all of these assertions require further study.

WPFO was first classified as a separate species from The Eastern Prairie Fringed Orchid (EPFO), *Platanthera leucophaea* in1986, EPFO is also classified as threatened (Sheviak & Bowles 1986, U.S. Fish and Wildlife Service 1999). WPFO is found only west of the Mississippi River, while EPFO is found east and west of the Mississippi River. WPFO and EPFO are very similar but can be distinguished on the basis of flower size (WPFO flowers are larger than EPFO flowers), flower structure (column shape and pollinia spacing) and fragrance (Sheviak & Bowles 1986). Hybridization is possible between EPFO and WPFO (Sheviak & Bowles 1986). However

cross-pollination between the species is not likely in wild populations because the two species have different floral morphologies preventing a common pollinator from cross pollinating the plants (Sheviak & Bowles 1986). Populations of EPFO and WPFO overlap in Iowa, but the amount of gene flow between these two species is unknown (Sheviak & Bowles 1986).

The range of WPFO as of 2011 extends from Manitoba to northeastern Oklahoma and includes six US states (U.S. Fish and Wildlife Service 2009). The majority of extant orchids are located in Red River of the North Ecoregion in eastern North Dakota and Western Minnesota (U.S. Fish and Wildlife Service 1996). WPFO populations in Manitoba were first recorded in 1987; these populations compose the largest extant metapopulation (Catling and Brownell 1987 cited in Environment Canada 2006). The southernmost populations in Oklahoma were first observed in 1979; these were documented until 1996 and may represent a temporary colonization (Phil Delphey, personal communication 2010). No known WPFO populations remain in South Dakota (U.S. Fish and Wildlife Service 1996).

2.2.2 Sampling and Populations

Eight populations of orchids ranging from 1 to 130 flowering individuals were sampled during July of 2009 for genetic analysis in (Table 2.1). I define a population as a recognizable group of plants that are closer to each other than to individuals in other populations. Distances among eight populations varied from 2.32 km to 117 km. Two populations: A Annex Ditch and Viking Ditch are located in eastern North Dakota and are part of the Sheyenne Grassland metapopulation. The populations are located in Ransom and Richland counties respectively (Figure 2.1). The remaining six populations are located in western Minnesota. Four of the Minnesota populations, Bicentennial, Hartke, Ulen, and Bluestem were located in Clay County. Syre and Dalby population are located in Norman County. The Minnesota populations are

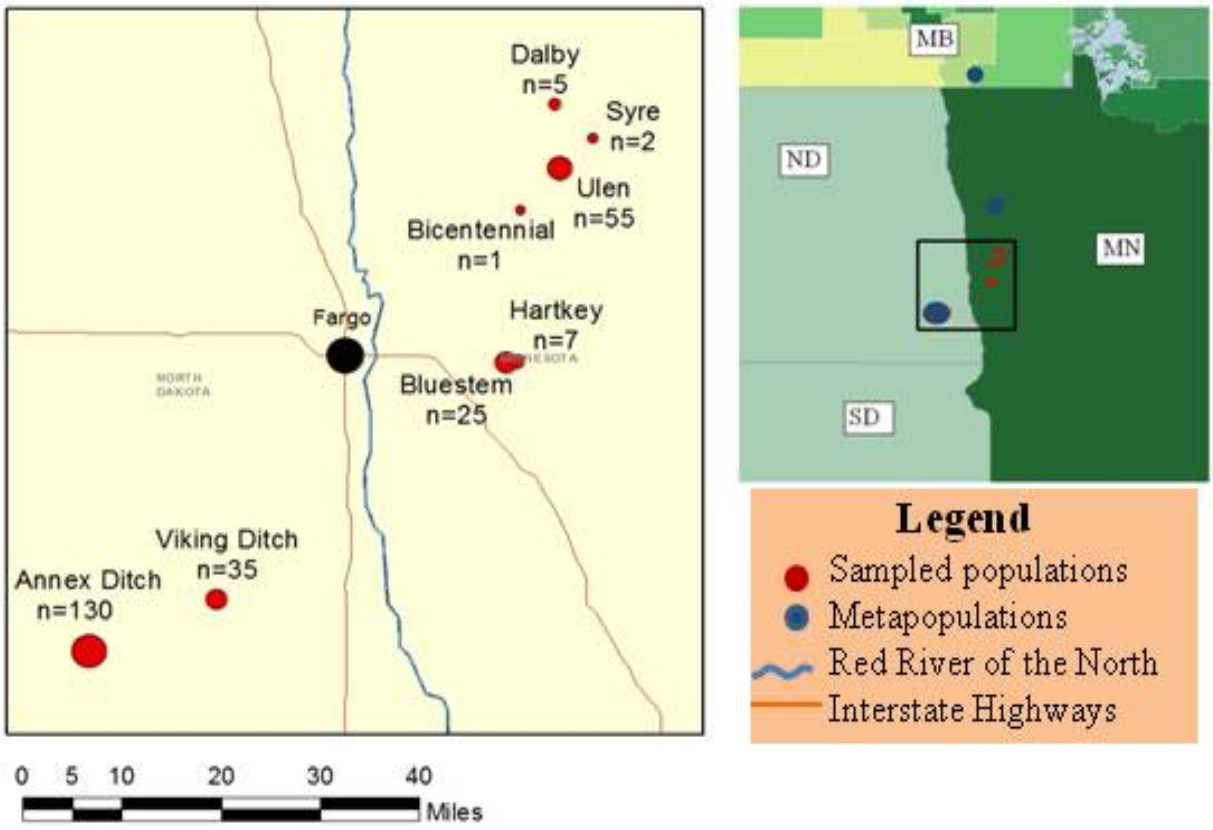

Figure 2.1. A map of the eight Western Prairie Fringed Orchid study populations. The size of red circles is proportional to the number of flowering individuals. The blue circles indicate the three extant conceptual metapopulations of WPFO. All populations are in ecoregion 251A designated in 2009 Western Prairie fringed orchid 5 year update.

located in remnant prairie patches left behind on the beach ridges of the Pleistocene Lake Agassiz. The populations were selected from a limited area to examine genetic diversity on a limited scale. The populations were sampled based on their presence and availability for sampling. Other populations were available in the Sheyenne Grassland; however we did not have permission to sample them.

At each population, the flowering plants were marked with metal tags placed approximately 2 inches north of the base of the plant with a roofing nail driven into the ground to allow the plants to be relocated in subsequent samplings. I also recorded the GPS coordinates of

each plant using a Magellan explorist 600 GPS unit (Carquefou Cedex, France), accurate to approximately 2 meters. An approximately 2 cm^2 leaf clip was taken from a healthy green leaf on each visible, flowering, individual plant. The leaf samples were immediately placed in microcentrifuge tubes, labeled, and frozen on dry ice until they were transferred to a -80^0C freezer at the end of the day.

2.2.3 DNA Extraction and Sample Genotyping

DNA extractions were carried out on all leaf samples from populations with less than 30 flowering individuals. In populations with greater than 30 individuals, 30 leaf samples were randomly selected using a random number generator (www.random.org) for extraction and analysis (Table 2.1). I used a Quiagen Dneasy Mini Kit (Qiagen Inc., Valencia CA) and approximately 5 mm^2 of each leaf sample. I ground each sample with liquid nitrogen in a microcenterfuge tube using a pellet pestle (Sigma Aldrich, St Louis MO) for approximately 30 seconds prior to the first step of the kit protocol. The final volume of extracted DNA solution was 100µl.

Table 2.1. The number of flowering individuals in each population in 2009 and the number sampled for genetic analysis.

Population	County	Approximate number of flowering individuals	Number sampled
A Annex ditch	Ransom	130	30
Viking ditch	Richland	35	30
Bluestem	Clay	25	25
Ulen	Clay	55	30
Bicentennial	Clay	1	1
Syre	Norman	2	2
Hartke	Clay	7	7
Dalby	Norman	5	5
Totals:		260	130

Microsatellite markers were used to evaluate the genetic diversity of the WPFO samples. Microsatellites are regions of the genome with sequence repeats that can vary enormously. As genetic markers they are very useful in conservation genetic studies due to their high rate of polymorphism (Allendorph and Luikart 2007). They have been used successfully in several population genetic studies in orchids (Swarts and Dixon 2009). A large amount of microsatellite polymorphism between populations has been observed even when there was little allozyme polymorphism (Hughes and Queller 1993).

The DNA template from each plant was used in PCR reactions to amplify microsatellite regions at six previously identified loci (Chapter 3) (Table 2.2). In order to fluorescently label the PCR products, I used a three primer "CAG tag" protocol (Oetting *et al.* 1995). One forward or reverse primer of each primer pair was tagged with a sequence complimentary to a binding site on a third primer that was 5' end labeled with a florescent tag primer (Table 2.2). Three labeled tags VIC (green), PET (red), NED (yellow), were used. Three loci 5, 7 and 17 were VIC labeled. Two loci, 2 and 27, were NED labeled. One locus, 2, was PET labeled.

