

**The role of hybridization in structuring species- and population-level
variation in *Sarracenia* (Sarraceniaceae)**

A Dissertation Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

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August 2012

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DEDICATION

This work is dedicated to:

Annie Furches, my wife and best friend, for being her best when I was at my worst.

Gabrielle Furches, my first lab assistant.

Zachry Furches, my second lab assistant.

Charles and Helen Furches, for everything.

ACKNOWLEDGMENTS

My Committee

Randall Small

Joe Williams

Ben Fitzpatrick

Sally Horn

My colleagues and coworkers

Mackenzie Taylor

Nicholas Buckley

Michelle Connely

Matthew Valente

Lisa Wallace

Joey Shaw

Dwayne Estes

ABSTRACT

The North American Pitcher Plants (*Sarracenia*) have long fascinated naturalists, not only for their carnivorous habit, but also their ability to form countless combinations of hybrids with apparent ease. While their ability to form hybrids has been widely recognized, the role hybridization plays in shaping genetic and morphological variation in natural populations has been virtually ignored. This study examined the extent and effects of hybridization in the genus using both non-coding chloroplast sequences and nuclear microsatellite markers.

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INTRODUCTION

In Chapter 1, “Nuclear and chloroplast genomes reveal contrasting patterns of diversity in North American Pitcher Plants (*Sarracenia*),” I provide the foundation for the study by examining broad-scale patterns of all eleven *Sarracenia* species across the southeastern United States using both non-coding chloroplast sequences and nuclear microsatellites. In Chapter 2, “Genetic diversity in three endangered pitcher plant species (*Sarracenia*) is dramatically lower than in widespread congeners,” I examine genetic diversity in the three Federally endangered species of the genus. The only two *Sarracenia* species that exist completely allopatrically – *S. oreophila* and *S. alabamensis* – and in *S. jonesii*, which exists allopatrically in all but a small portion of its range, are unlikely to be subject to hybridization. In Chapter 3, “Hybridization leads to interspecific gene flow in *Sarracenia*,” I examine a single field site consisting of three species using nuclear microsatellites. By comparing this site to sympatric sites of the same three species, I show that hybridization is leading to interspecific gene flow in the genus.

CHAPTER 1

NUCLEAR AND CHLOROPLAST GENOMES REVEAL CONTRASTING PATTERNS OF DIVERSITY IN NORTH AMERICAN PITCHER PLANTS (*SARRACENIA*)

Abstract

The North American Pitcher Plants (*Sarracenia*) have long fascinated naturalists, not only for their carnivorous habit, but also their ability to form countless combinations of hybrids with apparent ease. While their ability to form hybrids has been widely recognized, the role hybridization plays in shaping genetic and morphological variation in natural populations has been virtually ignored. Our study examined 265 individuals of all eleven species of *Sarracenia* across the southeastern United States, and included multiple species per geographical location and multiple individuals per species at each geographical location. Utilizing three non-coding chloroplast regions and six microsatellite loci, we found that chloroplast haplotypes were widely shared across species, but often confined to relatively small, contiguous geographic areas. We used a simulation analysis to examine whether haplotype-sharing was likely due to ancestral polymorphisms or hybridization. Our results indicate haplotype-sharing due primarily to hybridization. Microsatellite data suggest nuclear genomes are much less permeable to introgression, with clear species delineation in most circumstances. The contrasting data sets indicate that hybridization is uneven in the genus, with some species showing little or no evidence of interspecific gene flow, and others showing evidence of uni- or bi-directional gene flow.

Introduction

The role of hybridization in plant evolution and speciation has long been debated, and has gained considerable attention in recent years (Arnold 2006; Mallet 2007). Some authors see hybridization as unimportant, holding the view that because dispersal of hybrids is compensated for by selection against hybrid offspring, their evolutionary role is minimal (Barton & Hewitt 1985; Mayr 1992). Others view it as a potent evolutionary force (Anderson & Stebbins Jr 1954; Stebbins 1959; Rieseberg 1991; Arnold & Hodges 1995; Rieseberg 1997; Rieseberg *et al.* 2003) leading to species fusion (Seehausen *et al.* 1997), gene flow between species (Cruzan & Arnold 1994), or hybrid speciation (Rieseberg 1991). Theoretical models have investigated the evolutionary potential of hybrids, and numerous studies on model systems such as *Helianthus* (Rieseberg 1991; Rieseberg 1997; Rieseberg *et al.* 1999; Lexer *et al.* 2003; Rieseberg *et al.* 2003), and *Iris* (Arnold 1993; Cruzan *et al.* 1993; Arnold 1994; Carney *et al.* 1994; Cruzan & Arnold 1994; Arnold & Hodges 1995; Burke *et al.* 1998; Johnston *et al.* 2001; Johnston *et al.* 2003) have contributed greatly to our understanding of both the mechanisms and impacts of hybridization. However, important questions remain regarding the nature and maintenance of species boundaries in hybridizing taxa, as well as the nature and extent of the contribution of hybridization to morphological and genetic variation both within and between species.

Previous studies of hybridization have often focused on single species pairs, comparing morphological and genetic variation in sympatric vs. allopatric portions of the species' ranges to expedite detailed assessment (Arnold 1994;

Johnston *et al.* 2001; Palme *et al.* 2004; Wu 2005). It is not unusual, however, for hybridization to span multiple species within a genus, and such situations provide an opportunity to assess the role and impact of hybridization in a wider organismal context. As an example of this situation we studied the North American pitcher plant genus, *Sarracenia* L.

Sarracenia is a genus of carnivorous, rhizomatous, perennial herbs found primarily along the southeastern coastal plain of the United States, with a few populations in the extreme reaches of the southern Appalachians and a single species extending north into New England and Canada (Figure 1.1; Schnell 2002.) They are primarily found in acidic soils of sphagnum bogs, mountain seeps, and longleaf pine savannas (McDaniel 1971). There are slight variations in flowering period among species, which begins in early March in some species along the Gulf Coast. Timing is later in the northern ranges and may be as late as June in Canada (Schnell 2002). Pollination studies in *Sarracenia flava* (Schnell 1983) and *S. purpurea* (Mandossian 1965) have indicated bees of the genera *Bombus* and *Augochlorella* as pollinators along with the fly *Fletcherimyia fletcheri* (Ne'eman *et al.* 2006). With no obvious dispersal mechanisms and large gaps between suitable habitats, long distance dispersal of the small seeds has been hypothesized to be dependent upon migrating birds (Harper 1918; Ellison & Parker 2002). The group has been popular in cultivation since the nineteenth century due to their unusual morphology, carnivorous habit and the ability to form a variety of hybrids, including complex hybrids and backcrosses (Moore 1874; Masters 1881; Harper 1918; Russell 1918; Bell 1952; Bell & Case 1956).

