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Confirming Hybridization Status Between Two North American Pitcher Plants: *Sarracenia jonesii* and *Sarracenia purpurea* var. *montana*

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Abstract

Habitat loss and modification due to factors such as climate change, as well as human activity such as poaching and overcollection, have led to many plant species becoming rare or endangered. These activities have also reduced population genetic diversity, which significantly influences the ability to adapt to environmental changes. *Sarracenia* species (North American pitcher plants) are rare, perennial carnivorous plants typically found in bogs whose soils are very acidic and nutrient poor. *Sarracenia jonesii* (Jones' pitcher plant; federally endangered) and *Sarracenia purpurea* var. *montana* (mountain purple pitcher plant; federal species of concern) co-occur in some habitats, and the presence of phenotypic intermediates in these sites led scientists to believe that hybridization has occurred. Although the intermediates have morphological traits of both *Sarracenia jonesii* and *Sarracenia purpurea* var. *montana*, this potential hybridization had not been investigated with molecular genetic tools. The purpose of this research was to determine levels of genetic diversity in populations of these rare plants, then confirm or deny hybridization between these two species. Tissue samples from the two *Sarracenia* species and their potential hybrids were non-destructively collected from three sites in western North Carolina, and DNA was extracted. Two sets of informative microsatellite loci were used to analyze DNA extracts; one indicated population level diversity, while the other determined hybrid ancestry. Fragment analysis was used to assess allele sizes, and results were analyzed using two R packages. Genetic diversity within populations was relatively high, though it varied among sites and species. Hybrids overrepresented ancestry of the less common species at their site of origin. Results could help conservation biologists to preserve both species as separate, diverse taxa and make management decisions, particularly during plants' reproductive seasons.

1. Introduction

Habitat loss and modification due to factors such as climate change, as well as human activity such as poaching and overcollection, have led to many plant species becoming rare or endangered. Genetic diversity significantly influences a population's ability to adapt to environmental change and might be particularly crucial for imperiled species, and genetic studies of rare and plant species allow conservation biologists to assess the importance of genetic diversity for preservation of species[1,2]. For instance, rare plant species usually present lower levels of genetic diversity and hybridization than common plant species[3]. In endangered and rare plant populations, genotypic and allelic diversity is positively correlated with population size[1]. Even if a rare plant species presents high levels of genetic diversity, which is not common, it may still be at risk of extinction due to stresses such as climate change[4], human activity, and specificity of habitat preferences[5].

Hybridization often occurs between different species of plants belonging to the same genus when two parental species (P) produce offspring (F_1 hybrids). Later on, hybrids can breed with each other (creating F_2 hybrids), or they can go back and breed with their parental species in a process known as backcrossing. Hybridization of plant species can have positive outcomes like speciation[6,7] and adaptive genetic diversity[8], but it can also have negative outcomes such as introgression with non-native species[9,10] and loss of genetic diversity in imperiled species[11].

North American pitcher plants (genus *Sarracenia*), a group of carnivorous plants, encompass many rare and imperiled species[12]. *Sarracenia* are in the Sarraceniaceae family, and the genus contains 8 - 11 species as well as multiple subspecies[13]. *Sarracenia* are perennial and are typically found in saturated soils that are highly acidic and low in nitrogen quantity and availability. They obtain most of their nitrogen from insects captured in their pitchers, modified tubular leaves which sometimes store digestive juices and hydrolytic enzymes at the bottom; photosynthesis is done by both pitchers and by phyllodia[14]. Several southern *Sarracenia* species are listed as threatened or endangered at either the federal or state levels as a result of poaching and overcollection (NatureServe). Another important threat to these plants is habitat loss from agriculture or development, or changes to their habitat in response to lack of fire, which has led to habitat fragmentation and degradation[2]. In contrast to most rare plant species, all extant *Sarracenia* species hybridize freely with each other when in sympatry[15]. This might not be surprising, since they diverged from each other less than three million years ago due to phenomena like glaciation[13].