A PCR reaction master mix consisted of 8.8µl of DD H_2O, 4µl 5X buffer, 1.2µl $MgCl_2$, 0.8µl dNTP, 1 µl of CAG-sequence primer at 0.5µM, 1µl of non CAG-sequence primer at 5µM, 1µl of fluorescently-labeled primer at 5µM (Integrated DNA Technologies), 0.2 µl of "Hotstart Gotaq" polymerase (VWR International) per reaction. I added this mixture to 2µl of template DNA for a final volume of 20µl. The microsatellite amplification was performed on an Eppendorph AG 22331 thermocycler using the following touchdown profile: initial denaturization of 94^0 for 2 min, 16 cycles of (1) a denaturization step at 94^0 C for 30 sec, (2) an annealing step starting at 65^0C for 30 seconds in the first cycle and decreasing 0.5^0 C each subsequent cycle, and (3) an elongation step of 72^0 C for 30 seconds. The initial 16 cycles were

Table 2.2. Summary of the six microsatellite loci used in *P. praeclara* populations.

Primer	Forward Sequence	Reverse Sequence	Repeat Type	Range In Base Pairs
2	ATGAGGGTCTTCACGCATGT	CAGTCGGGCGTCATCACCAC GGGATCTCCTTCCAAT	CT	177-205
5	TACCCGAGTTCCTTGCTGAC	CAGTCGGGCGTCATCACCTC TCGACAACAACCAGT	CT	204-220
7	ACCCTCGTAGATCGTTTCGG	CAGTCGGGCGTCATCAGTGG ATTTCGTGTGCCTT	AG	239-245
12	CAGTCGGGCGTCATCAGGTGC GGTCACTAACTTTGA	GGCGCAACCCACATTGATT	AC	246-382
17	GCATGTCTCAAGCTCTCACG	CAGTCGGGCGTCATCATCGC TCTCATTTCCACCG	GTTT	311-323
27	CAATGGTTGTGCTCTGAATGAC	CAGTCGGGCGTCATCACCGG TTCCAACAAAGTGC	GT	433-451
Fluorescently labeled primer: 5-CAGTCGGGCGTCATCA-3				

followed by 20 cycles of a denaturization step (1) of 94^0 C for 30 seconds, (2) an annealing step at 57^0 C for 30 seconds, an elongation step (3) of 72^0C for 30 seconds, with a final 4^0C hold.

I tested for successful amplification of each reaction by conducting agarose gel electrophoresis. Four µl of PCR product from each reaction was mixed with one µl of 5x loading dye and loaded in individual wells in a 2% agarose gel. The gel was run at 100 volts for 2 hours, the gel was stained in an approximately 10 mg/mL ethidium bromide solution and photographed under UV light in a Flurochem FC2 analyzer (Alpha Innotech) to verify a successful amplification.

PCR products were analyzed for fragment length using an Applied Biosystems 3730 analyzer at the Plant Microbe Genomics Facility at Ohio State University. I sent samples individually in separate wells to the Plant Microbe Genetics Facility for fragment analysis. The genomics facility provided me with the raw data in electropherogram format. I then used the software Peakscanner v1.0 (applied biosystems) to visualize the results.

Because *Platanthera praeclara* is a diploid plant, I visually assayed the electropherogram output of each plant sample to assign two allele fragment sizes to each microsatellite gene locus. If there were two distinct peaks the individual was determined to be a heterozygote, if there was only one distinct peak the individual was determined to be a homozygote. To accurately and consistently determine the allele sizes, rules were made and consistently followed for each locus (Appendix 1). To determine the consistency and accuracy of the results DNA samples from 55 individuals that were initially scored as homozygotes one or more of loci 7, 17 and 27 were amplified and analyzed twice.

2.2.3 Estimating Genetic Diversity

The data were examined using Microchecker 2.2.3 to investigate the presence of errors in

the data set and to determine the presence of null alleles or stuttering (Oosterhout *et al*. 2004). I calculated population-specific and locus-specific summary statistics of genetic diversity: observed number of alleles per locus and alleles per population (N_A), the number of effective alleles (N_E) per population and locus, number of private alleles (A_P), frequency of rare (A_R) alleles by population, allelic richness (R_S) by population, and gene diversity by locus (H_T) using GENALEX v 6.1 (Peakall & Smouse 2006) and FSTAT v 2.9.3.2 (Goudet 1995). I used GENEPOP v 4.0.10 (Raymond & Rousset 1995, Rousset 2008) to calculate the observed (*Ho*) and expected (H_E) heterozygosities in all populations and loci. A sequential Bonferroni correction was used to determine significant heterozygote deficits (Rice 1989). GENEPOP v 4.0.10 was used to determine the presence of linkage disequilibrium among loci with a sequential Bonferroni correction. The statistics program R v 2.13.2 (R Development Core Team 2008). was used to measure the correlation between population sample size and the population summary statistics Small populations (N< 25) were excluded from the analysis of allelic richness to avoid bias introduced by too few samples per population.

2.2.4 Estimating Population Differentiation and Inbreeding

Wrights F statistics: F_{IS}, F_{IT} and F_{ST} (Allendorf and Luikart 2007) were calculated over all loci to determine the degree of genetic divergence among populations and the characteristics of allele and genotype frequencies within populations using FSTAT v 2.9.3.2. The four small populations (N< 25) were excluded from these analyses due to possible sampling bias from the small sample size. F_{IS} was a calculated for each population with the exception of Bicentennial sample that consisted of one individual. The statistics program R v 2.13.2 was used to determine correlations the between F_{IS}, and population sample size using a Pearson correlation test. F_{ST} values were calculated for each locus. FSTAT v 2.9.3.2 was also used to calculate the overall

Nei's genetic distance (G_{ST}) (Nei 1973). An analysis of molecular variance AMOVA was undertaken in GENALEX 6 to compare the genetic variation within and among populations. Pairwise F_{ST} values were calculated between populations using GENEPOP v 4.0.10, and these were correlated with the pairwise geographic distances using a Mantel t test in GENALEX 6 to test for genetic isolation by distance. The pairwise number of migrants (N_M) between population pairs were calculated with GENEPOP v 4.0.10 using the private alleles method (Slatkin 1985) and from the pairwise F_{ST} values using the formula $N_M = 1/4\ (1/ F_{ST} -1)$ (Slatkin 1995).

STRUCTURE v 2.3.1 (Evanno *et al.* 2005, Pritchard *et al.* 2000) was used to compare the allele frequencies among populations and identify genetic clusters among our study populations. Optimal K among the 8 populations was determined using the admixture model with allele frequency correlated among populations. I ran Structure analysis with K values of 1 to 8. The standard conditions of the analyses were a burn in period of 10000 cycles with 100,000 final replications. All eight populations were included in this analysis. Results from structure were uploaded into Structure Harvester v 0.6.8 (Earl and vonHoldt 2011) to produce the Delta K values (Evanno *et al.* 2005).

2.3 Results

2.3.1 Genetic Diversity

Significant linkage disequilibrium was not found at any of the six loci when a Bonferroni correction was used. Comparisons among all six populations indicated mild differences in genetic variability and heterozygote deficiencies. I assessed genetic diversity by locus in a total of 115 individuals from the four large ($N \geq 25$) populations using six microsatellite loci. The total number of alleles per locus (N_A) varied from 3 to 38 with a mean number of 12.5 alleles per locus (Table 2.3). The overall gene diversities (H_T) varied from 0.132 to 0.95 with an average of

0.674 per locus (Table 2.3). The observed heterozygosity (H_O) varied from 0.098 to 0.758 by locus (Table 2.3) and was lower than expected heterozygosity (H_E) at all six loci. The effective number of alleles (N_E) and mean number of alleles per locus were highest in the relatively large A annex population and smallest in the three small populations in Minnesota. Both variables were positively correlated with sample size (Table 2.4). The numbers of private alleles and rare alleles per locus were higher in the four large populations compared to the small populations as well. Allelic richness (R_S) in the four large populations varied from 5.6 in Bluestem to 7.6 in A annex (Table 2.4). Observed heterozygosity (H_O) was lower than expected heterozygosity (H_E) in all populations (Table 2.4). In addition, the observed heterozygosity per locus was significantly lower than expected at three loci in three of the four large populations: Bluestem, Ulen and Viking. Consistent with these heterozygote deficiencies, positive F_{IS} values were found in populations ranging from 0.124 to 0.24 (Table 2.4) suggesting some degree of inbreeding.

Table 2.3. Genetic diversity by locus in four Western Prairie Fringed Orchid populations: Bluestem, Ulen, A-annex and Viking ditch. The values are number of alleles (N_A); number of effective alleles (N_E); observed homozygosity (H_O); observed heterozygosity (H_E); overall gene diversity (H_T), population differentiation $F_{IS,}$ $F_{ST,}$ F_{IT}, and G_{ST}.
**indicates significant departure from HWE after Bonferroni correction in two populations.