Natural hybridization among species combined with morphological variation within species has led to conflicting taxonomies within the genus. From eight to eleven species are recognized, and numerous subspecies and varieties have been published (Case & Case 1976; Naczi & Soper 1999; Schnell 2002). The source of the most intense taxonomic debate is a group known as the '*Sarracenia rubra* complex' (Case & Case 1976; Schnell 1977; Schnell 1979). For the purposes of this study, the more widely accepted taxonomic treatment will be used. Under this taxonomic treatment, eleven species are recognized. *Sarracenia jonesii* and *S. alabamensis* are recognized at the species level rather than subspecies of *S. rubra*. (Case & Case 1976; Godt & Hamrick 1998).

Many *Sarracenia* species are threatened by poaching, and all are threatened by habitat loss due to land conversion for agricultural usage, tree farming, lowering of water tables, and/or fire suppression (personal observation, Folkerts 1982; Schnell 2002). Longleaf pine (*Pinus palustris*) savannas, for example, a primary habitat of *Sarracenia* in the southeastern coastal plain, have been reduced by over 97% since settlement by Europeans began (Kush et al. 2004). As a result, three species are currently on the Federal Endangered Species list, two are listed as rare/threatened and several receive protection at the state level (Fish & Wildlife Service 2011).

Hybridization has long been of interest in *Sarracenia*. In an 1874 issue of *The Gardener's Chronicle*, Dr. David Moore described '*Sarracenia moorei*', "the first hybrid *Sarracenia* which has yet flowered" (Moore 1874). He described the plant as a cross between *S. flava* and *S. leucophylla*, stating it was "as nearly as possible intermediate between those two noble species of the genus" (Moore

1874). An article in that same publication in 1881 described four different hybrids, including *S. melanorhoda* (*[S. purpurea* X *S. flava]* X *S. purpurea*), a complex, backcross hybrid (Masters 1881). Russell (1918) made detailed anatomical comparisons of three hybrids and their parent species. He reported that “hybrid forms, in comparison with their parents, are intermediate in relation in almost all details” (Russell 1918). However, without the advantages of genetic analyses, studies of hybrids in natural environments were necessarily limited to those individuals that could be readily identified morphologically as hybrids. Later Bell (1952) and Bell and Case (1956) described a total of 19 *Sarracenia* hybrids known at that time.

Subsequently the focus of study in *Sarracenia* shifted to taxonomy, and despite the intense interest in hybridization for nearly a century, little work has been done in the last 50 years. This is particularly surprising given that hybridization has often been cited as an obstacle to any attempt at determining the phylogeny of *Sarracenia* (McDaniel 1971; Bayer *et al.* 1996; Neyland & Merchant 2006). While several attempts have been made using traditional morphological characters (McDaniel 1971; Schnell 1978c), flavonoids (McDaniel 1971; Romeo *et al.* 1977), petal extract chromatography (Schnell 1978b), and more recently DNA-based methods (Bayer *et al.* 1996; Neyland & Merchant 2006), interspecific relationships within *Sarracenia* have yet to be resolved. Relationships of the five subspecies of the *Sarracenia rubra* complex are particularly problematic, having been described as three separate species (Case & Case 1976) or one species with five subspecies (Schnell 1978a; Schnell 1979).

Bayer et al. (1996) cited a lack of informative characters for the poor resolution obtained by their study using nuclear ribosomal ITS sequences. The study by Neyland and Merchant (2006) used a portion of the 26S large subunit rRNA gene in conjunction with the ITS-2 spacer. Lack of informative characters was again cited by the authors as a potential reason for lack of resolution and low bootstrap support. In addition to generally poor resolution, these studies obtained conflicting results. For example, Bayer et al. (1996) found *S. alata* to be sister to all other species of *Sarracenia* and *S. purpurea* sister to *S. leucophylla* while Neyland and Merchant (2006) found *S. purpurea* to be sister to all other species, with *S. alata* closely related to a polytomy containing *S. rubra* and *S. oreophila*. Both studies cited hybridization as an issue of concern in determining species relationships within *Sarracenia*.

Nearly every pair of species that exists in close proximity in nature has been found to hybridize, and complex (i.e., multi-species) hybrids are well-known in cultivation (Bell 1952; Bell & Case 1956; D'Amato 1998). To date, nearly all 'geographically possible' (i.e. two species having at least some portion of their ranges that overlap) simple hybrids of *Sarracenia* have now been recorded in nature (Russell 1918; Bell 1952; Bell & Case 1956). However, the role of hybridization in generating morphological and genetic diversity within and among species is not well-understood. At one end of the spectrum, hybridization may be rare and generate only F1 hybrids that may confound taxonomic studies, but contribute nothing to genetic or morphological diversity within species. Alternatively, hybridization may be an important 'bridge' that allows for gene flow

between species that results in much of the morphological diversity observed within species.

To address these alternatives, we conducted a comprehensive molecular study using both bi-parentally inherited nuclear markers (microsatellites) and uniparentally (maternally) inherited non-coding chloroplast DNA sequences. In addition, we sampled multiple individuals per population and multiple individuals per species to sample the full range of geographic and intraspecific variation. A confounding factor in the examination of hybridization, particularly in recently diverged taxa, is distinguishing between allele/haplotype-sharing resulting from introgressive hybridization versus retention of ancestral polymorphisms (Charlesworth 2010; Koblmüller *et al.* 2010; Mims *et al.* 2010). While difficult to disentangle, the issue can be addressed when species are found both sympatrically and allopatrically under the assumption that if hybridization is responsible for shared alleles, individuals of different species should share more alleles when they are found at the same location than those found at different locations (Mims *et al.* 2010).

Study Goals

Given the widely accepted view that hybridization is a prominent process in *Sarracenia* a primary goal of our study was to identify patterns of genetic variation within and among species using both chloroplast- and nuclear-encoded molecular markers. To assess evidence for hybridization we sampled individuals of all known *Sarracenia* species and included multiple populations of all species and multiple individuals from all populations. Once the initial patterns had been

discovered we used analytical approaches to attempt to identify the underlying processes. Specifically, we sought to assess (1) whether genetic variation was taxonomically or geographically structured, (2) whether or not allele sharing between species was due to hybridization or retention of ancestral polymorphisms, and (3) whether similar patterns and processes are present in all potentially hybridizing species or if unique scenarios were present in specific species pairs.

If hybridization is uncommon, or more specifically, if backcrossing is uncommon, we expect to find little or no admixture of nuclear genomes based on microsatellite data. We would also expect chloroplast sequences from individuals of different species found in sympatry to be no more similar than sequences from those same species found in different locations. However, if hybridization and backcrossing are common, we would expect to see a high degree of admixture at microsatellite loci and chloroplast sequences of different species that are more similar in sympatric populations. A third, more complex scenario, is also possible - one in which similarities among some species are due to ongoing hybridization while others are no longer experiencing gene flow but retain ancestral polymorphisms.