Sarracenia purpurea var. *montana* (mountain purple pitcher plant) is a carnivorous perennial pitcher plant that is a federal species of concern. Found in about 20 populations, it occupies wetlands and bogs in the southern Appalachian Mountains of North Carolina, South Carolina, and Georgia[16,17]. *Sarracenia purpurea* var. *montana* pitchers have a lid, peristome, tube, keel, and sheath. Pitchers collect and store water from the rain, creating an ideal aquatic ecosystem for its prey[18]. *Sarracenia jonesii* (Jones' pitcher plant), also known as the mountain sweet pitcher plant, is found in ten small populations. Four of those populations are in North Carolina, and the other six are in South Carolina. Out of the six populations found in South Carolina, four are protected by the state[12]. At least 16 populations have been extirpated, and this plant has been listed as endangered since 1988[19].

Sarracenia jonesii and *Sarracenia purpurea* var. *montana* grow sympatrically in some western North Carolina sites, and *Sarracenia jonesii* has also been intentionally outplanted into *Sarracenia purpurea* var. *montana* populations. At sites where they co-occur, scientists have observed morphological hybrids that phenotypically resemble both *Sarracenia* species. Insect-mediated cross-pollination, perhaps facilitated by bees, is a likely cause of hybridization[2]. Studying the presence and rate of hybridization between two *Sarracenia* species is important for conservation management decisions, as species may have significantly different genetic make-ups if they are found in sympatry with species with which they can reproduce[20].

My study focuses on understanding patterns of genetic diversity and hybridization patterns of these two pitcher plant species. First, genetic diversity within species and sites is unknown. Since genetic diversity is usually low in imperiled species, we hypothesized that genetic diversity in the three sites studied would be low. Although morphologically some pitcher plants have been found to resemble both *Sarracenia jonesii* and *Sarracenia purpurea* var. *montana*, this potential hybridization had not yet been genetically confirmed. We hypothesized that due to the high probability of hybridization between *Sarracenia* species that hybridization of morphological intermediates would be genetically confirmed at all three sites.

2. Methods

2.1 DNA Extraction

One hundred twenty-two tissue samples of *Sarracenia jonesii* and *Sarracenia purpurea* var. *montana* (Table 1), as well as their hybrids, were collected from three sites in western North Carolina (Table 2) in which both species are sympatric. The tissue was collected non-destructively from each plant's keel, preserved in tubes with silica, and stored at -20 ºC until extraction. Then, a modified CTAB method[21] was used to extract DNA from tissue samples. DNA was quantified and qualified with a NanoDrop ND-1000™ Spectrophotometer. Samples with concentrations greater than 10 ng/μl were diluted by 50% with 1X TE buffer.

Table 1. Number of pitcher plant species (Sp = *Sarracenia purpurea* var. *montana*, Sj = *Sarracenia jonesii*, Hy = hybrid) from each population from which the tissue samples were collected and analyzed. Species identity at the collection stage was based only on morphology.

Population	**Species**	**Number of Plant Replicates**
CM	Sp	28
CM	Sj	8
CM	Hy	3
MB	Sp	12
MB	Sj	15
MB	Hy	1
RL	Sp	21
RL	Sj	20
RL	Hy	16

Table 2. Descriptive table of the three sites analyzed.

Site	When did sympatry begin?	Dominant species
CM	*S. jonesii* introduced into *S. purpurea* populations ~2014	*Sarracenia purpurea* var. *montana*
MB	Unconfirmed	*Sarracenia jonesii*
RL	Have coexisted since 1960 or earlier	neither

2.2 Polymerase Chain Reaction and Agarose Gel Electrophoresis

An effective method to detect hybridization status between two plants is the use of microsatellites, genetic markers that are typically intergenic and vary widely within and among populations[2,22]. A total of seven informative microsatellite loci, published by Rogers et al. (2010), were used for this process. Two sets of primers were used: one to determine population level genetic diversity, and the other one to determine hybrid index (Table 3). In order to determine if certain genetic markers were only found in one or both plant samples, polymerase chain reaction was performed in 13 μl reactions, containing 1X Promega GoTaq Green™ MasterMix, 0.30 μM of forward primer, 0.30 μM of reverse primer, and 5μl of DNA with a concentration of about 10ng/μl. PCR water was added to the total volume of 13 μl.

Table 3. Primer information of all the primers used in *Sarracenia purpurea* var. *montana*, *Sarracenia jonesii*, and their potential hybrids.