Locus	N_A	N_E	H_O	H_E	H_T	F_{ST}	F_{IS}	F_{IT}	G_{ST}
2	14	4.88	0.683	0.792	0.836	0.0414	0.1563	0.1927	0.033
5	7	3.32	0.683	0.695	0.721	0.0216	0.0368	0.0564	0.017
7	4	2.62	0.362**	0.597	0.682	0.1246	0.4035	0.4824	0.103
12	38	10.3	0.758**	0.888	0.95	0.0589	0.1708	0.203	0.046
17	3	1.16	0.098	0.112	0.132	0.1682	0.0795	0.281	0.132
27	9	3.36	0.512**	0.653	0.72	0.0903	0.235	0.3039	0.071
Mean	12.5	4.27	0.516	0.622	0.674	0.0692	0.1903	0.2447	0.055

Table 2.4. Comparison of genetic diversity of seven *Platanthera praeclara* populations. Number of private alleles (A_P): Number of rare alleles per locus (frequency less than 0.1) (A_R); Observed number of alleles (N_A); number of effective alleles (N_E), allelic richness (R_S), Mean alleles per locus, inbreeding coefficient, (F_{IS}) observed heterozygosity (H_o), expected heterozygosity (H_E). The Pearson correlation coefficient between each variable and population sample size is shown in the bottom row. ***indicates significant departure from HWE after Bonferroni correction at three loci.

Population ID	*N*	A_P	A_R	N_A	N_E	R_S	*Mean alleles per locus*	F_{IS}	H_O	H_E
Dalby	5	0	0.83	20	2.3	-	2.5	0.52	0.300	0.529
Syre	2	0	0.0	12	1.9	-	1.5	0.4	0.417	0.354
Ulen	30	5	4.17	56	4.8	6.62	7.0	0.23	0.497***	0.632
Hartke	7	0	0.83	19	2.4	-	2.4	0.3	0.389	0.495
Bluestem	25	4	2.67	34	3.1	5.57	5.7	0.24	0.458***	0.588
Viking	30	6	3.33	42	4.2	6.63	7	0.17	0.543***	0.638
A annex	30	9	6.5	49	5.0	7.55	8.2	0.12	0.565	0.633
Mean	19	3.4	2.62	41.9	4.2	6.60	4.9	0.28	0.453	0.553
Pearson Correlation		r= 0.92 p=0.003	r= 0.98 p=0.000	r=0.83 p=0.021	r=0.90 p=0.005	r=0.90 p= 0.104	r=0.87 p=0.011	r= -0.76 p=0.044	r=0.79 p=0.034	r=0.90 p=0.006

Moreover, F_{IS} values increased with decreasing population size (r = -0.76, P = 0.044). However, analysis using Microchecker indicated the likelihood of null alleles at two loci: locus 7 in the Ulen and Viking populations and locus 12 in Viking and Bluestem populations (Table 2.5). Allelic richness in the four large populations was not significantly correlated with sample size (r = 0.90 P= 0.104) but expected heterozygosity did positively correlate with sample size (r = 0.90, P= 0.006).

Table 2.5. Proportion of null alleles by locus and population indicated in Microchecker. The value from the Brookfield 1 method is given from populations and loci that had significant null alleles.

Locus	Population	Null Frequency
12	Bluestem	0.188
12	Viking	0.0644
7	Ulen	0.2097
7	Viking	0.1653

2.3.2 Genetic Divergence Among Populations

There was little evidence of genetic divergence among the six populations we characterized. An AMOVA partitioned the total genetic variation into within populations (90%) and among populations (10%). The G_{ST} value was 0.055 indicating the majority of variation occurred within populations. Genetic differentiation was weak across the populations in this study with an overall F_{ST} value of 0.0692. However, some loci (e.g. 17, 7) showed a greater tendency to diverge among populations than other loci (Table 2.3).

Pairwise comparisons of populations were generally consistent with a pattern of little divergence among populations. Pairwise F_{ST} values were generally low, ranging between 0.0222 and 0.1268 (Table 2.6).

However, the Bluestem population had higher pairwise F_{ST} values than other population (Table 2.6). The average estimated number of migrants between populations per generation (N_M) was 3.363 when calculated from the F_{ST} value and 2.563 when calculated using the private allele's method (Slatkin 1985). The pairwise number of migrants was greater than one for every population pair (Table 2.6). There was no correlation between genetic and geographic distance according to a Mantel test (r=0.2793, p=0.31).

According to the Evanno *et al.* (2005) method of optimal STRUCTURE analysis, a K value of 2 was optimal indicating that there were two detectable genetic clusters resulting from the STRUCTURE analysis (Figure 2.2). All eight populations surveyed had elements of both clusters. However, the Bluestem population was distinct from all other populations in the degree

Table 2.6. Pair wise F_{ST} values (below diagonal) between populations and pairwise number of migrants (above diagonal) calculated from the F_{ST} values compared. Distances between populations were as follows: Bluestem-A Annex=85.2 km; -Ulen=30.2 km; -Viking=61.3 km; A Annex-Ulen=109.9 km; -Viking=24.6 km; Ulen-Viking=87.6 km.

	Bluestem	A Annex	Ulen	Viking
Bluestem	0	1.722	2.037	2.322
A Annex	0.1268	0	11.011	5.969
Ulen	0.1093	0.0222	0	7.612
Viking	0.0972	0.0402	0.0318	0

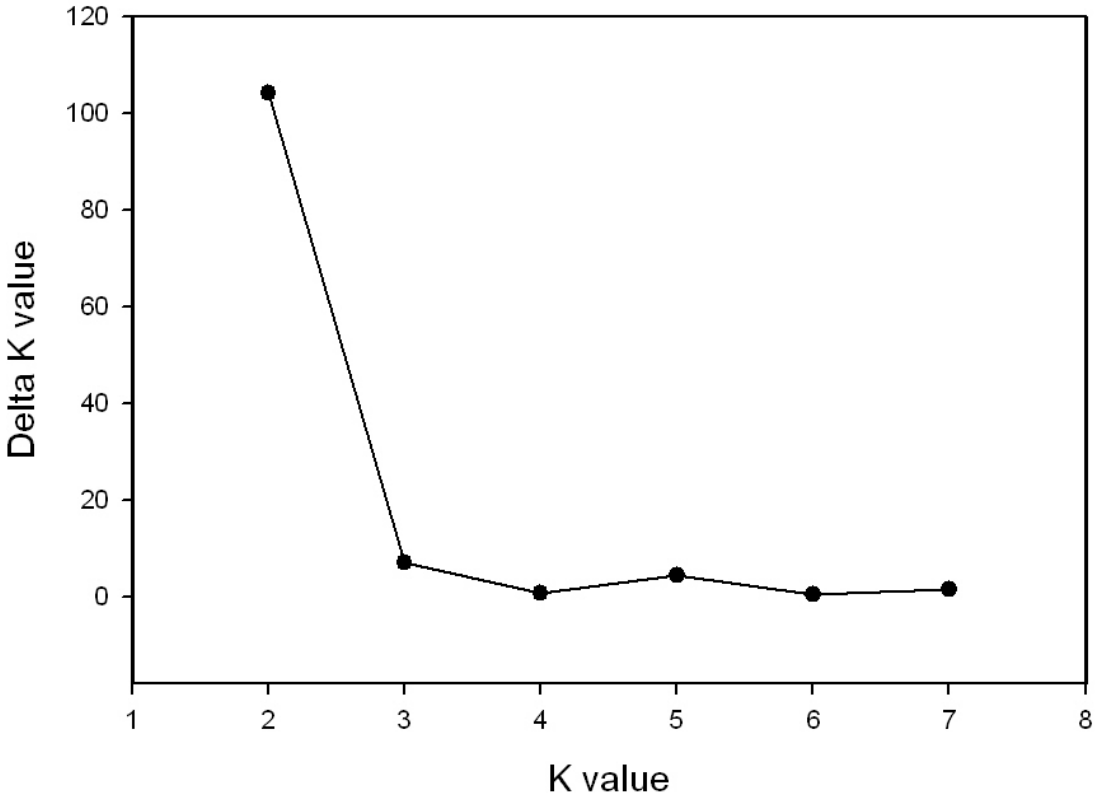

Figure 2.2. Plot of the Delta K value associated with differing group sizes (K) based on Evanno *et al*. (2005) method of determining optimal group size from Structure analysis.

