**Genetic Diversity of *Cypripedium parviflorum* in Door County, Wisconsin**
**Jason Miller**

**Abstract**

*Cypripedium parviflorum* is one the 12 *Cypripedium* species found in North America and while being found across almost all North America, current populations are under threat from poaching and loss of habitat from human expansion. By utilizing microsatellite markers to assess the levels of genetic diversity within and amongst populations will give insight into a species adaptability and potential for long term survival. Currently there have been no SSR primers developed for this species so initial testing began with microsatellite loci developed for four closely related *Cypripedium* species and while some loci positively amplified DNA samples, the subsequent sequencing revealed that the microsatellite motif was not present.

**Introduction**

Conservation biologists use estimates of genetic diversity as an indication of the ability of populations to adapt to changing environmental conditions. For rare or endangered species genetic diversity plays an important role in population viability versus extinction and the lower the genetic diversity is for a rare species the more vulnerable it becomes to stresses both biological and environmental (Pandey et al., 2015).

While orchid conservation efforts in Door County, Wisconsin, have had a resurgence in recent years, there is still no data on the levels of genetic variation and diversity for populations of *Cypripedium parviflorum*. Having this information would allow conservation managers to create species-specific management plans that would benefit the plant population and hopefully result in greater success in long-term orchid restoration.

Many orchid species which used to be more widespread are now found in small, fragmented populations due to both poaching and habitat loss (Krupnick et al., 2013). This isolation, coupled with small population sizes, can result in the loss of genetic diversity and inbreeding (Frankham et al., 2002). Due to the loss of genetic diversity, fragmented populations are adversely affected and their ability to respond to environmental changes is reduced (Frankham, 2005), potentially leading to extirpation or even extinction. Genetic diversity is based on mutation rates, mating systems, population size, bottlenecks, and levels of migration or gene flow amongst populations. Understanding the genetic flow within and among isolated orchid populations will identify if populations are truly isolated or if there is sufficient gene flow amongst the populations.

Currently there have been no population genetic studies conducted on *Cypripedium parviflorum* within Wisconsin or the Great Lakes Region. In order to assess the population genetics of wild populations, microsatellites, or simple sequence repeats (SSRs) are commonly used. However, SSRs are species specific and at the time of this research none have been identified for this species. When primers have not been developed for a specific species, it is common to test the applicability of primers previously published for closely related species due to the high cost of developing species-specific microsatellites. A review of the current literature revealed that SSRs have been developed for other *Cypripedium* species including two Asiatic species: *C. tibeticum* (Li et al., 2017) and *C. japonicum* (Yamashita et al., 2019). In addition to these two species, primers have also been developed for the European lady's slipper, *C. calceolus* (Minasiewicz and Znaniecka, 2014) and the North American Kentucky lady's slipper, *C. kentuckiense* (Pandey and Sharma, 2013). Therefore, this study aims to test the applicability of the primers developed for these closely related *Cypripedium* species.

**Materials and methods**

*Study species*

Yellow lady's slipper orchid (*Cypripedium parviflorum* Salisb.) is a perennial herbaceous orchid native to much of the United States and quite common throughout Wisconsin. This orchid has had pollinators found in *Agapostemon*, *Andrena*, *Apis*, *Ceratina*, *Eristalis*, *Osmia* and *Lasioglossum* documented visiting flowers (NAOCC, 2011). This species is highly variable with four accepted varieties (NAOCC, 2011). In addition to these varieties, this species hybridizes easily therefore making identification challenging. Within Wisconsin the varieties *makasin* and *pubescens* are the only two documented species with the former being of special concern (WI rare plants), however, both have been observed in Door County. Due to the highly variable nature of this species it was not differentiated between the two for this study.