Materials and Methods

Plant Collection

Due to the nature of the wetland habitats in which most *Sarracenia* are found, field sites tend to have relatively distinct, unambiguously-defined boundaries. This is often due to natural occurrences, such as a slight change in

elevation resulting in decreased soil saturation, or anthropogenic causes, such as fire suppression or conversion to pasture (personal observation). A point of clarification is needed due to the varying levels of sympatry in which these species are found. Throughout this paper, the term 'population' will be used to indicate a single *Sarracenia* species at a single geographic location. The term 'field site' will be used to refer to a specific geographic location that may include as few as one or as many as five *Sarracenia* species.

All necessary permits and/or landowner permission were obtained in advance of collection of plant material. A total of 265 individuals, representing all species, was sampled from 34 field sites throughout the southeastern United States (Figure 1.2). Plants were sampled haphazardly while attempting to maximize distance among individuals and include members of all species at a given field site. Due to threats facing many *Sarracenia* populations, to minimize impact, only a single pitcher of each species from each field site was taken as a voucher specimen and deposited at the University of Tennessee herbarium (TENN). Additionally, photographs were taken of all specimens. While there are some relatively consistent morphological differences among flowers of different species, field identification is most easily done in combination with, or solely by, leaf (i.e. pitcher) morphology (Schnell 1978; Schnell 2002). In locations in which multiple species were present, individuals were chosen with leaf morphological characteristics most consistent with species descriptions.

Material for DNA extraction was obtained by removing approximately 1-2 cm² from the pitcher hood or ala and stored on ice for the duration of the field trip

(up to 7 days) before being transferred to -80° C storage. DNA was extracted using the Dneasy Plant Mini Kit (Qiagen®, Valencia, CA).

Non-coding Chloroplast DNA Amplification

Initially, seven non-coding chloroplast regions – *rpl32-trnL*, *trnQ-5'rps16*, *psbD-trnT*, *psbJ-petA*, *3'rps16-5'trnK*, *atpI-atpH*, and *trnS-trnG* – were screened for variation. These regions have been shown to be highly variable across a wide range of angiosperm species (Shaw *et al.* 2005; Shaw *et al.* 2007). Single representatives of five *Sarracenia* species were sequenced for all seven regions and the three regions with the greatest variability were chosen for use in this study – *3'rps16-5'trnK*, *atpI-atpH*, and *trnS-trnG*. All 265 samples were amplified at these three regions using the following PCR protocol: 95°, 1 min; 35× (95°, 1 min; 55°, 1 min; 65°, 1 min.); 72°, 2 min. PCR products were then visualized by running on a 1.5% agarose gel for approximately 30 minutes at 96 mV.

Sequencing was performed on an Avant 3600 Genetic Analyzer (Applied BioSystems, Foster City, CA) at the University of Tennessee Molecular Biology Resource Facility (MBRF). Initial examination of sequences, forming of contigs, and examination of ambiguous base calls were performed with SEQUENCHER v. 4.9 (Gene Codes Corporation, Ann Arbor, MI). Automatic alignment was performed using CLUSTAL X (Larkin *et al.* 2007) and further manual alignment was made using MESQUITE v. 2.72 (Maddison & Maddison 2009).

Alignment and Processing

Sequences from the three non-coding chloroplast regions were concatenated to produce a cpDNA dataset with a total aligned length of 3,869 base pairs [*3'rps16-5'trnK* (1,084 bp), *atpI-atpH* (1,235 bp), *trnS-trnG* (1,550 bp)]. Sequences from all 265 individuals were then condensed to 91 unique haplotypes using the program Collapse 1.2 (Posada 2006). Sequence gaps (indels) were then coded manually based on the guidelines of Simmons and Ochoterena (2000). This produced an additional 34 characters for a total of 3,903 characters.

Analyses

To examine patterns of variation in chloroplast sequences, a phylogram was produced by first independently analyzing each of the three sequence regions as well as the set of characters from indels with MrModeltest v2.2 (Nylander 2004) to determine the most appropriate model of evolution based on Akaike Information Criterion (AIC) scores. The partitioned dataset was then analyzed with MrBayes v3.1.2 (Mau & Newton 1997; Huelsenbeck *et al.* 2001) under multiple parameter scenarios until a combination was found that produced both convergence of Markov chains and sufficient chain swapping (see Mau & Newton 1997; Huelsenbeck *et al.* 2001). The final analysis was then performed with the following parameters: generations = 5,000,000; burn-in fraction = 0.25; temperature = 0.1; sample frequency = 1000; chains = 4. To illustrate relationships of haplotypes, we performed a haplotype network analysis using TCS v1.21 (Clement *et al.* 2000).

The distribution of genetic variation was then examined to assess patterns across species and/or field sites. First, aligned, concatenated sequences were converted into an Arlequin v3.5 haplotype file using FaBox (Villison, 2007). Two AMOVA were then performed. In the first analysis, populations were placed into groups based on taxonomy, and again examined at three hierarchical levels – (1) within populations, (2) among populations but within species, and (3) among species. In the second analysis, populations were placed into groups based on geographic location (i.e. field sites, $n = 35$) and analyzed to determine the distribution of genetic variation at three hierarchical levels – (1) within populations, (2) among populations but within field sites, and (3) among field sites. To test for the presence of phylogeographic structure in the genus, G_{ST} and N_{ST} were calculated and compared using the program PERMUT v1.0 (Pons & Petit 1996). If N_{ST} , which takes genetic similarities among haplotypes into consideration, is significantly larger than G_{ST} , which only considers haplotype frequencies, this indicates the presence of phylogeographic structure (Pons & Petit 1996; Burban *et al.* 1999). However, this analysis makes no indication as to the cause of the phylogeographic structure. To address this issue, we chose four species pairs for closer examination. These species were chosen because each pair has an overlapping range in which they could be examined in sympatry as well as some part of their range in which they could be examined in allopatry. The pairs used were *S. alata* and *S. leucophylla*, *S. flava* and *S. minor*, *S. flava* and *S. psittacina*, and *S. minor* and *S. psittacina*. We first created a table of pairwise ϕ_{ST} values for each of the four sympatric populations of *S. alata* and *S. leucophylla*, as well as all allopatric populations. Then, using a custom-written

code in R (<http://www.r-project.org>) developed and described in Mims et al. (2010), we created a null distribution by randomly sampling four *S. alata* and four *S. leucophylla* populations, forming four random pairs. The average pairwise ϕ_{ST} values were calculated for each of 10 000 replicates. The fraction of randomizations with mean ϕ_{ST} values less than or equal to the observed mean for sympatric populations was then used as an estimate of the one-tailed *P*-value for a null hypothesis of no relationship between genetic differentiation and sympatry. Rejection of this null hypothesis supports hybridization, rather than ancestral polymorphisms, as the cause of haplotype sharing between species. The same process was repeated for the other three species pairs.