Name	**Information**	**Use**	**Fluorescent Tag**	**Annealing Tº (ºC)**
Sarr005	Variable within populations	Population level genetic diversity	NED	56
Sarr010	Allele size varies between species	Population level genetic diversity and hybrid index	6FAM	52
Sarr029	Only amplifies for *S. purpurea*	Hybrid index	VIC	56
Sarr035	Absent in *S. jonesii*	Hybrid index	None	56
Sarr042	Absent in *S. jonesii*	Hybrid index	None	54
Sarr045	Absent in *S. purpurea*	Population level genetic diversity	None	52
Sarr050	Variable within populations	Population level genetic diversity	PET	52

To test for cross-contamination, a negative control was always used, consisting of a PCR cocktail with PCR water instead of DNA. After mixing DNA with the PCR cocktail, samples were centrifuged for 30 s then placed in the BIO-RAD T100^{TM} Thermal Cycler to begin the PCR program. Samples were denatured at 94°C for 4 min, followed by 35 cycles of PCR amplification consisting of denaturation at 94°C for 1 min, annealing at the primer's specific annealing temperature (Table 3) for 1 min and extending at 72°C for 1 min. A final extension was performed at 72°C for 10 min. Samples were then held at 12ºC until confirmatory electrophoresis was performed.

After DNA samples from every pitcher plant sample were analyzed at presence / absence loci (Table 3), fluorescently labeled forward primers were used in remaining PCR cocktails. The procedure for performing PCR and gel electrophoresis followed that outlined above. After PCR, products were run on 2% TBE agarose gels to test for the absence or presence of amplicons. Finally, gels were visualized under UV light and photographed using a UVP BioDoc-It$^{2®}$ Imager.

2.3 Fragment Analysis and Data Analysis

PCR products that amplified successfully were multiplexed and mixed with Hi-Di Formamide (Applied Biosystems) and GeneScan™ 500 ladder (containing LIZ dye). Samples were then mailed to the Genomic Sciences Laboratory at North Carolina State University. Fragments were analyzed and binned using Geneious 2021.1. In order to determine allelic diversity, we used the R package *polysat*[23]. The R package *HIest* was used to calculate hybrid ancestry (S) and heterozygosity (H)[24]. To predict hybrid generation, information from Fitzpatrick (2012) was used (Table 4).

Table 4. Expected ancestry (S) and heterozygosity (H_1) of hybrids, from Fitzpatrick 2012

Class	S	H_1
P1	0	0
P2	1	0
F1	1/2	1
F2	1/2	1/2
B1	1/4	1/2
B2	3/4	1/2

3. Results

Alleles unique to only *Sarracenia purpurea* var. *montana* or to *Sarracenia jonesii* amplified with every primer used for population level genetic diversity. In addition, hybrid status of morphological intermediates between the *Sarracenia* species was genetically confirmed at all three sites.

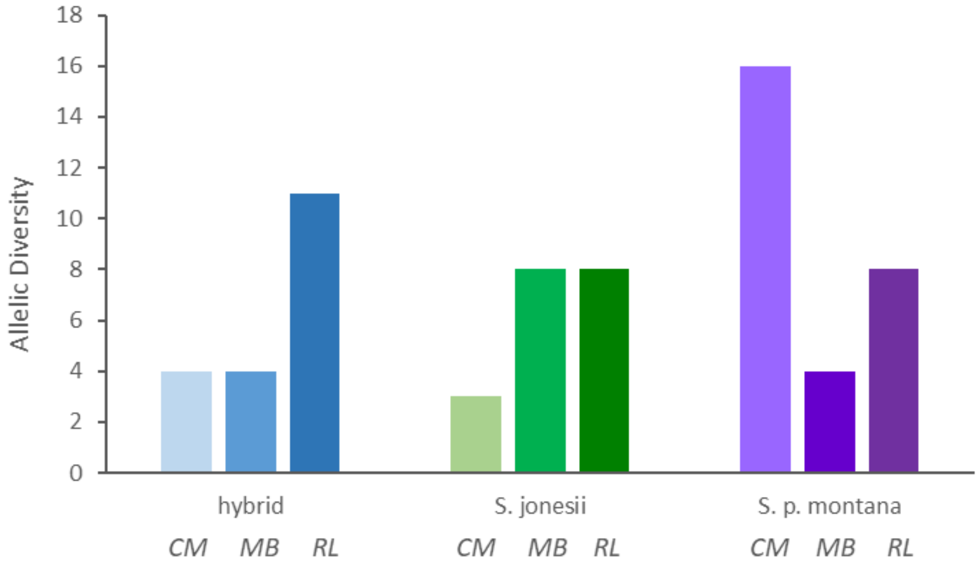

Figure 1. Bar graph of allelic diversity

Figure 1. Data from primer Sarr005 show the allelic diversity of each taxon at each of the three sites. Allelic diversity varies by species and location, and it is relatively high overall.

