University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
May 2018

Population Genetic Structure and Hybridization within Western North Carolina *Sarracenia* (Pitcher Plants)

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Abstract

Sarracenia (pitcher plants), a genus of carnivorous perennial herbs, includes many species of conservation concern. S*arracenia* species can hybridize when in sympatry, with seemingly few pre-zygotic barriers to cross-fertilization. Two pitcher plant species, *S. jonesii* (mountain sweet pitcher plant) and *S. purpurea* var. *montana* (mountain purple pitcher plant), are native to western North Carolina bogs, and others, including *S. flava* (yellow pitcher plant) and *S. leucophylla* (white pitcher plant), have been introduced to the region. This study examined the genetic composition of phenotypically hybrid plants and *S. pupurea* var. *montana* individuals from a site in which these four species co-occur. Plants were non-destructively sampled, DNA was extracted, and samples were PCR-amplified at 5 diagnostic (hybrid) or 5 variable (*S. purpurea* var. *montana*) microsatellite loci; after fragment analysis, microsatellite lengths were quantified in Geneious©. Calculations of hybrid indices showed that all individuals contained *S. jonesii* and *S. purpurea* var. *montana* DNA. The contribution of *S. jonesii* DNA to hybrid plants ranged from 20 - 60% and *S. purpurea* var. *montana* DNA ranged from 20 - 40%. The latter result is of particular concern, as *S. purpurea* var. *montana* is being considered for federal listing. Genetic diversity indices for *S. purpurea* var. *montana* individuals showed moderate levels of allelic and genotypic diversity. Ongoing experiments are investigating genetic diversity within and among *S. jonesii* and *S. purpurea* var. *montana* sites in western North Carolina, to better understand population dynamics and prioritize conservation work.

1. Introduction

The biological species concept defines species as a group of populations with the ability to interbreed and produce viable, fertile offspring[3].This species concept does not address the formation of hybrids, offspring of two individuals from different species or varieties. Hybrids range from fully fertile to sterile, in their ability to produce their own offspring. There are many different barriers to hybridization, and even closely related species are often incapable of producing viable hybrids due to pre- or post-zygotic reproductive barriers. These include hybrid inviability or sterility, phenological differences, changes in morphology, and gamete incompatibility[9, 13, 17]. The process of hybridization can be costly and inefficient, with the possibility of producing maladapted individuals. This generates selective pressures to decrease the amount of gene flow among species[9], and such hybrid incompatibility helps reinforce reproductive barriers that allow species differentiation[11]. The process by which selective pressures against hybridization increase reproductive isolation between species in sympatry is known as reinforcement[9].

Hybridization has the potential to form new lineages and can have long lasting evolutionary consequences. Despite the many barriers that prevent successful hybridization, plants have often been observed in nature to readily hybridize with each other[22]. There are multiple ways in which hybridization can affect the evolution of plant species. When two plant species are crossed together, the resulting hybrid offspring often have increased growth rates, reproductive output, and biomass at maturity. This phenomenon is known as hybrid vigor or heterosis. Similar to this is the process of transgressive segregation, in which phenotypic traits in later hybrid generations fall outside the range of the parental

variation; though this process can have a negative or positive effect on the hybrid lineage[8]. These different processes show how hybridization can allow new lineages to adapt to ecological niches and express new phenotypes. Hybrids even allow the parental species to share genetic information between each other via mating across generations, a process known as introgression[8]. Hybridization can drive diversification in plant species and allow the new lineages to colonize more extreme niches than their parents. One such example is the plant genus *Helianthus* (sunflowers), in which there are three known hybrid lineages that live in habitats outside of the range of the parent species, showing the adaptive novel phenotypes that hybridization produces[19].