Microsatellite Amplification and Fragment Analysis

A subset of 115 individuals representing all eleven species was chosen for microsatellite analysis. Based on results from chloroplast sequences, this subset of individuals was chosen because they provided an adequate representation of all taxa while minimizing monetary costs. Microsatellites were amplified as described in Koopman et al. (2009). Of the nine loci developed for *Sarracenia alata*, six successfully cross-amplified in all eight remaining *Sarracenia* species (Table 1.1). PCR products were then visualized by running on a 1.5% agarose gel for approximately 30 minutes at 96 mV. Individuals were genotyped by electrophoresing fluorescently labeled products through an Avant 3600 Genetic Analyzer (Applied BioSystems, Foster City, CA) at MBRF. Fragment sizes were determined by comparison to the ROX500 size standard in PeakScanner software (Applied Biosystems).

Microsatellite Analysis

To assess patterns of variation in microsatellites, Bayesian analysis was performed using STRUCTURE 2.2.3 (Pritchard et al. 2000). Because of possible hybridization, an admixture model was used. The number of inferred genetic clusters (K) was determined by running 20 simulations of 10^6 steps at each value of K ranging from two to 17. One set of simulations was performed using a correlated allele frequencies model, and another set was performed with allele frequencies uncorrelated (see Pritchard et al, 2000). To determine proper K value, mean likelihood values were examined as originally described by Pritchard et al (2000) as well as using the method of Evanno (2005). When allele frequencies were uncorrelated, there was no clear K , while under the correlated allele frequencies model, both analyses overwhelmingly indicated 11 as the K value with the highest likelihood. The simulation was then run under these parameters at $K = 11$ for 5×10^6 steps with the initial 5×10^5 discarded as “burn-in.”

Results

Non-coding chloroplast DNA sequence variation

The Bayesian analysis of non-coding chloroplast data is shown in Figure 1.3. Branch tips are labeled with the haplotype number followed by an indication of which species are represented. *Heliamphora nutans* served as the outgroup. Haplotypes '1' through '91' represent between one and 26 *Sarracenia* individuals. Multiple haplotypes were found in each of the eleven *Sarracenia* species – as many as 21 in *S. psittacina* and as few as three in *S. oreophila* and *S.*

alabamensis. Most haplotypes found in multiple individuals were also present in multiple species. Only *Sarracenia oreophila* and *S. alabamensis* formed resolved monophyletic groups, and the phylogram shows no clear taxonomic pattern. Notably, *S. oreophila* and *S. alabamensis* are also the only two species whose geographic ranges are entirely disjunct from other *Sarracenia* species. The haplotype network analysis (Figure 1.4) further demonstrates haplotype sharing among species. The analysis produced 28 haplotype groups consisting of one to 68 individuals. With one exception (30 individuals of *S. oreophila*), the eight haplotypes with more than ten individuals were found in multiple species.

The two AMOVA provided a quantitative examination of the distribution of genetic variation in *Sarracenia* (Table 1.2). The combination of analyses showed greater variation exists among populations within a given species than among populations within a given field site. In the first analysis, in which populations were grouped by species, the distribution of sequence variation was as follows: 25.77% among species, 54.26% among populations within species, and 19.98% within populations. In the second analysis, in which populations were grouped by geographic location (field sites), the distribution of genetic variation was as follows: 34.02% among field sites, 45.59% among populations within field sites, and 20.38% within populations. The examination of phylogeographic structure yielded an N_{ST} that was significantly larger than G_{ST} ($N_{ST} = 0.523$, $G_{ST} = 0.387$, $p < 0.05$), indicating closely related haplotypes were more often found in the same geographic area than would be expected by chance (Palme *et al.* 2004). When four species pairs were examined to determine if populations of two different species in sympatry are more similar than allopatric populations of the same

species, results were mixed (Figure 1.5). When *Sarracenia alata* and *S. leucophylla* are found in sympatry, populations are significantly more similar than random population pairs ($P = 0.008$), indicating that recent hybridization is likely responsible for sharing of haplotypes, rather than ancestral polymorphisms. When *S. flava* and *S. minor* were examined, populations in sympatry were found to be no more similar than random pairs ($P = 0.428$), indicating ancestral polymorphisms may explain haplotype sharing in these species. In the third comparison, sympatric populations of *S. flava* and *S. psittacina* were much more likely to share haplotypes than random population pairs ($P = 0.022$), again supporting the alternative hypothesis of recent hybridization as an explanation for haplotype sharing. Finally, when populations of *S. minor* and *S. psittacina* were examined, we failed to reject the null hypothesis of ancestral polymorphisms as the cause of haplotype sharing ($P = 0.060$).

Microsatellite marker variation

After the K value of eleven was chosen, a final analysis was performed for 5×10^6 iterations with 5×10^5 discarded as “burn-in.” Out of the 115 individuals examined, 68 were inferred as belonging to a given cluster with a proportion of ≥ 0.90 , 85 with a proportion of ≥ 0.80 , and 102 with a proportion of ≥ 0.60 (Figure 1.6). Of the thirteen taxa examined (eleven species, including three *S. rubra* subspecies), the following taxa were placed entirely into a single cluster: *S. minor* (Cluster 8), *S. oreophila* (Cluster 7), *S. flava* (Cluster 5), *S. alata* (Cluster 10), *S. alabamensis* (Cluster 4), *S. rubra* ssp *wherryi* (Cluster 1), *S. rubra* ssp *gulfensis* (Cluster 11), *S. jonesii* (Cluster 9), and *S. rubra* ssp *rubra* (Cluster 9).

Exceptions included *S. psittacina* (Clusters 3 and 6), *S. leucophylla* (Clusters 1, 2, and 6), *S. rosea* (Clusters 3 and 10), and *S. purpurea* (Clusters 2, 3, and 10). Also of note, three individuals of *S. rubra* from west-central Georgia were divided among Clusters 1, 4, and 9.

Discussion

Even prior to the availability of molecular techniques, few questioned the placement of the genus *Sarracenia* within the family Sarraceniaceae along with the South American Sun Pitchers (*Heliamphora*), and the Cobra Lily (*Darlingtonia*). Morphological (MacFarlane 1893), cytological (Bell 1949; Hecht 1949), and palynological (Thanikaimoni & Vasanthy 1972) studies had long supported this placement long before it was further confirmed by modern molecular studies (Bayer *et al.* 1996; Neyland & Merchant 2006). Relationships within *Sarracenia*, however, have been difficult to decipher, and hybridization is likely a contributing factor to the lack of success in this area.