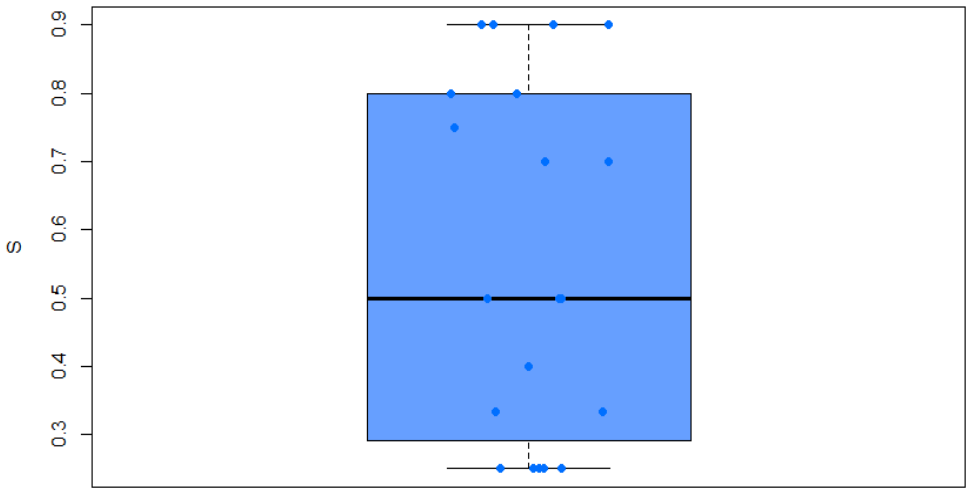

Figure 2. Hybrid ancestry boxplot

Figure 2. This boxplot shows hybrid ancestry (S). The y axis shows ancestry (S) of all hybrids studied, where 1.0 means that a plant is pure *Sarracenia purpurea* var. *montana* (all *S. purpurea* alleles) and 0.0 means that a plant is a pure *Sarracenia jonesii* (all *S. jonesii* alleles). Across all three sites, the median hybridization index is 0.50, an equal representation of both parents' alleles. These results imply that the hybrids could be F_1 or F_2 (Table 3). Clusters of dots closer to 0.0 and 1.0 suggest that proportion of *Sarracenia jonesii* and *Sarracenia purpurea* ancestry was variable.

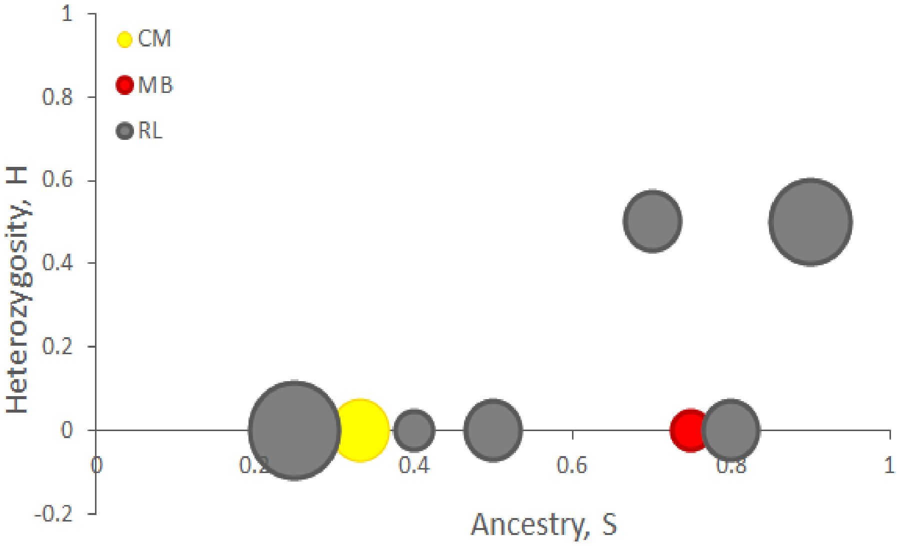

Figure 3. Bubbleplot of Heterozygosity (H) vs. Ancestry (S)

Figure 3. This bubbleplot examined heterozygosity vs. ancestry to more accurately predicting the generation of hybrid plants. The size of the bubble indicates the relative abundance of individuals with the same combination of heterozygosity and ancestry. Low heterozygosity levels across a range of ancestry predict that most hybrids are not from the F_1 or F_2 generations.