*Field methods*

Data collection took place from June 7th through July 26th following emergence of the plants from dormancy. Sampling occurred at various sites throughout Door County including Appel's Bluff (*n* = 14), The Ridges Sanctuary (*n* = 14), Newport State Park (*n* = 9), Peninsula State Park (*n* = 9; Fig1 1), Toft Point (*n* = 7), and Potawatomi State Park (*n* = 2). An additional 6 collections were made along public roadsides for a total of 61 collections (Fig. 2). At each location, Miller collected GPS coordinates, five to six single-hole punches of leaf tissue and assigned each sample a unique identification number. Additional morphological data was collected on flowering individuals in an effort to delineate between the two varieties occurring in this region.

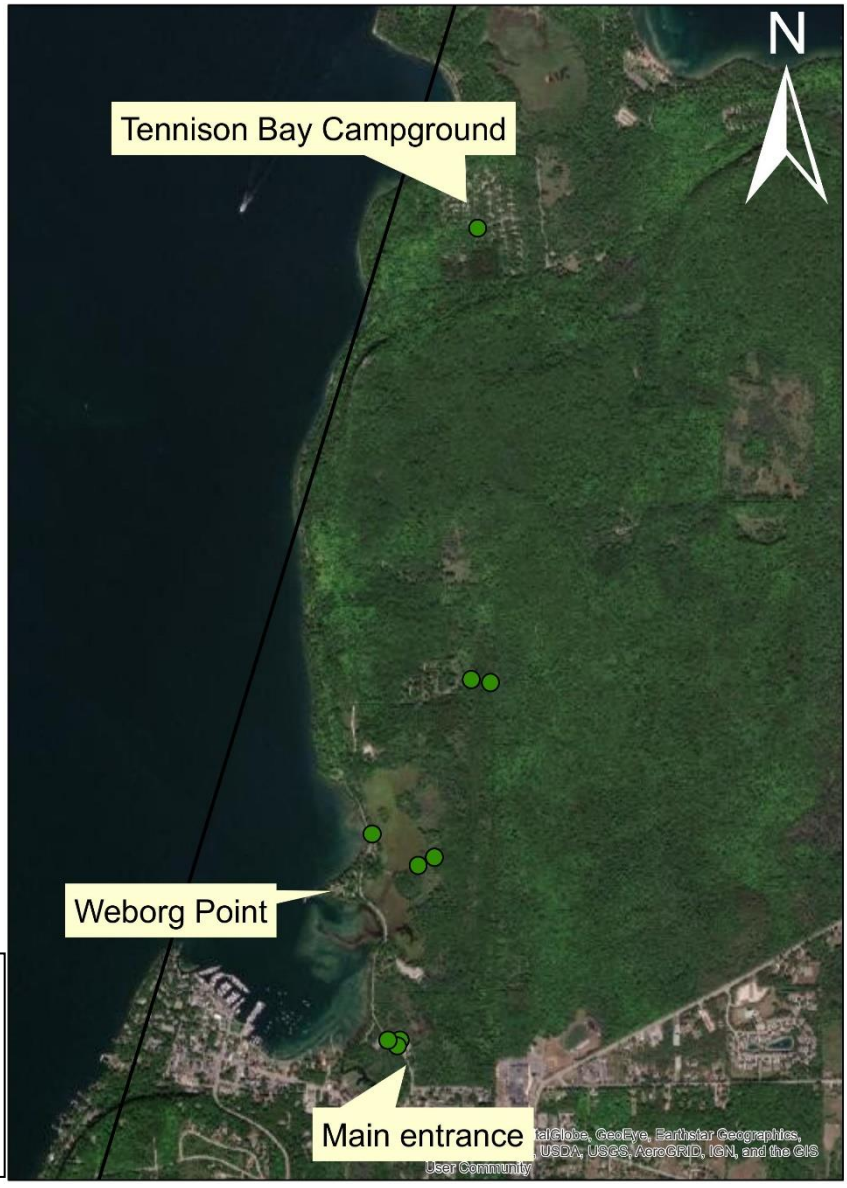


Figure 1. Map of Peninsula State Park showing the locations where collections were made.