It is widely recognized that hybridization is ubiquitous in *Sarracenia*, yet when visiting most field sites morphological hybrids can be difficult to find (personal observation; George Folkerts, personal communication). Surprisingly, however, no study has addressed the role that hybridization plays in the genus. In this study, we sought to gain insight into hybridization in the genus by examining both chloroplast sequences and nuclear markers in a large number of individuals from all species across a broad geographic area. Our data reveal extensive chloroplast haplotype sharing from both recent hybridization and

ancestral polymorphism, but a more cohesive nuclear genome, albeit with signs of admixture.

Current Study

It has long been recognized that chloroplast haplotypes of hybridizing plant species may exhibit geographic rather than taxonomic patterns (Whittemore & Schaal 1991; Rieseberg & Soltis 1992; Soltis *et al.* 1992; Jackson *et al.* 1999; Wu & Campbell 2005). Even a low level of hybridization followed by subsequent backcrossing can result in introgression of chloroplast genomes between species (Jiggins & Mallet 2000; Minder *et al.* 2007). These backcross events are an important step, however, as they can provide the 'bridge' necessary for gene flow between species (Jiggins & Mallet 2000). If no backcrosses occur, gene flow will not occur between species. At the other end of the spectrum, a complete breakdown of barriers to gene flow among species will result in species fusion (Seehausen *et al.* 1997). In a complex system such as *Sarracenia*, patterns of chloroplast variation among species and across geographic areas can provide a insight into species dynamics, particularly when compared with nuclear markers. Given the number of species in the genus, the overlapping yet variable flowering times, and differences in pitcher morphology, it is unlikely that all species are hybridizing equally.

Non-coding Chloroplast Sequences

Extensive haplotype sharing was found throughout the genus, with two notable exceptions – *Sarracenia oreophila* and *S. alabamensis* (Figure 1.4).

These two species are also the only two taxa with no geographic overlap with other *Sarracenia*, existing allopatrically throughout their respective ranges. When viewed geographically, haplotypes were found in one to seven contiguous field sites. The two AMOVA further illustrate the geographic nature of chloroplast variation (Table 1.2). Populations of different species at the same field site are more similar than populations of the same species at different field sites (45.59% of variation among populations within field sites, versus 54.26% among populations within species). The test for phylogeographic structure was also positive, with N_{ST} significantly greater than G_{ST} , indicating closely related haplotypes were more likely to be found in the same geographic area. Hybridization is not the only explanation for such geographic structure, however. Sharing of haplotypes may also result from ancestral polymorphisms that existed before speciation occurred (Muir & Schlotterer 2005). We therefore chose four pairs of species for closer examination to determine if populations of two species found in sympatry are more similar to those same two species found separately (Figure 1.5). Sympatric populations of *S. leucophylla* and *S. alata* were significantly more similar than populations of those same two species in different geographic areas. The same was true for *S. flava* and *S. psittacina*. However, the same could not be said for sympatric populations of *S. flava* and *S. minor*, where the null hypothesis of haplotype sharing due to ancestral polymorphisms could not be rejected. The comparison of *S. minor* and *S. psittacina* revealed a low, but not statistically significant, P -value of 0.06, again failing to reject the null hypothesis. These results are consistent with field observations. Field sites where both *S. leucophylla* and *S. alata* are present in large numbers often show

a wide range of morphological variation with many individuals that appear to be hybrids or backcrosses (personal observations, Schnell 2002, Folkerts 1982). A morphological hybrid of *S. flava* and *S. minor*, however, has not been observed at any field sites visited by the authors. This is consistent with a combination of hybridization and ancestral polymorphisms producing the patterns seen in the genus.

Microsatellites

The microsatellite data show a pattern that, like the chloroplast data, is consistent with differential rates of hybridization (Figure 1.6). In most cases, all individuals of a given species were assigned to the same cluster (*S. minor*, *S. oreophila*, *S. flava*, *S. alata*, *S. alabamensis*, *S. rubra* ssp *wherryi*, *S. rubra* ssp *gulfensis*, and *S. rubra* ssp *rubra*/*S. jonesii*), while four species had individuals assigned to two (*S. psittacina* and *S. rosea*) or three clusters (*S. leucophylla* and *S. purpurea*). While 85 individuals had 80% or more of their genome assigned to a single group, the remaining 30 showed stronger signs of admixture. These included one or more individuals of *S. alata*, *S. flava*, *S. leucophylla*, *S. psittacina*, *S. rosea*, and *S. rubra* ssp *wherryi*.

Hybridization

The two data sets reveal a nuclear genome that is much more consistent with taxonomic designations than the chloroplast genome. Even with relatively few microsatellite loci, most individuals of most species were assigned to their expected group. However, the presence of admixture in many individuals, in

combination with chloroplast haplotype sharing, indicates ongoing gene flow among at least some members of the genus. Our analyses show that this interspecific gene flow is likely not uniform throughout the genus. When we compared populations of *S. leucophylla* and *S. alata*, they exhibited much greater chloroplast haplotype similarity in sympatry than is expected from ancestral polymorphisms alone (Figure 1.5). These two species also exhibited greater admixture in microsatellite analyses (Figure 1.6). This is consistent with our field observations. In many field sites where these two species are found, a wide range of virtually continuous morphological diversity can be seen. Additionally, *S. alata* and *S. leucophylla* both begin flowering in early March, with *S. alata* continuing into early-to-mid April and *S. leucophylla* continuing until late April (Schnell 2002). This is in stark contrast to our analyses of *S. minor*. In microsatellite analyses, all individuals of *S. minor* were assigned to the same cluster with an average value of > 0.95 , forming the single most genetically cohesive cluster (Figure 1.6). In fact, *S. minor* formed an even stronger group than *S. oreophila*, a species that is only found in areas ≥ 80 km from any other *Sarracenia* species. When we compared chloroplast sequences of sympatric populations of *S. minor* and *S. psittacina* or *S. minor* and *S. flava*, we found them to be no more similar than allopatric populations of the same species (Figure 1.5). These findings are also consistent with field observations, as not a single *S. flava* X *minor* or *S. minor* X *psittacina* morphological hybrid was found in any of our site visits. Flowering phenology may again provide insight into these results. Despite having the southernmost range, *S. minor* flowers later than any other southeastern species, initiating only in late March in the southern portion of

the range (where no other *Sarracenia* species are found) and lasting until mid-May in the northern portion (personal observation, Schnell 2002). While some overlap exists, this is later than *S. flava*, which begins in mid-March its southern range and lasts until mid-April further north. More overlap exists with *S. psittacina*, which also begins in late-March and lasts until May. Further demonstrating the complex nature of this system are *S. flava* and *S. psittacina*. There is some overlap in flowering (mid-March to mid-April in *S. flava*; late-March until May in *S. psittacina*). When we examined chloroplast sequence similarities in these species, we found that sympatric populations were significantly more similar ($P = 0.022$, Figure 1.5). Interestingly, they exhibited very different microsatellite patterns. While *S. psittacina* was highly admixed and split between two clusters, *S. flava* formed a strong cluster (Figure 1.5). One plausible scenario responsible for this discordance is uneven gene flow between these two species. Studies of *Populus* (Martinsen *et al.* 2001) and others (Archibald *et al.* 2004; Minder *et al.* 2007) have shown that hybridization and subsequent backcrossing can lead to uneven gene flow between species. This can occur if one species preferentially or exclusively acts as the pollen donor, or if hybrids preferentially backcross with one parental species. Gene flow occurring preferentially from *S. flava* to *S. psittacina* could be responsible for the differences seen in the microsatellite data.