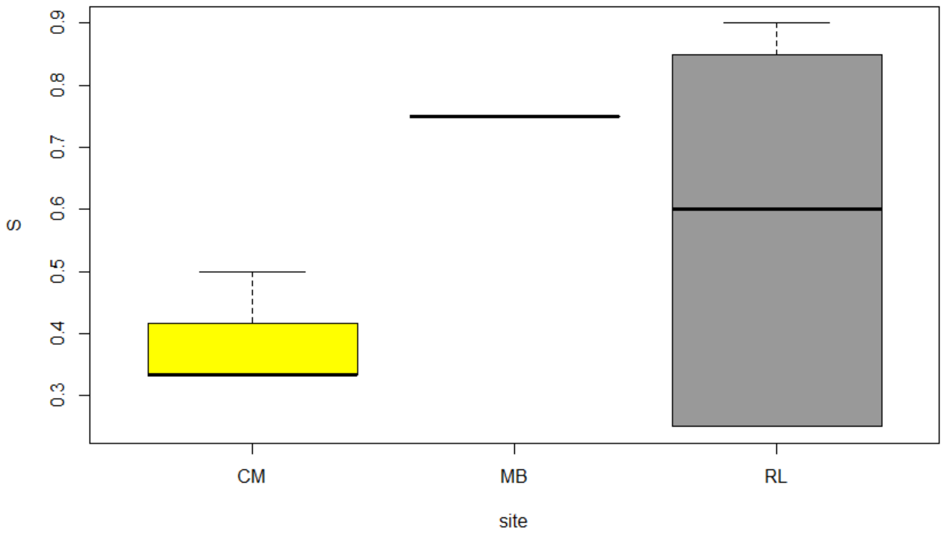

Figure 4. Hybrid ancestry boxplots for each population

Figure 4. Boxplots were created for each site, with ancestry (S) ranging from 0.0 (pure *Sarracenia jonesii*; all *S. jonesii* alleles) to 1.0 (pure *Sarracenia purpurea* var. *montana*; all *S. purpurea* alleles). At site CM, hybrids have more *S. jonesii* ancestry. At site MB, hybrids have more *S. purpurea* ancestry, while at site RL site hybrids have a broad range of ancestry.

4. Discussion

The results of this study indicate high amounts of allelic diversity overall in all three sites studied, which is surprising since generally allelic diversity is low for rare or endangered plant species[1]. However, allelic diversity for a species is higher at sites where that taxon is most abundant (Table 2). CM has the most population level diversity for *Sarracenia purpurea* var. *montana*, which is the numerically dominant species there. MB has more diversity for *Sarracenia jonesii* than *Sarracenia purpurea* var. *montana*, and *Sarracenia jonesii* is the numerically dominant species there. Hybrid diversity is highest at RL, which has more phenotypic hybrids than other sites. This could be due to a larger gene pool, with more opportunity for allelic diversity[25]. The reason why allelic diversity varies among taxons in all three sites could be due to the location of these populations, as edge populations tend to present less allelic diversity than more centrally located populations. These could lead to future problems, as populations and species with less genetic diversity might face higher risks of extinction than the ones with higher diversity levels[5].

An examination of ancestry alone predicted that many hybrids in all three sites are early generation hybrids (F_1 or F_2), as median S is 0.5. However, analysis of hybrid ancestry with both S and heterozygosity shows strong evidence of backcross between the hybrids and their parental species *Sarracenia purpurea* var. *montana* and *Sarracenia jonesii*. Heterozygosity levels for most samples are significantly lower than they would be for early generation hybrids. These results, however, could be skewed as four of five taxonomically informative loci only indicated presence and absence of a genetic marker; this left only one locus that could be used to calculated heterozygosity. Another explanation for potential discrepancies is that Fitzpatrick's metrics[24] were developed for diploid species, and it is likely that *Sarracenia* species are tetraploid. Fragment analyses of these data often revealed up to four different alleles at a single microsatellite locus.