The genus *Sarracenia* (pitcher plants) has species that naturally hybridize when in sympatry both in the wild and *ex situ*. Hybridization is complex within the genus, with interspecific gene flow being common, rare, or even absent in some species and uneven or unidirectional in others[4]. Some barriers to hybridization include differences in flowering phenology and allopatry of species ranges which lessen rates of interspecific hybridization among species. Almost every species in the genus will produce a hybrid when paired together in close proximity in nature, and especially when cultivated[4]. Hybrids tend to be phenotypically intermediate to parent species yet, the consequence of hybridization for fitness is unknown. Identifying hybrid species based on phenotype can be difficult in sites with rampant hybridization or introgression. The role that hybridization plays in generating morphological and genotypic diversity is not well understood, and there has been much debate in regards to the phylogeny of the genus[4].

Sarracenia contains 11 perennial herbaceous species found throughout North America. It is one of three genera of carnivorous plants belonging to the family Sarraceniaceae; the other two are *Darlingtonia* in North America and *Heliamphora* in South America[12]. Most *Sarracenia* species are located in the wetlands of the coastal plain of the southeastern US[17]. Pitcher plants are able to photosynthesize all of their carbohydrates but require supplemental insects and other small animals in their diet to obtain nitrogen. They commonly grow in acidic environments such as acid bogs and similar wetlands, with poor soils with low availability of nitrogen and other minerals[12]. In order to capture their prey, *Sarracenia* typically have specialized leaves that act as funnels that are filled with water into which prey fall and drown. The prey is then enzymatically digested by the pitcher plant in some individuals. In others, the prey will eventually be broken down into nutrients through a complex food web that the pitcher plant hosts[18]. In order to attract both pollinators and prey, *Sarracenia* have colorful flowers with specialized glands, also found on the pitcher, which produce large amounts of nectar[12]. Pollination is mainly facilitated by the bumblebee (*Bombus* spp.), the honey bee (*Apis mellifera*), and to a smaller extent the fly *Fletcherimyia fletcheri*[4, 12]. All species produce waxy, hydrophobic seeds that are distributed by water or by air and can reproduce asexually via rhizomes[12].

Most *Sarracenia* species are located in the southeastern United States, where human land use conflicts with high levels of biodiversity; as a result several *Sarracenia* species are threatened due to a variety of factors[15]. Agricultural activity, over-collection, invasive species, and pollution are largely responsible for reductions in population size, and as a result several species of the genus are listed as endangered[10]. Federal rules for listing a species as endangered rely on five different factors outlined in the Endangered Species Act of 1973, including: damage to the species habitat; overutilization of the species due to commercial, recreational, educational, or scientific reasons; disease or predation; and other natural or artificial factors that endanger the species' continued existence[20]. Two natives of western North Carolina bogs are *Sarracenia jonesii* (mountain sweet pitcher plant) and *Sarracenia purpurea* var. *montana* (mountain purple pitcher plant). *Sarracenia jonesii* is listed as endangered, while *S. purpurea* var. *montana* is a Federal Species of Concern (FSC)[20]. Two congeners that have been introduced to the region include *S. flava* (yellow pitcher plant) and *S. leucophylla*, neither of which is federally listed.

Species that are rare in numbers and have a narrow geographical range tend to be low in genetic diversity[5]. The opposite is true for widespread species, which typically have larger population sizes that help maintain allelic diversity and greater genetic structure. *Sarracenia purpurea* (*sensu lato*) is the most widespread species in the genus, ranging from Florida as far north as Newfoundland, and as such has the greatest genetic diversity[7]. *Sarracenia jonesii* are found in only ten small populations scattered in North Carolina and South Carolina[6]. Human activity resulting in habitat loss can reduce population sizes and have a greater effect on genetic diversity in this species. *Sarracenia jonesii* has been observed across its geographic range to be low in diversity as revealed by allozyme, non-coding chloroplast, and microsatellite markers[5, 6, 17].

Microsatellites are used to better understand population dynamics by acting as molecular markers for genetic diversity[21]. A previous study published 25 microsatellite loci that showed variability among and within *Sarracenia* species in order to help facilitate conservation genetic analyses of the genus[17]. Microsatellites are short tandem repeats in non-coding DNA regions; they are 2-10 base pairs long. The number of repeats varies in length among individuals, as the specific microsatellite loci are not subject to natural selection. These variations are analyzed using PCR and fragment analysis, and the resulting data can reveal genetic diversity among populations[14].