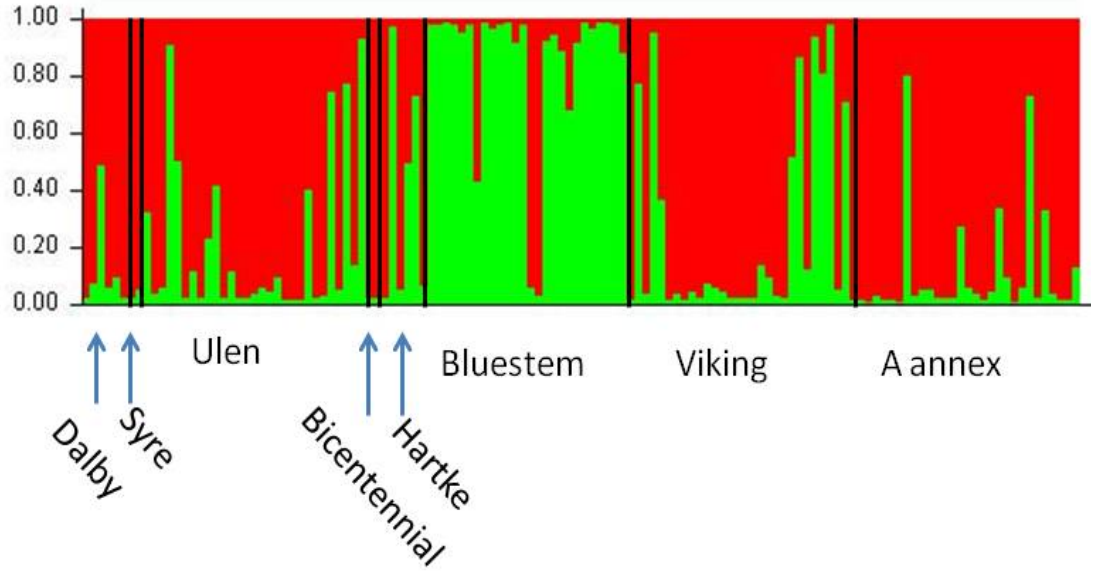

Figure 2.3. Bar plot of Structure results using a K value of 2. The portion of individual genotypes assigned to each of the two Structure clusters are indicated by red versus green. Populations are sorted by decreasing latitude from left to right.

were calculated without the Bluestem population the overall G_{ST} Value was 0.021 and the F_{ST} value was 0.032, indicating that approximately half of the genetic diversity among populations was due to the Bluestem population.

2.4 Discussion

We found very little evidence of significant genetic divergence among the populations sampled in North Dakota and Minnesota. The overall F_{ST} value of 0.0692 showed weak population differentiation. Pairwise comparisons of differences between populations yielded similar results with relatively low F_{ST} values and estimates of the number of migrants (N_M) universally greater than 1 (mean = 3.36). An N_M value greater than one is considered sufficient to prevent genetic drift and divergence between populations (Slatkin 1985). These results are comparable to those found by Sharma (2002) and Pleasants and Klier (1995). The AMOVA analysis showed a similar result, classifying 90% of genetic variation within rather than among populations. A Mantel t test on the pairwise genetic and geographic distances among populations found no correlation between genetic and geographic distance ($r = 0.2796$, $p = 0.31$) supporting the conclusion of no genetic structure among the populations.

One explanation for no evidence for genetic structure is that there may be significant gene flow occurring among WPFO populations at the geographical scale of this study. This may be occurring from seed dispersal or movement of pollen among populations by pollinators. Hawkmoths can fly hundreds of kilometers, for example hawkmoths native to the tropics have been found in New England (Tuttle 2007). However, it has been shown that hawkmoths move pollen most commonly among flowers on the same plant and between neighboring plants (Nilsson *et al*. 1992, Johnson *et al.* 2004). Nectar rewarding orchids may cause pollinators engage in geitonogamy as pollinators visit flowers on the same plant sequentially as they forage

for nectar (Zu 2011). As in other orchid species the Hawkmoth pollinators are not specific to WPFO, this may affect pollen transfer over long distances among populations (Cuthrell 1994).

A second explanation could be the transfer of seeds among populations. The maximum distance and level of seed travel is unknown in WPFO. Research on orchid seed dispersal has shown highly differing rates of ability for seeds to travel. Studies have determined the great majority of orchid seeds travel very short distances within approximately one meter or less from the parent plant **(**Machon *et al*. 2003, Jeráková J and Malinová 2007). A smaller amount of orchid seed, that can be sufficient for colonization, can travel much farther, hundreds or even thousands of kilometers (Arditti and Ghani 2000). It is not clear what the situation with WPFO is: WPFO seeds are much smaller than many other orchid seed types. WPFO seeds are approximately 3 μm long by 2 μm wide; the smallest seeds listed in review by Arditti and Ghani (2000) were approximately 0.15 mm long. WPFO habitat is highly limited and it is possible that micro sites where WPFO seeds can germinate are uncommon in its habitats (Jeráková and Malinová 2007). WPFO seeds also appear to have a low germination rate (Alexander *et al.* 2010b).

Another possible explanation is that the original pattern of colonization by WPFO, prior to fragmentation, was panmictic across the range of this study and there has been little change over time despite recent habitat fragmentation. Although seed set in WPFO is low, it is the primary method of reproduction, while vegetative propagation can also occur (Armstrong *et al*. 1997). A plant with a relatively short life span that is seed dependent, with low seed set would be expected to genetically diverge more quickly than a plant with a longer lifespan that can propagate and persist vegetatively.

Other orchid species have been shown to be either reliant on seed or vegetative

reproduction. Roberts (2003) reviewed two orchid species, *Cypripedium calceolus* and *Ophrys sphegodes,* that mainly rely on either seed or vegetative reproduction. *Cypripedium calceolus* has been determined to be dependent on vegetative reproduction, with the plants having a distinct clumping pattern up to 70 cm in diameter. Most populations of *Cypripedium calceolus* consist of a small number of genets with several ramets that have a lifespan of up to 100 years (Kull 1988, Kull 1999). *Ophrys sphegodes* is an example of a seed dependent orchid with which very few new plants are found near (within 10 cm) of parent plants. The individuals of *Ophrys sphegodes* have significantly shorter life spans than *Cypripedium calceolus* with an average of half life of 1.5-2.3 years (Hutchings 1987).

The life history of WPFO is likely similar to *Ophrys sphegodes*, as it has been shown to rarely produce more than one ramet is seed dependent and may have a comparable lifespan. (Sieg 1995). In this study there were only two cases where ramets were found closer than approximately 5 cm of each other, one of these was determined to be a clone. The distances among the WPFO individuals were highly variable; there were several cases in which individuals were within one meter of each other.

However, WPFO does not appear to be diverging at a population level. A significant overlap in generations may possibly act against genetic differentiation if flowering plants are consistently a mix between new and plants that are re-emerging. The true number of individuals may be higher in populations than is understood, with several plants not emerging every year. It is possible gene flow is occurring among the populations via either pollination or seed.

There is some evidence that one population is relatively distinct from the others in this study. The highest pairwise F_{ST} values were found in the Bluestem population compared to the three other large populations. Despite a low overall F_{ST} value, the STRUCTURE analysis

identified two genetically distinct genetic clusters (Figure 2.2). The majority of the individuals in the Bluestem population were classified as genetically distinct from the majority of individuals in other populations (Figure 2.3). The Bluestem population also had the highest pairwise F_{ST} values in Sharma (2002) study when compared to seven other WPFO populations using allozyme loci. The Bluestem population may be genetically differentiated from the other study populations despite being the nearest to the geographic center of the study populations. The divergence of this population cannot be explained geographically; this may reflect a separate colonization event from a different ancestral group of plants. It is not clear why this population would be genetically isolated based on either seed dispersal or pollinator isolation due to location or size.

When compared to studies using microsatellites in other orchid species, the overall F_{ST} value is comparable (Gusstafson 2000, Gusstafson and Sjogen-Gulve 2002, Swarts *et al.* 2009, Solvia and Widmer 2003, Mant *et al.* 2005) (Table 2.7). When compared with other microsatellite studies of plants with a mixed selfing outcrossing breeding system, the average F_{ST} overall is 0.26 (Table 2.7) compared to 0.0692 for WPFO. This suggests that WPFO populations may be undergoing outcrossing and the overlap in generations may prevent genetic divergence.

Studies in other plant species have shown significant genetic divergence correlating with geographic distance. A study by Ægisdóttir *et al.* (2009) in *Campanula thyrsoides* found a G_{ST} value of 0.53. A mantel test showed a clear correlation with genetic distance $r = 0.62$, $p < 0.001$; the STRUCTURE analyses with a K values of 2 and 3 both showed a clear genetic structure correlating with its range. A similar trend was found by Fan *et al.* (2010) in *Litchi chinensis.* An overall F_{ST} of 0.269 was found, as well as a significant correlation between geographic and genetic distance ($r = 0.655$, $p = 0.002$). A STRUCTURE analysis with K values of 2 and 3 both showed genetic structure that matched the geographic structure.

Table 2.7. F_{ST} and F_{IS} values compared with seven other orchid species and with two examples of plant species with significant genetic structure. The the *Fst* value from Mant *et al*. (2005) is the value calculated with three listed species. The value form Nybom (2004) was a value given for plants with a mixed selfing and outcrossing breeding system.

Study	Species and Family	*Fst*	*Fis*
Ross 2012	*Platanthera praeclara* *Orchidaceae*	0.0692	0.19
Gustafson 2000	*Gymnadenia conopsea* *Orchidaceae*	0.06	-
Gusstafson and Sjogen-Gulve 2002	*Gymnadenia odoratissima* *Orchidaceae*	0.19	0.149
Swarts *et al.* 2009	*Caladenia huegelii* *Orchidaceae*	0.047	0.22
Solvia and Widmer 2003	*Ophrys sphegodes* *Orchidaceae*	0.063	-
Mant *et al*. 2005	*Ophrys sphegodes* *Ophrys exaltata* *Ophrys garganica* *Orchidaceae*	0.075	-
Nybom 2004	5 species	0.26	-
Ægisdóttir *et al.* 2009	*Campanula thyrsoides* *Campanulaceae*	0.53 (Gst)	0.022
Fan *et al.* 2010 .	*Litchi chinensis* *Sapindaceae*	0.269	0.229

A lower level of genetic structure was found in Western Prairie Fringed Orchid (F_{ST} = 0.0692, G_{ST} = 0.055), there was no correlation between geographic in genetic distance among populations in a mantel test (r = 0.2793 P = 0.31). The STRUCTURE analysis did not show a correlation between geographic and genetic distance.