It was surprising that, at each site, the less abundant taxon contributes disproportionately to hybrid ancestry. At the CM site, *Sarracenia purpurea* var. *montana* is the most common taxon, but hybrids are more *Sarracenia jonesii* (they present more *Sarracenia jonesii* specific alleles), at the MB site, *Sarracenia jonesii* is more common numerically than the other taxons, but the hybrids are more *Sarracenia purpurea* var. *montana* (they present more *Sarracenia purpurea* var. *montana* specific alleles), and at the RL site, both species are similarly abundant, so hybrids have a broad range of ancestry. As in Furches et al. (2013), all species do not contribute equally to hybridization. Possible reasons for the significance of these unexpected results include pollinator preference, natural selection, or avoidance of competition with parental species.

4.1 Further Studies And Implications

Hybrid status of morphological intermediates between the *Sarracenia* species was genetically confirmed at all three sites. However, analysis was not complete on all plant-primer combinations, which is why further steps should include complete analyses of population genetic data. Additional primers could reveal more distinctions among sites and species. More tests must be run using more taxonomically informative loci indicating heterozygosity values and hybrid ancestry. This will be necessary to confirm the backcrossing of the early generation hybrids with their parental species. Further studies could also involve the confirmation of ploidy of *Sarracenia* species, as there is not a lot of research done on this topic.

Findings of this research will be important so that we can provide conservation recommendations to conservation biologists and the US Fish and Wildlife Service in order to protect *Sarracenia purpurea* var. *montana* and *Sarracenia jonesii*. This will help preserve these two *Sarracenia* species and make conservation management decisions, as plant species might have significantly different genetic make-ups if they are found in sympatry with species with which they can reproduce. Care should be taken when choosing the correct restoration site to outplant these species[26] (Zaya et al. 2015). To better understand hybridization patterns in *Sarracenia purpurea* var. *montana* and *Sarracenia jonesii* populations, further steps will require the observation of pollination in *Sarracenia jonesii* and *Sarracenia purpurea* var. *montana*. This could reveal the role of pollinators in hybrid production and inform management approaches.

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6. References

1. Szczecińska M, Sramko G, Wołosz K, Sawicki J. 2016. Genetic Diversity and Population Structure of the Rare and Endangered Plant Species *Pulsatilla patens* (L.) Mill in East Central Europe. *PLoS One.* 11(3)
2. Koopman M.M, Carstens B.C. 2010. Conservation genetic inferences in the carnivorous pitcher plant *Sarracenia alata* (Sarraceniaceae). *Conservation Genetics*. 11(5): 2027-2038
3. Furches M.S, Small, R.L, Furches A. 2013. Genetic diversity in three endangered pitcher plant species (*Sarracenia*; Sarraceniaceae) is lower than widespread congeners. *American Journal of Botany*. 100(10): 2092-2101
4. Ellison A.M, Butler E.D, Hicks E.J, Naczi R.F.C, Calie P.J, Bell C.D, Davis C.C. 2012. Phylogeny and Biogeography of the Carnivorous Plant Family Sarraceniaceae. *PLoS ONE*. 7(6)
5. Stone B.W, Ward A, Farenwald M, Lutz A.W, Wolfe A.D. 2019. Genetic diversity and population structure in Cary's Beardtongue *Penstemon caryi* (Plantaginaceae), a rare plant endemic to the eastern Rocky Mountains of Wyoming and Montana. *Conservation Genetics.* 20: 1149–1161
6. Grant B.R, Grant P.R. 1998. Hybridization and speciation in Darwin's finches. *In endless forms: species and speciation*. 367-378
7. Mavarez J, Salazar C.A, Bermingham E, Salcedo C, Jiggins C.D, Linares M. 2006. Speciation by hybridization in Heliconius butterflies. *Nature*. 441:868-871
8. Arnold M.L, Martin N.H. 2009. Adaptation by introgression. *J Biol*. 8:82
9. Fitzpatrick B.M, Shaffer H.B. 2007. Hybrid vigor between native and introduced salamanders raises new challenges for conservation. Proceedings of the National Academy of Sciences. 104: 15793–15798.
10. Fitzpatrick B.M, Johnson J.R, Kump D.K, Smith J.J, Voss S.R, Shaffer H.B. 2010. Rapid spread of invasive genes into a threatened native species. Proceedings of the National Academy of Sciences. 107: 3606–3610.
11. Placyk J.S, Fitzpatrick BM, Casper G.S, Small R, Reynolds R.G, Noble D.W.A, Brooks R.J, Burghardt G.M. 2012. Hybridization between two gartersnake species (Thamnophis) of conservation concern: a