Our study demonstrates the complex nature of species interactions in North American Pitcher Plants. The entire genus can not be generalized as “freely hybridizing,” and allele-sharing among species can not be entirely explained by ancestral polymorphisms. This is not only an oversimplification, it

is, as our data show, also incorrect. Interspecific gene flow in *Sarracenia* may be common between some species, rare or absent in others, and uneven or unidirectional in others. *Sarracenia* presents an excellent system in which we can study multi-species interactions and a wide range of interspecific gene flow.

Future Directions

Our next step in this investigation is to investigate hybridization on a local scale. Using additional microsatellite loci to provide greater resolution, we will examine field site in Alabama consisting of three species – *S. alata*, *S. leucophylla*, and *S. rubra* ssp *wherryi*. We hope this study will provide greater insight into the frequency of hybridization, backcrosses, and possibly even complex crosses.

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Appendix 1 – Figures and Tables

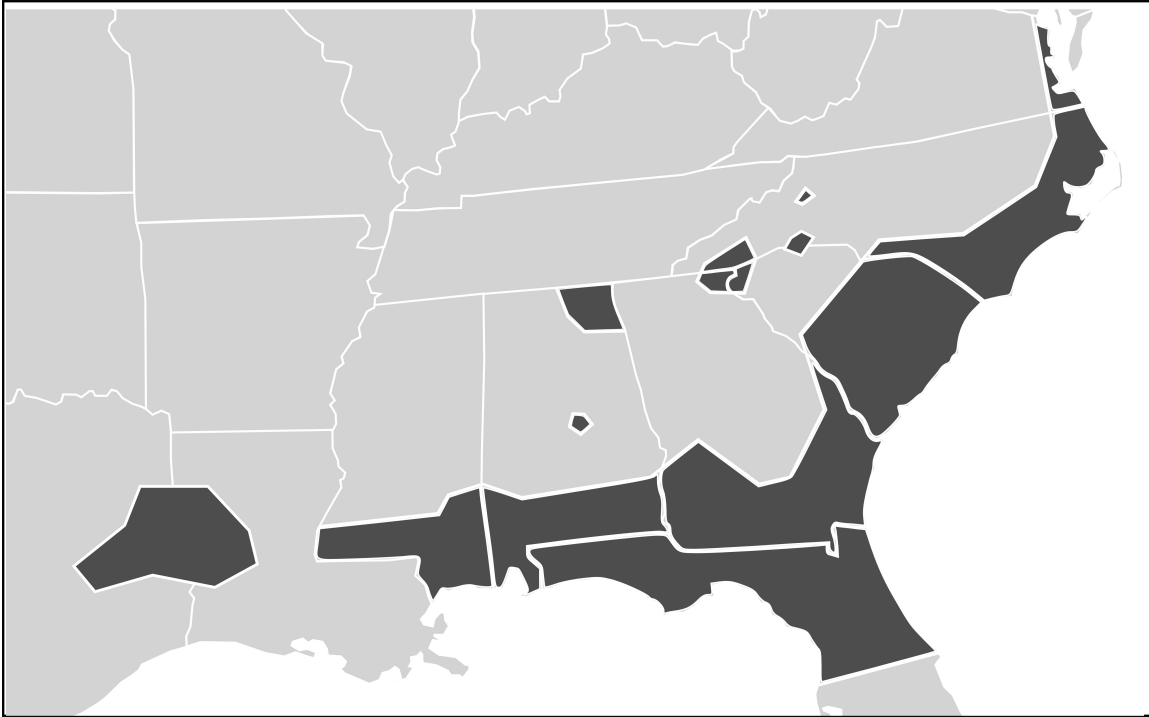


Figure 1.1 – Range of the genus *Sarracenia* in the southeastern United States.

The northern range of *S. purpurea* is not shown.