A second goal of my study was to determine the genetic diversity of WPFO populations by assessing genetic diversity and inbreeding. The overall observed heterozygosity (H_O), expected heterozygosity (H_E) and number of alleles per population (N_A) were comparable to other orchid species (Gusstafson 2000, Gusstafson Sjogen-Gulve 2002, Swarts *et al.* 2009, Campbell *et al.* 2007) (Table 2.8). All WPFO of the populations were comparable in levels of genetic diversity; no population was shown to be significantly genetically impoverished. When compared with other microsatellite studies in plants, WPFO is comparable in both observed and expected heterozygosity (Nybom 2004) (Table 2.8). Both previous allozyme studies in WPFO

Table 2.8. Measures of genetic diversity: observed heterozygosity (*Ho*), expected heterozygosity (*He*) and number of alleles per locus (*Na*) compared with other orchid species (Gustafson 2000, Gustafson 2002, Swarts 2009), other plant species (Nybom 2004), and two previous allozyme studies in WPFO. The values from Nybom (2004) are for plants with a mixed breeding system that allows for selfing.

Study	Species	*Ho*	*He*	*Na*
Ross 2012	*Platanthera praeclara*	0.516	0.622	12.5
Gustafson 2000	*Gymnadenia conopsea*	0.667	0.727	14
Gustafson 2002	*Gymnadenia odoratissima*	0.423	0.509	9.3
Swarts 2009	*Caladenia huegelii*	0.551	0.69	15.29
Nybom 2004	15 species	0.51	0.60	-
Pleasants and Klier 1995 (allozyme loci)	*Platanthera praeclara*	0.019	0.043	1.16
Sharma 2002 (allozyme loci)	*Platanthera praeclara*	0.071	0.072	2.09

compared both genetic diversity and genetic structure to other orchid species and determined WPFO was similar (Pleasants and Klier 1995, Sharma 2002) (Table 2.8). The overall genetic diversity measures indicate that genetic diversity and evolutionary potential are most likely not a limiting factor in WPFO populations in this study.

Significant inbreeding may be common in the populations in this study. There are significant deficits of heterozygotes as well as a positive F_{IS} values. These F_{IS} values are similar to other plant species that have breeding systems similar to WPFO that allow selfing and inbreeding (Swarts *et al.* 2007, Kettle *et al.* 2007, Michalski and Durka 2007, Fan *et al.* 2010) These *Fis* values are significantly higher than for plants that have breeding systems that prevent selfing or other forms of inbreeding (Ægisdóttir *et al.* 2009, Setsuko *et al*. 2007). This may indicate selfing or breeding between close relatives is occurring at a high rate in WPFO populations. The F_{IS} values negatively correlate with sample size indicating that smaller populations show higher levels of homozygotes; this could be caused by genetic drift or inbreeding.

Null alleles may have contributed to the positive F_{IS} at two loci: 7 and 12. The only locus that showed significant heterozygote deficits and no evidence of null alleles was locus 27. Locus 7 had the highest F_{IS} value of any locus. Null alleles were present in all populations that showed significant heterozygote deficits. The population with the highest F_{IS} score was Dalby that showed no evidence of null alleles. The Wahlund effect, caused by genetic structure within a population, where individuals from different populations are treated as being from the same population, could be causing significant heterozygote deficits. The Wahlund effect could possibly be more pronounced in populations with more scattered individuals or in small populations if they attract fewer pollinators.

Inbreeding has been shown to have a direct effect in seed production in EPFO; when flowers were selfed seed production significantly decreased (Wallace 2002). This could lead to a decrease in recruitment and population numbers. Other potential consequences are an overall loss of genetic diversity and fitness. The effects of inbreeding depression have been shown in increase extinction risk in many species **(**Frankham 2005). It has been speculated this is the cause of the loss of several small WPFO populations (Fox 2008).

WPFO is vulnerable to inbreeding via both selfing and geitenogamy; small population sizes can also lead to mating between closely related plants. Hawk moth pollinators are nectar rewarded and may excessively cross the same plant and in small populations cross closely related individuals (Nilsson *et al*. 1992, Fritz and Nilsson 1994, Brys *et al.* 2008). Inbreeding may decrease seed production further lowering numbers of orchids leading to a loss of genetic diversity and a potential extinction vortex (Frankham 2005).

The microsatellites were more polymorphic than the allozymes used in previous WPFO studies. The Plesants and Klier (1995) study had an average of 1.16 alleles per locus. Sharma's study showed 2.09 alleles per locus compared to 12.5 alleles per locus in this study. The expected heterozygosity (*He*) in this study was higher than in the Pleasants and Klier (1995) and Sharma (2002) allozyme studies: 0.622 compared to 0.043 and 0.072, respectively (Table 2.8) The microsatellites were polymorphic enough to allow all individual plants to be identified. For example, a single clone was identified, which consisted of three ramets found in an approximately 5 cm^2 area.

This project was a first step in using microsatellites to assess the genetics of WPFO. To further access the genetics of the populations of WPFO in this study these developed loci will have to be evaluated over a longer time scale. The year to year population fluctuations in

numbers of individuals per population can be large and it is not clear if this will cause changes in genetic structure form year to year. All population genetic studies in WPFO have been limited to a single season. The geographic range and number of populations in this study were limited. The range of WPFO covers a large area and populations consist of differing numbers of individuals with varying ranges among the populations. Populations will have to be characterized over a larger geographic range to determine if larger scale genetic structure is present. Many factors such as presence of pollinators can have large effects on the genetic structure of other populations.

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CHAPTER. 3 THE DEVELOPMENT OF MICROATELLITE MARKERS FOR WESTERN PRAIRIE FRINGED ORCHID

3.1 Introduction

The Western Prairie Fringed Orchid (*Platanthera praeclara*) is one of a handful of North American orchid species in the *Platanthera* genus. It is also one of the rarest, earning it a federally threatened status. The historical range of *P. praeclara* is throughout the upper Great Plains in close association with tallgrass prairie. Large scale conversion of the original tallgrass prairie to agriculture is believed to be the leading cause of local extirpation and widespread reductions in the numbers of *P. praeclara* (Sheviak and Bowles 1986).

This particular species of *Platanthera* is diploid, self-compatible and typical of plants adapted to hawk moth pollination (Sheviak and Bowles 1986). Previous surveys of genetic diversity of *P. praeclara* found little evidence of divergence among populations or extreme reductions in genetic diversity within populations (Pleasants and Klier 1995). However, these conclusions were based on the use of allozyme markers. In contrast, microsatellite markers are characterized by higher levels of allelic variation and often provide an improved measure of genetic variation both within and among populations (Allendorph and Luikart 2007) Here we present the results of the development of microsatellite markers for *Platanthera praeclara* in the hopes of providing a tool for further study of this species and genus.

3.2 Methods and Results

Leaf material was collected from a total of 115 plants in 4 populations near Fargo, North Dakota. Genomic DNA was extracted from frozen leaf material prepared using a DNeasy miniprep kit (Qiagen). DNA was then serially enriched twice for microsatellites using 3 probe

mixes (mix 2 = $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$; mix 3 = $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$; mix 4 = $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$) following Glenn and Schable (2005). Briefly, DNA was digested with restriction enzymes *Rsa*I (New England Biolabs) and then ligated to double-stranded SuperSNX linkers (SuperSNX24 Forward 5' GTTTAAGGCCTAGCTAGCAGCAGAATC and SuperSNX24 Reverse 5'-GATTCTGCTAGCTAGGCCTTAAACAAAA). Linker-ligated DNA was denatured and hybridized to biotinylated microsatellite oligonucleotide mixes, which were then captured on magnetic streptavidin beads (Dynal). Unhybridized DNA was washed away and remaining DNA was eluted from the beads, amplified in polymerase chain reactions (PCR) using the forward SuperSNX24 as a primer, and cloned with TOPO-TA Cloning Kits (Invitrogen). Inserts were PCR amplified and sequenced with M13 forward and reverse primers using the BigDye Terminators v3.1 (Applied Biosystems) and ABI-3130xl capillary sequencer. Sequences from both strands were assembled and edited in Sequencer 4.6 (Genecodes). Microsatellites were identified using MsatCommander version 0.8.1 (Faircloth 2008) and primers designed with Primer3. One primer from each pair was modified on the 5' end with an engineered sequence (CAG tag 5'-CAGTCGGGCGTCATCA-3') to enable use of a third primer in the PCR (identical to the CAG tag) that was fluorescently labeled for detection.