In this study we used microsatellite DNA analysis to examine the genetic composition of phenotypically hybrid individuals at a site in western North Carolina where four different *Sarracenia* species co-occur. We surveyed six of

the published microsatellite loci known to distinguish among *Sarracenia* species in order to calculate hybrid indices. Our goal was to determine the degree to which these species hybridize, and provide population genetic data integral to conservation and restoration strategies for these species. We hypothesized that different hybrid individuals would vary in their parentage. This study also analyzed five variable *S. purpurea* microsatellite loci to determine the genetic variation within and among *S. purpurea* var. *montana* individuals. These individuals were collected from three different sites across two different counties in western North Carolina. We expected low genetic diversity among *S. purpurea* var. *montana* individuals due to population isolation and small population sizes.

2. Methods

Samples of 11 phenotypically hybrid *Sarracenia* individuals from a single population in Transylvania County, NC, were non-destructively collected from a site in which four different congeners (*S. flava, S. jonesii, S. leucophylla*, and *S. purpurea* var. *montana*) co-occur. Tissue was cut from leaf keels so as not to induce whole-plant mortality and whole genomic DNA was extracted using modified Qiagen DNeasy® Plant Mini Kits©. For each extract, six microsatellite loci able to discriminate between *S. jonesii* and *S. purpurea* var. *montana* (SARR02, SARR028, SARR032, SARR035, SARR040, and SARR042)[17] were PCR-amplified using the following recipe: 16 µl of Promega 2X Master Mix©, 1 µl 10 µM Eurofins© M13-labeled forward primer, 1 µl 10 µM Eurofins© M13 6-FAM fluorophore, 2 µl 10 µM reverse primer, 18 µl of PCR water, and 2 µl of DNA extract. Samples were run in a Bio-Rad T100 Thermal Cycler© using the following protocol: 2 min at 94°C, 45 sec at 94°C, 1 min at 48°C, 45 sec at 72°C, then 34 cycles of the previous 3 steps, followed by a final extension at 72°C for 10 min. PCR products were checked via electrophoresis on 1% agarose gels, and confirmed products were multiplexed, mixed with the GeneScan 500© ladder, and shipped to the DNA Analysis Facility at Yale for fragment analysis. Fragments were quantified using Geneious© 11.2.0, and percent loci amplified were used to calculate hybrid indices. Note; data for one of the microsatellite loci amplified were discarded due to ambiguous results.

Tissue samples from 32 *S. purpurea* var. *montana* plants were collected from 3 sites in western North Carolina, and DNA was extracted using the procedure above. Extracted DNA samples were PCR-amplified using the following recipe: 16 µl of Promega 2X Master Mix©, 2 µl 10 µM IDT© fluorescent-labeled forward primer, 2 µl 10 µM Eurofins© reverse primer, 18 µl of PCR water, and 2 µl of DNA extract. Amplifications for *S. purpurea* var. *montana* individuals were done to loci SARR05, SARR07, SARR20, SARR58, and SARR60, and fragments were analyzed as above[17]. Population genetic indices were calculated using the *polysat* package in RStudio 3.1.0[1].

3. Results

Phenotypic hybrids contained 20 - 60% *S. jonesii* alleles and 20 - 40% *S. purpurea* var. *montana* alleles at diagnostic loci (Table 1). Note that data for Hybrid Individual 9 appear unusual since this plant showed alleles diagnostic for two species at one locus (SARR032; Table 1). Unique microsatellite alleles for *S. purpurea* var. *montana* individuals were found at each site tested (Table 2). Additionally, genotype diversity ranged from 0.6 – 1.8 across all three sites (Table 2). Pairwise F_{ST} values ranged from 0.05 – 0.15, indicating moderate genetic differentiation among all *S. purpurea* var. *montana* populations tested (Table 3).