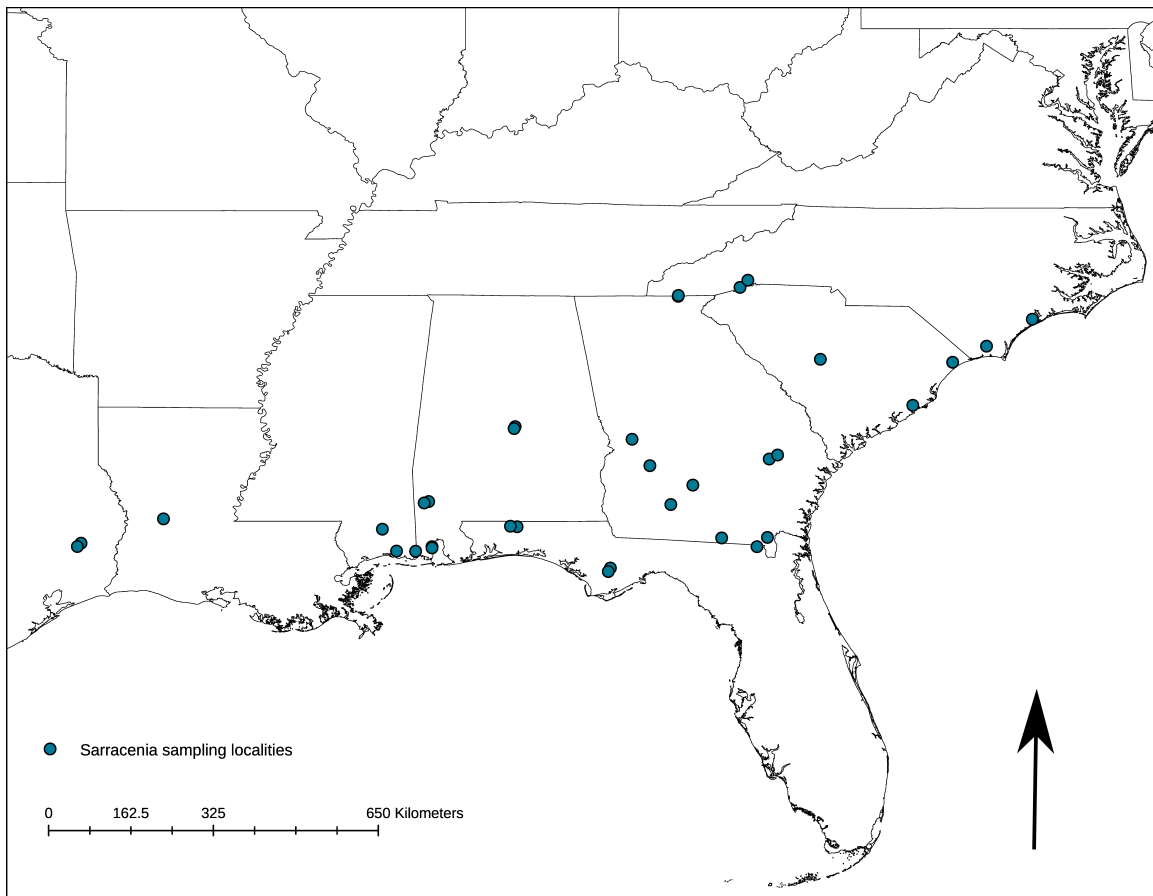


Figure 1.2 – Thirty-four field sites in which one or more *Sarracenia* species was sampled.

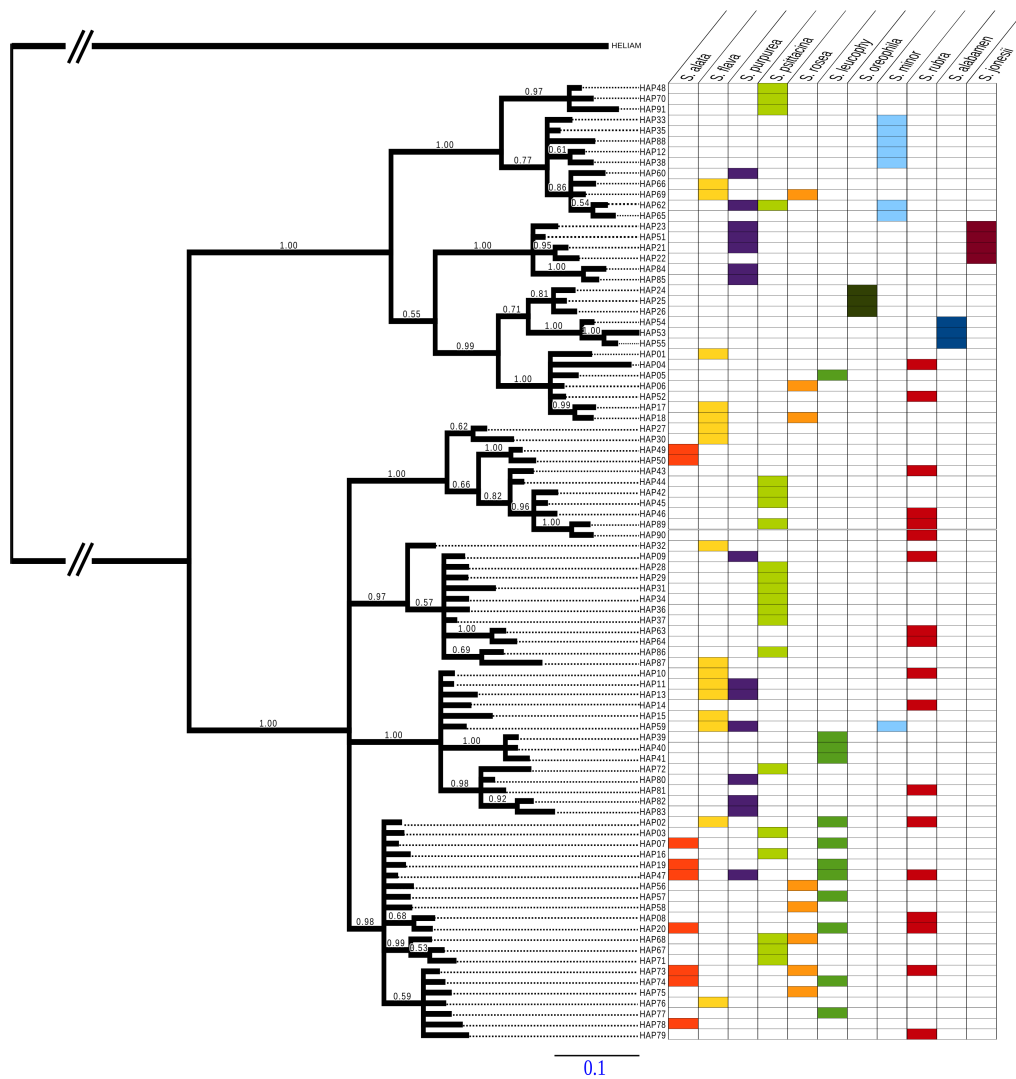


Figure 1.3 – Phylogram of 91 *Sarracenia* haplotypes produced from MrBayes analysis.