threat or an important natural interaction? *Conserv Genet*. 13:649–663

12. Godt M.J.W, Hamrick J.L. 1996. Genetic Structure of Two Endangered Pitcher Plants, *Sarracenia jonesii* and *Sarracenia oreophila* (Sarraceniaceae). *American Journal of Botany*. *83*(8): 1016–1023.
13. Ellison A.M, Davis C.C, Calie P.J, Naczi R.F.C. 2014. Pitcher Plants (*Sarracenia*) Provide a 21st Century Perspective on Infraspecific Ranks and Interspecific Hybrids: A Modest Proposal for Appropriate Recognition and Usage. *Systematics Botany*. 39(3): 939-949
14. Ellison A.M, Gotelli N.J, 2002. Nitrogen availability alters the expression of carnivory in the northern pitcher plant, *Sarracenia purpurea*. *PNAS*. 99 (7): 4409-4412
15. Furches M.S, Small R.L, Furches A. 2013. Hybridization Leads to Interspecific Gene Flow in *Sarracenia* (Sarraceniaceae). *American Journal of Botany*. 100(10): 2085-2091
16. Gray S.M, Akob D.M, Green S.J, Kostka J.E. 2012. The Bacterial Composition within the Sarracenia purpurea Model System: Local Scale Differences and the Relationship with the Other Members of the Food Web. *PLOS ONE*. 7(12)
17. Rogers W.L, Cruse-Sanders J, Determann R, & Malmberg R. L. 2010. Development and characterization of microsatellite markers in *Sarracenia L*. (pitcher plant) species. *Conservation Genetics Resources. 2*(1): 75-79
18. Hsu Y.-F, Yan J, Song Y, Zheng M. 2021. *Sarracenia purpurea* glycerol-3-phosphate acyltransferase 5 confers plant tolerance to high humidity in *Arabidopsis thaliana. Physiologia Plantarum*. 173(3):1221-1229.
19. U.S. Fish & Wildlife Service. 1990. Mountain sweet pitcher plant recovery plan. U.S. Fish & Wildlife Service, Atlanta, Georgia.
20. Rentsch J.D, Holland R.C. 2020. Population Genetic Structure and Natural Establishment of Hybrids Between *Sarracenia flava* and *Sarracenia minor* in Francis Marion National Forest. *Castanea*. 85(1): 108-121
21. Doyle J.J, Doyle J.L. 1987. A Rapid DNA Isolation Procedure For Small Quantities of Fresh Leaf Tissue. Phytochemical Bulletin. 19(1): 11-15
22. Hay N.M, Ulrey C, Kauffman G, Murrell Z.E, Estep M.C. 2019. The Genetic Diversity and Population Structure of *Geum radiatum*: Effects of a Past Augmentation of an Endangered Hexaploid. *Castanea*. 84(2): 273-288
23. Clark L.V, Jasieniuk M. 2011. PolySat: an R package for polyploid microsatellite analysis. Molecular Ecology Resources. 11: 562-566
24. Fitzpatrick B. 2012. Estimating ancestry and heterozygosity of hybrids using molecular markers. *BMC Evolutionary Biology*. 12:131
25. Tamaki, S., Isoda, K., Takahashi, M. *et al.* Genetic structure and diversity in relation to the recently reduced population size of the rare conifer, *Pseudotsuga japonica*, endemic to Japan. *Conserv Genet* 19, 1243–1255 (2018)
26. Zaya D.N, Leicht-Young S.A, Pavlovic N.B, Feldheim K.A, Ashley M.V. 2015. Genetic characterization of hybridization between native and invasive bittersweet vines (*Celastrus* spp.). *Biol Invasions* 17, 2975–2988