Table 1. Hybrid indices for 11 individuals. Indices were calculated using diagnostic microsatellite fragment length data from five loci known to distinguish among *Sarracenia* species[17]. The species of origin was scored for each locus.

Hybrid Individual	*S. flava*	*S. jonesii*	*S. leucophylla*	*S. purpurea*
1	-	60%	20%	20%
2	20%	20%	20%	40%
3	20%	40%	20%	20%
4	20%	40%	20%	20%
5	-	40%	20%	40%
6	50%	25%	-	25%
7	-	60%	20%	20%
8	20%	40%	20%	20%
9	-	50%	16.7%	33.3%
10	40%	20%	20%	20%
11	-	50%	-	50%

Table 2. Genetic diversity indices for *S. purpurea* var. *montana* sites calculated using the *polysat* package in RStudio. Data were input from 5 microsatellite loci known to be variable within *Sarracenia purpurea*[17].

Population	Population Size	# of Plants Genotyped	Total Alleles (A)	# Unique Alleles	Genotype Diversity
DB	297	10	20	1	1.8343720
HC	100+	11	19	2	0.6001661
RL	50+	11	24	4	1.5941667

Table 3. Pairwise F_{ST} values for *S. purpurea* var. *montana* sites, calculated using the *polysat* package in RStudio.

	DB	HC	RL
DB	0.00	-	-
HC	0.08285	0.00	-
RL	0.06181	0.07687	0.00

4. Discussion

Data for *S. purpurea* var. *montana* analyses revealed moderate levels of genetic diversity at all three sites in western North Carolina. This is likely a result of inbreeding within spatially isolated populations. The unique alleles discovered for each site could be the product of genetic drift. All populations of *S. purpurea* var. *montana* tested so far exist in the same watershed, and alleles could be spread between sites due to seed dispersal via rivers. Our values are consistent with those found for other threatened and endangered plant species[2, 7].

DNA from both *S. purpurea* var. *montana* and *S. jonesii* was found in all phenotypically hybrid individuals tested, demonstrating that introgression between these and other congeners occurs under field conditions. This rampant introgression could jeopardize the latter's Federal Species of Concern status, or block its eligibility for listing as a threatened or endangered species[20]. In addition, work by labmate Lila Uzzell showed that phenotypic hybrids produced more ovules than either *S. jonesii* or *S. purpurea* var. *montana*, and that their pollen and seed production was intermediate to parental types. *Sarracenia jonesii is* already vulnerable to extinction due to its small population sizes

and lack of genetic diversity[5, 6]. Continued production of fit hybrid offspring could create competition for space, nutrients, and pollinators. Thus, planting endangered species like *S. jonesii* in sympatry with other *Sarracenia* species is problematic as parental species could be lost to hybrid swarms.

Future work will focus on expanding microsatellite analysis for *S. purpurea* var. *montana* individuals by including additional plants and loci. In addition, the study will analyze microsatellite loci for phenotypic hybrids from additional protected sites in which only *S. jonesii* and *S. purpurea* var. *montana* co-occur. Together, this dataset will allow us to make conservation recommendations for these species to the United States Fish and Wildlife Service.

5. Acknowledgements

The author wishes to express his appreciation to Dr. Rebecca Hale, Professor Caroline Kennedy, Dr. Mara Alexander, Dr. H. David Clarke, Dr. Matthew Greene, and Dr. Emily Coffey. Chelsea Brown, Steven Jaslow, Lila Uzell, Christine Mowad, Magnolia Wilson, Danielle Pierson, Christian Muñoz, and Elise Powell contributed to sample collection, data analysis, and presentation of results. Special thanks to the North Carolina Plant Conservation Program, Cathy Reimer, and the Highlands Biological Station for specimens. This project was funded by grants from the UNC Asheville Undergraduate Research Program and the United States Fish and Wildlife Service.