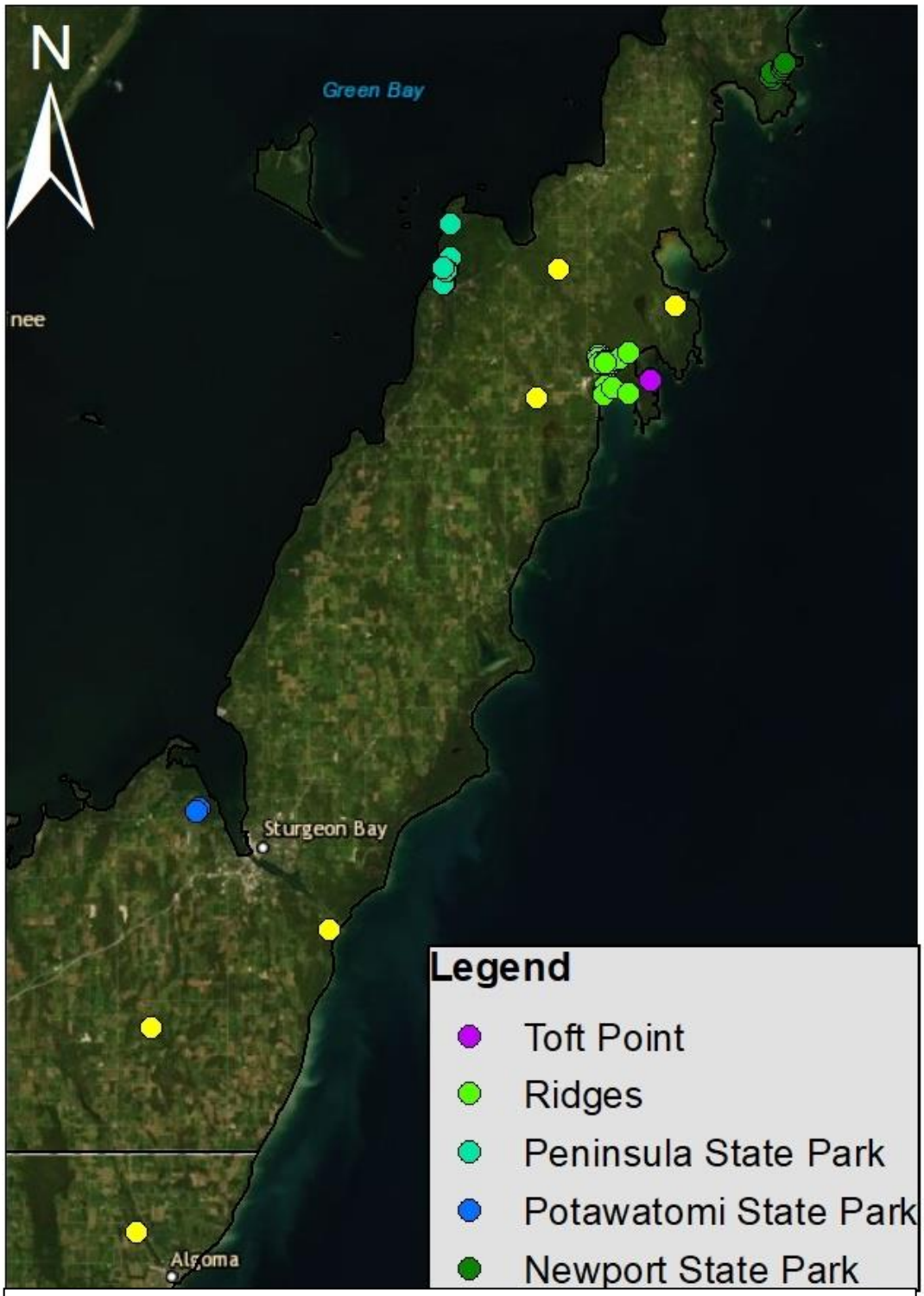


Figure 2. Map of Door County, WI showing the locations where collections were made. Yellow dots represent collections made along public roadsides.