PCR reactions were performed at a total volume of 20 µL. Each reaction was composed of 8.8µL of DD H_20, 4µL 5× GoTaq fexi buffer (Promega, Madison WI), 1.2 uL of 25mM $MgCl_2$, 0.8uL of dNTPs (5mM each), 1µL of 0.5 µM CAG tag modified primer (Integrated DNA Technologies, Coralville IA), 1µL of non CAG tag modified primer (5µM), 1uL of florescent tag (5 µM), 0.2 µL of GoTaq polymerase (Promega, Madison WI) and 2µL of template DNA for a

final volume of 20µL. The touchdown (Don et al. 1991) PCR profile consisted of an initial denaturization of 94^0 for 2 min, followed with 16 cycles of a denaturization step at 94^0 C for 30 s, an annealing temp starting at 65^0C for 30 s, and decreasing 0.5^0 C each cycle, and an elongation step of 72^0 C for 30 seconds. This was followed by 20 cycles of a denaturization step of 94^0 C for 30 s, an annealing step at 57^0 C for 30 s, an elongation step of 72^0C for 30 s, and a final elongation step of 5 min. PCR products were visualized on 2% agarose gels stained in ethidium bromide to verify a successful amplification. PCR products were analyzed for fragment length using an Applied Biosystems 3730 analyzer at the Plant Microbe Genomics Facility at Ohio State University. The data were visualized in electropherogram form using peakscanner v 1.0 software. The electropherograms were scored by eye to determine the alleles in each amplified sample. Rules were made for peak calling which were followed consistently for each locus. 31 loci were selected for characterization. A total of 16 loci amplified successfully and 10 were polymorphic (PP02, PP05, PP07, PP12, PP13, PP17, PP23, PP27, PP30, PP31). Significant linkage disequilibrium was found using Genepop v 4.0.10 (Raymond & Rousset 1995, Rousset 2008) leaving 6 loci for the population study of *P. praeclara* (PP02, PP05,PP07, PP12, PP17, PP27) (Table 1).

A total 115 Individuals were sampled from 2 populations in western Minnesota (Ulen and Bluestem) and 2 populations in eastern North Dakota (A annex and Viking). These samples were characterized with the 6 polymorphic loci that were not in linkage disequilibrium. H_O and H_E was calculated using GeneAlEx 6 (Peakall and Smouse 2006) the expected heterozygosity ranged from 0.112 to 0.888 by locus (Table 1) and 0.588 to 638 by population (Table 2). An AMOVA was calculated with GeneAlEx 6 which classified 90% of the genetic variation within

Table 3.1. Primer sequences of 16 polymorphic microsatellite loci developed for *Platanthera praeclara* The forward and reverse primer sequences, nucleotide repeat type, fragment size, and probe identification number are given.

Locus	*Primer Sequence 5' --> 3'*	Repeat	Size	Probe PUID
PP02	*F: ATGAGGGTCTTCACGCATGT* *R: CCACGGGATCTCCTTCCAAT**	CT	177-199	12324941
PP03	*F: TGGAGATCAACCACGCGATA** *R: ACTTCAGGTAAGCAGGCTTTG*	AAAC	189	12324946
PP05	*F: TACCCGAGTTCCTTGCTGAC* *R: CCTCTCGACAACAACCAGT**	CT	202-214	12324949
PP07	*F: ACCCTCGTAGATCGTTTCGG* *R: GTGGATTTCGTGTGCCTT**	AG	239-245	12324950
PP09	*F: CCATCTCTCCGTGGATACG** *R: GGACATGCACTAATCGGCAC*	GTTT	253-263	12324951
PP12	*F: GGTGCGGTCACTAACTTTGA** *R: GGCGCAACCCACATTGATT*	AC	256-328	12324936
PP13	*F: TTGTGGCGCTCGATCATCTT* *R: TTTCCCTCACCGCCTCTTT**	GT	248-318	12324937
PP15	*F: TCCGGGTTTCCTTTGACGTA** *R: AGGTGCTTCAACGATCCAAAC*	GTTT	293-298	12324938
PP17	*F: GCATGTCTCAAGCTCTCACG* *R: TCGCTCTCATTTCCACCG**	GTTT	311-323	12324939
PP19	*F:GCTTCACTGACATTTCTTGGGT* **R: TTCAGCAATCATTCCGCACA*	GTTT	335-339	12324940
PP20	*F: CGATCCGCGAGAGTGTAGAA* *R: GTCGCCTGTGAGTTTGGAGA*	AG	386	12324942
PP23	*F: GAAGTGTCCGCAGCTCTTTC* *R: CATCACGGTTGCGAGGTATC*	AG	368-392	12324943
PP27	*F: CAATGGTTGTGCTCTGAATGAC* *R: CCGGTTCCAACAAAGTGC**	GT	435-451	12324944
PP29	*F: TTTCATCAGCGCCAAGAACT* *R: TCCTACATTGGCCGCTACTC**	AGAT	465	12324945
PP30	*F: CTGAGCAAAGAGGCGTAGA** *R: TGCCACATTCCTGAGCTACC*	GT	475-507	12324947
PP31	*F: AGAAGGCACGGTTACTCAAA* *R:TCTCCATTCCTGAATCCTTGTG**	AC	503-517	12324948

Table 3.2. Six polymorphic loci in four populations of *Platanthera praeclara*. Number of alleles (N_a) observed heterozygosity (*Ho*) and expected heterozygosity (*He*) values are given by locus and population. The sample size is given in parentheses.

	N_a	H_o	H_e		N_a	H_o	H_e
Population Bluestem (N=25)				Population Ulen (N=30)			
PP02	8	0.680	0.759	PP02	8	0.567	0.771
PP05	5	0.696	0.718	PP05	5	0.586	0.674
PP07	3	0.280	0.463	PP07	3	0.300	0.645
PP12	10	0.480	0.824	PP12	19	0.900	0.923
PP17	2	0.292	0.353	PP17	1	0.000	0.000
PP27	6	0.320	0.407	PP27	6	0.630	0.779
Average	5.667	0.458	0.588	Average	7	0.497	0.632
Population A annex (N=30)				Population Viking (N=30)			
PP02	9	0.700	0.812	PP02	9	0.786	0.824
PP05	6	0.759	0.735	PP05	4	0.690	0.652
PP07	3	0.433	0.562	PP07	4	0.433	0.717
PP12	23	0.900	0.932	PP12	16	0.750	0.871
PP17	2	0.100	0.095	PP17	1	0.000	0.000
PP27	6	0.500	0.663	PP27	8	0.600	0.764
Average	8.167	0.565	0.633	Average	7	0.543	0.638

populations. The program Fstat 2.9.3.2 (Goudet 1995) was used to calculate the overall F_{st} which was low, $F_{st} = 0.069$. There was little genetic differentiation among populations which suggests the presence of gene flow among populations.

3.3 Conclusions

Of the 31 loci screened, six microsatellites were polymorphic, and not in linkage disequilibrium . Overall, there was considerable allelic variation at these loci, averaging between 5 and 8 alleles per locus. These populations represent a small portion of *Platanthera praeclara's* range and over a single year of sampling. These loci can be used in further conservation studies in further studies of *Platanthera praeclara* populations and populations of related orchids.

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CHAPTER 4. OVERVIEW OF WESTERN PRAIRIE FRINGED ORCHID POPULATION GENETICS

The goals and methods for conserving the western prairie fringed orchid (WPFO) were first outlined in the Western Prairie Fringed Orchid Recovery plan (US Fish and Wildlife 1996). This plan was updated in the 2009 Five Year Review Summary and Evaluation which covers many issues pertaining to WPFO management. It is a comprehensive source of information for the current state of the WPFO. Importantly, the 2009 report's coverage of the population genetics of WPFO is limited to Sharma (2002).

Here I summarize the state of knowledge of the population genetics of WPFO and Eastern Prairie Fringed Orchid (EPFO). Three projects on WPFO are covered: Pleasants and Klier (1995), Sharma (2002), and my study presented in Chapter 2. One project on (EPFO) Wallace (2002) is also covered. First, the state of the knowledge of WPFO population genetics is presented, and the results of the four studies are compared. Second, future directions for population genetic studies in WPFO and other *Platanthera* species are outlined.

4.1 Summary of WPFO Population Genetics Research

For the long-term sustainability of WPFO populations an important question is what are the consequences of fragmentation of WPFO habitat and small numbers of individuals in each population. The fragmentation and reduction of populations has genetic implications, mainly related to the loss of genetic diversity and evolutionary potential as well as genetic isolation and inbreeding (Frankham 2005). The two main questions addressed in the genetic studies of WPFO are:

-Is there an overall loss of genetic diversity that might affect the orchid on a species and population level?

-Are any of the remaining populations genetically isolated and therefore at risk for inbreeding depression?

The questions of loss of genetic diversity and risk of inbreeding depression are especially applicable as individuals in small, isolated populations are more likely to self or mate with close relatives and to be at risk for inbreeding depression (Ellstrand & Elam 1993). Loss of fitness through inbreeding is a potential problem in WPFO as inbreeding has been shown to cause a significant loss in seed viability and production in its sibling species EPFO (Wallace 2003).