6. References

1. Clark, Lindsay V and Jasieniuk, M. “POLYSAT: an R package for polyploid microsatellite analysis.” *Molecular Ecology Resources* 11.3 (2011): 562-566. Web.
2. Ellstrand, Norman C., and Diane R. Elam. "Population Genetic Consequences of Small Population Size: Implications for Plant Conservation." *Annual Review of Ecology and Systematics* 24.1 (1993): 217-42. Web.
3. Mayr, Ernst, Quentin D. Wheeler, and Rudolf Meier. *Species Concepts and Phylogenetic Theory: A Debate*. New York: Columbia UP, 2000. Print.
4. Furches, Marvin Steven, "The role of hybridization in generating species- and population-level variation in Sarracenia (Sarraceniaceae)." *University of Tennessee* (2012). Web.
5. Furches, M. S., R. L. Small, and A. Furches. "Genetic Diversity in Three Endangered Pitcher Plant Species (Sarracenia; Sarraceniaceae) Is Lower than Widespread Congeners." *American Journal of Botany* 100.10 (2013): 2092-101. Web.
6. Godt, Mary Jo W., and J. L. Hamrick. "Genetic Structure of Two Endangered Pitcher Plants, *Sarracenia jonesii* and *Sarracenia oreophila* (Sarraceniaceae)." *American Journal of Botany* 83.8 (1996): 1016. Web.
7. Godt, Mary Jo W., and J. L. Hamrick. "Genetic Divergence among Infraspecific Taxa of Sarracenia purpurea." *American Society of Plant Taxonomists* 23.4 (1998): 427-438. Web.
8. Goulet, Benjamin E., Federico Roda, and Robin Hopkins. "Hybridization in Plants: Old Ideas, New Techniques." *Plant Physiology* 173.1 (2016): 65-78. Web.
9. Hopkins, Robin. "Reinforcement in Plants." *New Phytologist* 197.4 (2013): 1095-103. Web.
10. Jennings, David E., and Jason R. Rohr. "A Review of the Conservation Threats to Carnivorous Plants." *Biological Conservation* 144.5 (2011): 1356-363. Web.
11. Johnson, N. (2008) “Hybrid incompatibility and speciation.” *Nature Education* 1(1):20
12. Les, Donald H. *Aquatic Dicotyledons of North America Ecology, Life History, and Systematics*. N.p.: CRC, 2017. Print.
13. Levin, Donald A. "The Origin of Reproductive Isolating Mechanisms in Flowering Plants." *Taxon* 20.1 (1971): 91. Web.
14. Nybom, Hilde. "Comparison of Different Nuclear DNA Markers for Estimating Intraspecific Genetic Diversity in Plants." *Molecular Ecology* 13.5 (2004): 1143-155. Web.
15. Ricketts, Taylor, and Marc Imhoff. "Biodiversity, Urban Areas, and Agriculture: Locating Priority Ecoregions for Conservation." *Conservation Ecology* 8.2 (2003): n. pag. Web.
16. Rieseberg, L. H., and J. H. Willis. "Plant Speciation." *Science* 317.5840 (2007): 910-14. Web.
17. Rogers, Willie L., Jennifer M. Cruse-Sanders, Ron Determann, and Russell L. Malmberg. "Development and Characterization of Microsatellite Markers in *Sarracenia l.* (pitcher Plant) Species." *Conservation Genetics Resources* 2.1 (2010): 75-79. Web.
18. Slack, Adrian. Carnivorous Plants. Cambridge, MA: MIT, 1980. Print.

19. Stevison, L. (2008) “Hybridization and gene flow.” *Nature Education* 1(1):111

20. "U.S. Fish and Wildlife Service." *Official Web Page of the U S Fish and Wildlife Service*. N.p., n.d. Web. Spring 2018.

21. Varshney, Rajeev K., Andreas Graner, and Mark E. Sorrells. "Genic Microsatellite Markers in Plants: Features and Applications." *Trends in Biotechnology* 23.1 (2005): 48-55. Web.

22. Whitney, Kenneth D., Jeffrey R. Ahern, Lesley G. Campbell, Loren P. Albert, and Matthew S. King. "Patterns of Hybridization in Plants." *Perspectives in Plant Ecology, Evolution and Systematics* 12.3 (2010): 175-82. Web.