*DNA extraction*

DNA was extracted from two single-hole punches of leaf tissue using Qiagen DNeasy Plant Mini Kit (Qiagen, USA) following the manufacturer's protocol and the quality of DNA was determined using Qubit flurometry. Polymerase chain reaction (PCR) amplification of the DNA samples was carried out in 10 µL reactions composed of 1.0 µL DNA, Promega GoTaq® Colorless Master Mix (Promega, Madison, Wisconsin, USA), 0.2 µM of each forward and reverse primers specific for each locus. Eighteen previously published microsatellite loci were tested (Table 1; Minasiewicz and Znaniecka, 2014; Pandy and Sharma, 2013; Yamashita et al., 2016; Li et al., 2017) using the following PCR conditions: 2 min initial denaturation at 95°C, followed by 35 cycles of denaturation at 94°C for 45 sec, a gradient annealing for 45 sec (ranging from 50°C to 60°C), and an extension at 72°C for 1 min. This was then followed by a final extension at 72°C for 5 min. As these primers were designed for other species, a gradient PCR test was conducted to determine the appropriate annealing temperature. A gradient PCR enables a single 8 unique annealing temperatures to be tested in a single run. Following the gradient PCR, the appropriate annealing temperature was determined for each specific locus and a 25 µL reaction was carried out on eleven of the samples to have an ample sample for DNA sequencing for verification of the microsatellite motif. PCR products were visualized using agarose gel electrophoresis following Grubisha et al. (2014) and were cleaned enzymatically using ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA) following the manufacturer's protocol. PCR products were sequenced at the University of Kentucky HealthCare Genomics Core Laboratory and sequences were viewed in Geneious version R8.1 (Biomatters Inc., Newark, New Jersey, USA) to verify the presence of the microsatellite motif.

Table 1. List of SSR primers tested on *C. parviflorum* DNA samples

| Species | Locus | SSR motif | Size (bp) | Acc # | # Alle | Bands present |
|---|---|---|---|---|---|---|
| Minasiewicz, Znaniecka (2014) | | | | | | |
| Cyp calceolus | Ccal_25 | (ATC)16 | 162–180 | KJ130951 | 8 | Y |
| Cyp calceolus | Ccal_50 | (AAG)10 | 122–134 | KJ130958 | 6 | N |
| Cyp calceolus | Ccal_24 | (ATC)13 | 118–123 | KJ130950 | 5 | Y |
| Cyp calceolus | Ccal_49 | (AAG)13 | 115–143 | KJ130957 | 6 | Y |
| Cyp calceolus | Ccal_53 | (AAG)13 | 164–185 | KJ130960 | 6 | Y |
| Cyp calceolus | Ccal_39 | (AC)15 | 154–164 | KJ130954 | 6 | Y |
| Pandey, Sharma (2013) | | | | | | |
| Cyp kentuckiense | Ck_1949 | (CTT)16 | 123–160 | KC990092 | 6 | N |
| Cyp kentuckiense | Ck_383 | (TGAA)11 | 151–175 | KC990095 | 5 | Y |
| Cyp kentuckiense | Ck_N844 | (CTT)12 | 320–377 | KC990098 | 8 | Y |
| Cyp kentuckiense | Ck_D140 | (AG)9 | 201–205 | KC990096 | 3 | Y |
| Cyp kentuckiense | Ck_NO9G | (CT)9 | 176–180 | KC990097 | 3 | Y |
| Li, Luo, Xu (2019) | | | | | | |
| Cyp tibeticum | M112 | (CT)10 | 232–304 | MF398589 | 20 | Y |
| Cyp tibeticum | M139 | (TC)11 | 213–231 | MF398592 | 9 | Y |
| Cyp tibeticum | M370 | (TC)24 | 271–339 | MF398595 | 15 | Y |
| Yamashita et al. (2016) | | | | | | |
| Cyp japonicum | CYPJ047 | (GCG)10 | 191–199 | LC73789 | 4 | Y |
| Cyp japonicum | CYPJ091 | (GA)23 | 125–131 | LC73796 | 4 | Y |
| Cyp japonicum | CYPJ218 | (TA)10 | 220–226 | LC73811 | 4 | Y |
| Cyp japonicum | CYPJ094 | (AG)11 | 128–157 | LC73797 | 5 | Y |