The first population genetics study of WPFO was undertaken by Pleasants and Klier in 1995. This study examined both Western and Eastern Prairie Fringed Orchid (EPFO) populations. Goals were to: (1) determine the overall genetic variation of WPFO, (2) determine the geographic pattern of the genetic variation in WPFO, and (3) determine the phylogenetic relationship between WPFO and EPFO. Fourteen WPFO populations were examined in five states and seven EPFO populations were examined in 2 states, with 11 allozyme loci (Table 4.1). The overall genetic diversity, measured by expected heterozygosity (H_E), was 0.043, which is low compared to other plants but comparable to other orchids (Pleasants and Klier 1995). Significant genetic structure was not found, the overall G_{ST} was 0.208 indicating approximately 80% of the genetic variation was within rather than among populations. WPFO and EPFO were not distinguishable using allozymes although they are clearly distinguishable morphologically.

A study by Sharma in 2002 was designed to answer these questions: (1) how does genetic variation within and among populations compare; (2) does population size correlate with genetic diversity; and (3) does genetic distance among populations correlate with geographic distance

Table 4.1. The results of four population genetics projects evaluating WPFO and EPFO populations. General information given is: the type of molecular marker, species, number of loci, and number of populations evaluated. The measures of population differentiation compared are *Fis* and *Fst*. The measures of genetic diversity compared are Observed heterozygosity (*Ho*) Expected heterozygosity (*He*) and number of alleles per locus (*Na*).

Study	Marker Type	Species	Number of loci	Number of Populations	*Fis*	*Fst*	*Ho*	*He*	*Na*
Plesants and Klier 1995	Allozyme	WPFO EPFO	11	14 (WPFO) 7 (EPFO)	-	0.208 (Gst)	0.019	0.043	1.16
Sharma 2002	Allozyme	WPFO	10	8	0.015	0.105	0.071	0.072	2.09
Wallace 2002	Allozyme	EPFO	7	7	0.745	0.754	0.008	0.033	1.18
Wallace 2002	RAPD	EPFO	7	10	-	0.26 (Gst)	-	0.159	-
Ross 2012	Microsatellite	WPFO	6	8	0.1903	0.0692	0.516	0.622	12.5

among populations. Eight populations in Minnesota were studied using 10 allozyme loci. The results were similar to the Pleasants and Klier (1995) study. A similar level of overall diversity was found compared to the Pleasants and Klier study $H_E = 0.072$. Sharma also compared other allozyme studies in orchids and found that WPFO was comparable. There was no significant genetic structure among populations. The G_{ST} value was 0.102 showing approximately 90% of the genetic variation was within populations. The overall F_{IS} value was 0.014, though most loci had excess heterozygotes. There was a significant correlation between population size and genetic diversity. A correlation between geographic and genetic distance was not found; a pairwise mantel test did not show a significant correlation. One further population genetics study was undertaken in EPFO by Wallace (2002). Seven populations located in Ohio were examined at twelve allozyme loci. The distances among the populations were variable, from less than 12 km to approximately 100 km. The expected heterozygosity (H_E) was found to be 0.033. In contrast to the Pleasants and Klier study the majority of genetic structure was among populations. The overall F_{ST} was 0.75 indicating a high level of divergence among populations. A high F_{IS} of 0.746 was also found; this was due do a lack of polymorphism in the allozyme loci. This contrasts with results from the Pleasants and Klier (1995) study that showed a much lower level of polymorphism among populations. Wallace also examined seven RAPD loci in seven populations in Ohio and thee populations in Michigan and determined a *Gst* value of 0.26 In both the allozyme and the RAPD study, the genetic structure among populations was not consistent with spatial distribution.

In my study I used 6 microsatellite loci in eight populations in Minnesota and North Dakota. The microsatellites were significantly more polymorphic than the allozyme markers. In the seven populations composed of more than one individual, the overall heterozygosity was

0.533. The markers were polymorphic enough to allow individual orchids to be genotyped. The overall diversity was found to be comparable to other orchid studies. Significant genetic structure was not found among the four large (N ≥ 25) populations, with an overall G_{ST} of 0.055 showing a similar level of within population genetic diversity as the allozyme studies. The number of migrants based on the F_{ST} value was 3.36. The overall F_{IS} was 0.1903 indicating excess homozygotes. Significant deficits in homozygotes were found in three populations. There was no correlation between genetic and geographic distance: a pairwise Mantel t test did not show a significant correlation between genetic and geographic distance.

All studies of WPFO and EPFO have evaluated a limited number of populations over a single year. The Pleasants and Klier study was the only study to examine the majority of the WPFO's range by evaluating 14 WPFO sites. WPFO is known to occur at approximately 175 sites, and all the studies examined a small minority of populations (US Fish and Wildlife 2009). This results in a very narrow view in the population genetics of the WPFO limited by populations and time.

Excess homozygotes and a positive inbreeding coefficient (*Fis*) were found using microsatellites at all populations and loci in WPFO. The level of homozygosity and inbreeding coefficient are comparable with plants that have crossing systems that allow for selfing.

4.2 Further Studies with Molecular Markers

There are two major directions that can be taken with the developed microsatellite loci. The first is to expand the study over a larger area, and to use the loci in a larger number of populations over a larger part of the WPFO's range. The loci can also be used in EPFO and other *Platanthera* species. The second approach is to examine a small number of populations or an

individual population thoroughly over a longer time frame. The goal of this approach can further elucidate the life history, life span, pollination biology and seed dispersal in WPFO.

4.2.1 Further Studies of Genetic Structure Over WPFOs Range.

Population studies with microsatellites could be undertaken using greater number of populations over a larger range to determine if any of the populations are genetically isolated or genetically impoverished. The only microsatellite study covers a very limited area of the range of the WPFO. The Bluestem population stands out in my study as unique; other not yet studied populations may likewise be genetically unique. There is the also the possibility of genetic structure correlating with geographic structure over a larger part of WPFOs' range.

Pleasants and Klier (1995) did not find a pattern over the majority of the range or any populations that were genetically "impoverished" compared to others. Microsatellites may be able to determine patterns of genetic diversity or genetic structure over a larger range. This was demonstrated as the Bluestem population was determined to be unique using microsatellites.

It would be interesting to do a comparative study between the WPFO and the EPFO. WPFO and EPFO are indistinguishable with allozyme loci (Pleasants and Klier 1995). They have been demonstrated to be most likely pollinator isolated. The microsatellite loci developed could determine if there is significant genetic distance between the two species. The developed microsatellites could also be applied to other species of the *Platanthera* genus to further determine phylogenic relationships and answer questions about their population genetics.

4.2.2 Further Studies Over a Long Time Scale.

The study of a limited number of populations over a long time scale can be highly informative in a number of areas. The principal areas are life history, seasonal changes in genetic

structure, and a further understanding of gene flow as well as an understanding of pollen and seed dispersal.

Despite several studies, the life history of WPFO is not well understood (Alexander 2006). WPFO plants emerge erratically, with no distinct pattern in phenological pattern. Due to this, surveys of orchid numbers are difficult. A previous study was undertaken for 7 years surveying orchid populations (Seig and King 1995). The surveying technique use counts of visible individuals: the absence of an above-ground stem was considered dormant or dead. The use of microsatellites can help determine if an individual is new or returning. Flooding has been demonstrated to kill WPFO individuals and other factors such as drought may cause long dormancy periods (Sieg and Woken 1999). The microsatellite loci can allow for a more accurate assessment of the number of orchids in a population and determine if plants are re-emerging or new individuals from seed.

It is claimed that the majority of orchid reproduction is from seed and vegetative reproduction is highly limited (Bowles 1983, Bowles and Duxbury 1986). In the future, studies of WPFO microsatellites could be used to determine the extent of clonal reproduction by genotyping all visible individuals. Populations could be evaluated over a long period of time to determine the level of clonal reproduction.

More information is needed regarding the duration of stages in the life cycle of WPFO. For example, Richarson *et al.* (1997) cited by Alexander (2006) stated that above ground growth in WPFO is not visible until five years after germination. Paternity analysis could be used to determine if that is the case. This could also elucidate the importance of the seed bank to the persistence of WPFO populations.

Sieg and King (1995) found the majority of plants live approximately 3 years. This study was limited to the Sheyenne National Grassland and is based on visible plants. Sather (1991) cited by Alexander (2006) indicated, based on visible plants, evidence for a longer life span of individuals in Minnesota. Microsatellites can determine this by sampling both vegetative and flowering plants in a population repeatedly over a long time span because the genotypes of plants can be compared from year to year. Average lifespan may vary by population and environmental conditions.

. The number of flowering plants is known to vary from year to year. For example the Bicentennial population was limited to one individual in 2009, but 30 WPFO individuals were found in 2011 (Rhett Johnson, Personal Communication 2011). Significant genetic changes could take place from year to year in the genetic structure within and among populations. This is very important as many genetic studies are limited to a single year. It is possible that measures of genetic diversity vary from year to year. This approach could present a more realistic view of the population genetics of WPFO.

A close examination of a small number of populations can further elucidate gene flow within and among populations. The rate that pollinators travel among populations is unknown. However, it is known that pollinators visit both large and small populations (Fox 2008). If all individuals in a population are genotyped, gene flow could be determined by the presence of new plants with new genotypes.