*Results*

Of the SSR primers tested, only two did not positively amplify the DNA samples of *Cypripedium parviflorum.* There were five samples that resulted in visible double bands, suggesting heterozygous alleles, or extremely faint bands when viewed using agarose gel electrophoresis. Faint bands could be the result of low quality or quantity of DNA in a specific sample whereas multiple bands (>2) could indicate the primer used is amplifying more than one area of the DNA. Six samples had single clear single bands and the PCR product was subsequently cleaned and sent off for sequencing. Upon receiving the completed sequences, they were imported into Geneious and viewed to determine if the particular motif was present for each sample. While snippets of the motif were present in each sample, the motif in its entirety was not present, thus not enabling the use of the microsatellite for assessing the genetic diversity.

**Discussion**

Given the high cost of developing species specific SSRs, it is common practice to test primers that have been developed for closely related species. Although the DNA sequencing showed that the microsatellite motif for the tested loci was not present in these samples funding has been secured to begin the development of primers specific for *Cypripedium parviflorum*. Therefore, this research will continue with the goals of assessing the genetic diversity of the populations in northeast Wisconsin.

**Literature Cited**


Frankham R, (2005). Genetics and extinction. *Biological Conservation* **126:** 131-140.

Frankham R., J.D. Ballou, D.A. Briscoe, (2002). *Introduction to conservation genetics* Cambridge University Press, Cambridge.

Grubisha LC, Dowie NJ, Miller SL, Hazard C, Trowbridge SM, Horton TR, Klooster MR (2014) Rhizopogon kretzerae sp. nov.: the rare fungal symbiont in the tripartite system with Pterospora andromedea and Pinus strobus. *Botany* **92**: 527-534.

Krupnick GA, McCormick MK, Mirenda T, & Whigham DF, (2013). The status and future of orchid conservation in North America. *Annals of the Missouri Botanical Garden* Vol 99, No 2: 180-198.

Li J., Luo Y., Xu L., (2017). Development of microsatellite markers for *Cypripedium tibeticum* (Orchidaceae) and their applicability to two related species. *Application in Plant Sciences* **5**(12): 1700084.

Minasiewicz J, Znaniecka, JM, (2014). Characterization of 15 novel microsatellite loci for *Cypripedium calceolus* (Orchidaceae) using MiSeq sequencing. *Conservation Genetics Resources* **6**: 527-529.

North American Orchid Conservation Center, NAOCC, (2011). Cypripedium parviflorum (Yellow lady's slipper): Go Orchids. https://goorchids.northamericanorchidcenter.org/species/cypripedium/parviflorum/

Pandy M, Richards M, Sharma J, (2015). Microsatellite-based genetic diversity patterns in disjucnt populations of a rare orchid. *Genetica* **143**: 693-704.

Pandy M, Sharma, J, (2013). Characterization of microsatellite loci for *Cypripedium kentuckiense* (Orchidaceae). *Conservation Genetics Resources* **5**: 1031-1033.

Wisconsin's rare plants, (2019). https://dnr.wi.gov/topic/endangeredresources/plants.asp

Yamashita Y., Izuno A., Isagi Y., Kurosawa T., Kaneko S., (2016). Isolation and characterization of novel microsatellite loci for the endangered orchid *Cypripedium japonicum* (Orchidaceae). *Applications in Plant Sciences* **4**(2): 1500097.