Studies have shown that the dispersal of pollen and the majority of seed in orchids many be highly limited (Nilsson *et al*. 1992, Johnson *et al*. 2005, Jeráková and Malinová 2007). However, it has been shown, for example, that small amounts of orchid seed can disperse over long distances hundreds or thousands of kilometers (Arditti and Ghani 2000).

Paternity analysis could also be carried out on plants to determine the rate of crossing within and among populations. This could be an excellent tool for determining pollinator behavior: the identity of the parent plants can be determined showing the crossing compared to distance among plants.

4.3 Conclusions

Despite the limitations of the population genetic studies in WPFO thus far, it should be considered a limited number or orchids have been evaluated using genetic markers (Swarts and Dixon 2009, Forrest *et al*. 2004). Compared to the vast majority of other orchid species WPFO is one of the most heavily studied orchids, as three projects have evaluated its population genetics. Many other research projects have been carried out on several aspects of WPFO biology. There are still aspects of WPFO that are not well understood and can possibly be elucidated with further microsatellite studies. The application of the developed loci to other species such as EPFO can add to the body of knowledge in orchid genetics. If further studies are undertaken with WPFO more insights could be gained into orchid pollination and reproduction.

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APPENDIX A. CRITERIA FOR INTERPRETING ELECTROPHROGRAMS

General Rules

1. Decide on Peaks

Use the computer to call the peaks, and call the electropherogram yourself using peak scanner. Initially at 0.5 base pairs or greater round up; at less than 0.5 base pairs round down to call the peak.

Example: Peaks sizes at 165.6 and 172.3 become 166 and 172 respectively.

2. Decide on the Bins.

A pattern of peaks that generally follows the repeat type should emerge; your bins should generally match the repeat types. Generally the electropherogram peaks and thus the bins have and even or odd repeat. This tends to shift if a locus has a large range in fragment sizes. If the repeat is two base pairs the bins must be at least two base pairs apart. If the repeat is four base pairs the bins must be four base pairs apart.

Example: A peak pattern may look like 144 145 146 148 150 151 152 164 166 167 168 169 171 173 174 180 when initially rounded. This would correspond to a binning pattern of 144 146 148 150 152 154 164 167 169 171 173 180.

3. Match the Electropherograms to Bins

Go through all the electropherograms in peak scanner and fit the peaks to the bins. A new bin can be established for a peak that matches the repeat type. You will have to redo some of the

rounding done in step 1 to group the peaks into bins, this is essentially the point: to group the peaks into the correct sizes.

Observe for peaks like those of step three that have to be rounded more than 0.5 BP consistently. If the majority have to be rounded more than 0.5 BP, then change the bin to match them, but the bin must be the correct number of base pairs from the other bins. The majority of peaks determine where to call the bin.

Example: in a two base pair repeat, if the bins are established to be 163 165 167 169 171 173 then peaks at 165.6 and 173.3 become 165 and 173 respectively. In a four base repeat the bins would be 163 167 171 175. In this case peaks at 165.6 and 173.3 would be binned in 167 and 175 respectively.

Miscellaneous

The height of the general "noize" in the electropherogram appears to top out at a height of 300. The height of general noise associated with specific peaks can be much higher.

Summary

There may be cases were single nucleotide polymorphisms are real and the bins could be a single nucleotide apart. The current technique may not be accurate enough to determine these consistently; the single polymorphism can be "noise" from inconstant PCR or inaccuracy in the electrophoresis. Single nucleotide differences among peaks are treated as noise here.

Locus Specific Rules

Locus 2. The spacing is good, the peaks follow an odd repeat, 183 185 ect. I simply rounded all the peaks to the nearest number. There is a tall single peak in front of the main peak that is noise and is ignored.

Locus 5. Generally good spacing following an even pattern 202 204 ect. The peaks generally round to an even repeat. There is a large poly a tail before the peak. A third smaller peak can be seen on some electropherograms, it is ignored.

Locus 7. Generally high peaks sometimes with a large poly a tail in front of the peak. Generally follows an odd pattern 239 241 ect. Several peaks are near the rounding cut off, ie 239.48 (rounded to 239). Some peaks are over the cutoff for rounding such as, 241.52. In this case I looked at the other peaks. 239.48 is about 2 base pairs from 241.52. I called them at 239 and 241 respectively. Other peaks, some large, are visible at approximately 295 base pairs. This most likely represents another locus that is not amplifying consistently. It is ignored.

Locus 12. Multiple peaks, difficult to call but multiple peaks have the shape a single peak. The repeat is even. Each set of peaks is treated as a single peak; I called the largest peak in each set.

Locus 13. Multiple peaks, difficult to call but peaks have the shape a single peak. Each set of peaks is treated as a single peak; I called the largest peak in each set.

Locus 17. One large peak, generally the base pair repeat is 4 base pairs, the peaks must be 4 base pairs apart. Several peaks were 319.91 318.83 ect. There were consolidated to 319. The other sizes are 311 and 323.

Locus 23. Clear peaks; mainly even for example 370 and 380 base pairs, not much noise.

Locus 27. Peaks are clear, there is a large poly a tail on all peaks, in some cases the tail is large enough to give the appearance of a double peak. The second, larger peak is called at the fragment size. The majority of the peaks round to an odd repeat, several round to an even repeat this is seen especially in homozygotes.

Locus 30. Pattern has four peaks for each fragment, I called the last, usually largest, peak.

Locus 31. Odd repeat pattern, good spacing, and clear peaks

APPENDIX B. TABLE OF INDIVIDUAL ALLELES

Table B.1. The fragment sizes and frequencies for all loci and populations are given. The loci and fragment sizes are given in the left column. The number of individuals (N) genotyped at each locus is given for each locus.

	Bluestem	A annex	Ulen	Viking	Bicentennial	Dalby	Syre	Hartke
Locus								
N	25	30	30	28	1	6	2	6
2								
177	0.100							
179		0.017	0.033					
181		0.100		0.054				
183	0.020	0.283	0.167	0.161		0.167	0.250	0.083
185	0.400	0.167	0.317	0.286		0.333		0.667
187	0.020	0.233	0.283	0.125	1	0.500		0.167
189	0.180	0.100	0.133	0.196			0.750	
191		0.067		0.089				
193	0.060		0.017					
195	0.040		0.033	0.018				
197			0.017	0.054				0.083
199	0.180	0.017						
203				0.018				
205		0.017						
N	23	29	29	29	1	6	2	6
5								
202	0.022	0.017		0.069				
204	0.283	0.345	0.431	0.293				0.583
206	0.370	0.241	0.207	0.155		0.400	0.500	
208	0.239	0.276	0.310	0.483		0.500	0.500	0.417
210	0.087		0.034			0.100		
214		0.103	0.017					
220		0.017						
N	25	30	30	30	1	6	2	6
7								
239	0.120	0.283	0.417	0.217	0.500	0.250	0.500	0.083
241	0.180	0.583	0.367	0.333	0.500	0.667	0.500	0.583

Table B.1. Continued

243	0.700	0.133	0.217	0.333		0.083		0.333
245				0.117				
N	25	30	30	28	1	6	2	6
12								
246				0.018				
250		0.017						
252				0.018				
256		0.033						
258				0.018				
260						0.083	0.250	
262		0.100						
266		0.150		0.232				
268			0.067					
270	0.140	0.017						
272	0.020	0.017	0.017					
274		0.017						
276	0.020							
278		0.033	0.033					0.083
280		0.033	0.083			0.083		
282	0.240		0.133			0.083		
284		0.050	0.083	0.107				0.083
286		0.017	0.050	0.018		0.083		
288		0.050	0.017			0.167	0.250	0.167
290		0.017		0.179				0.083
292	0.180	0.033		0.071				0.250
294	0.240	0.050	0.083	0.018				
296	0.060	0.067	0.133	0.018				
298		0.100	0.017	0.018				
300		0.033					0.250	
302		0.033						
304		0.033	0.017	0.107	1.0	0.500		
306	0.060	0.033		0.018				
308			0.033	0.107			0.250	
310			0.050					
312	0.020							
314			0.017					
316			0.050	0.036				0.333

Table B.1. Continued

318		0.050	0.050	318		0.050		
320			0.033					
322			0.033					
328		0.017						
332	0.020							
382				0.018				
N	24	30	28	28	1	6	2	6
17								
311	0.229					0.100		
319	0.771	0.950	1.000	1.000	1.000	0.900	1.000	1.000
323		0.050	0.000	0.000				
N	25	30	27	25	1	6	2	6
27								
433		0.017		0.020				0.333
435	0.760	0.483	0.315	0.380	0.500			0.250
437		0.017					1.000	
441	0.040		0.056	0.100				
443	0.020	0.067	0.037	0.220		0.250		0.417
445	0.040	0.133	0.204	0.080	0.500	0.500		
447		0.283	0.185	0.020		0.250		
449	0.040		0.204	0.160				
451	0.100			0.020				
Mean Heterozygosity								
Ho 0.458		0.565	0.497	0.543	0.333	0.300	0.417	0.389
He 0.588		0.633	0.632	0.638	0.167	0.529	0.354	0.495
Mean allels per locus 5.66667		8.1667	7	7	0.875	2.5	1.5	2.375