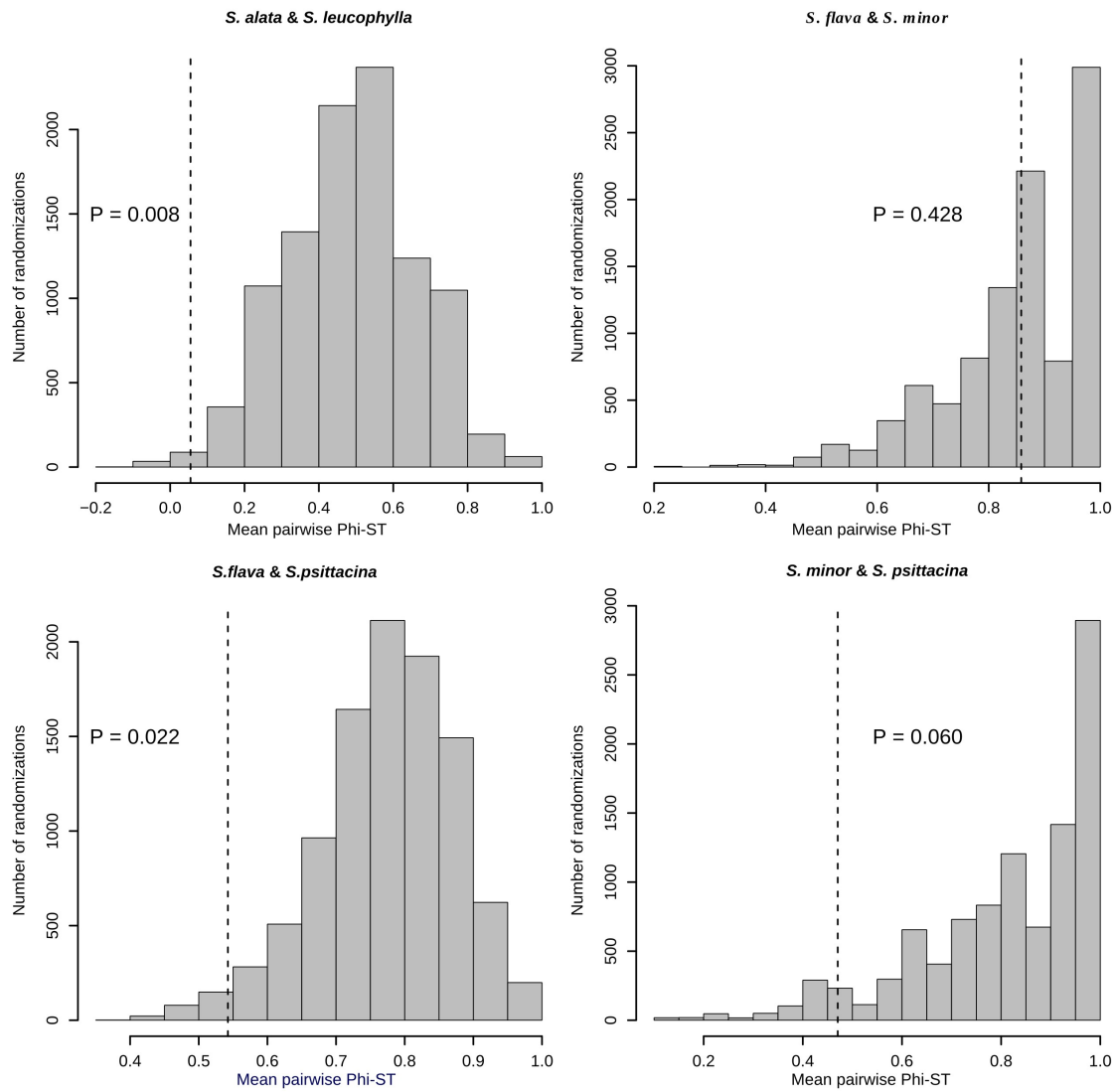


Figure 1.5 – Genetic differentiation (cpDNA) of selected *Sarracenia* species where they co-occur compared to a simulated distribution of of random population pairs

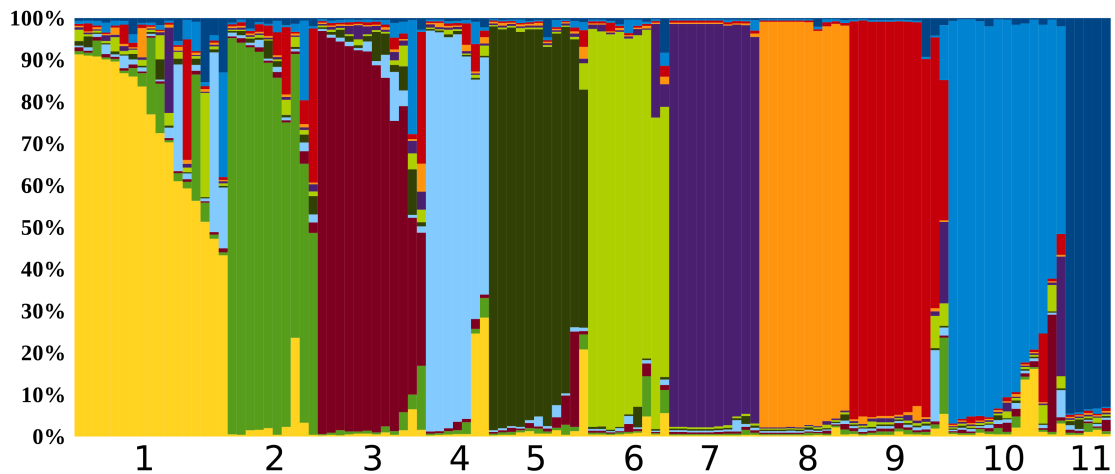


Figure 1.6 – STRUCTURE analysis of 115 *Sarracenia* individuals representing all eleven species. Of the thirteen taxa examined (eleven species, including three *S. rubra* subspecies), the following taxa were placed entirely into a single cluster: *S. minor* (Cluster 8), *S. oreophila* (Cluster 7), *S. flava* (Cluster 5), *S. alata* (Cluster 10), *S. alabamensis* (Cluster 4), *S. rubra* ssp *wherryi* (Cluster 1), *S. rubra* ssp *gulfensis* (Cluster 11), *S. jonesii* (Cluster 9), and *S. rubra* ssp *rubra* (Cluster 9). Exceptions included *S. psittacina* (Clusters 3 and 6), *S. leucophylla* (Clusters 1, 2, and 6), *S. rosea* (Clusters 3 and 10), and *S. purpurea* (Clusters 2, 3, and 10). Also of note, three individuals of *S. rubra* from west-central Georgia were divided among Clusters 1, 4, and 9.

Table 1.1 – Microsatellite loci used in this study.

Locus	Repeat Unit	Size Range (bp)	N _A	H _O	H _E	F _{ST}
S-05	AG	224 – 231	7	0.029	0.233	0.710***
S-07	CT	203 – 293	50	0.550	0.798	0.185***
S-18	GTTTT	195 – 238	23	0.381	0.494	0.496***
S-36	GTTT	194 – 198	3	0.000	0.191	0.727***
S-44	GT	164 – 171	9	0.184	0.330	0.638***
S-47	GGAAA	197 – 228	16	0.330	0.598	0.337***
Overall			18	0.246	0.441	0.490***

Table 1.2 – AMOVA

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of Variation	Phi Statistics	<i>P</i> -value
Among Species	8	551.022	1.95935	25.77	PhiCT = 0.25766	< 0.0001
Among populations within species	50	836.451	4.1258	54.26	PhiSC = 0.73087	< 0.0001
Within populations	167	253.713	1.51924	19.98		
Total	225	1641.186	7.60439			

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of Variation	Phi Statistics	<i>P</i> -value
Among Field Sites	28	979.302	2.53578	34.02	PhiCT = 0.34023	< 0.0001
Among populations within field sites	30	408.172	3.39807	45.59	PhiSC = 0.69104	< 0.0001
Within populations	167	253.713	1.51924	20.38		
Total	225	1641.186	7.45309			

CHAPTER 2

GENETIC DIVERSITY IN THREE ENDANGERED PITCHER PLANT SPECIES (*SARRACENIA*) IS DRAMATICALLY LOWER THAN IN WIDESPREAD CONGENERS

Abstract

Narrow-ranging or rare species often exhibit levels of genetic diversity lower than their more common or widespread congeners. These taxa are at increased risk of extinction due to stochastic threats associated with natural events as well as anthropogenic effects such as habitat degradation and destruction. Here we examined current and historical threats to maintenance of genetic diversity in three Federally endangered species in the genus *Sarracenia*. We used three non-coding chloroplast regions – *trnS* – *trnG*; *atpI* – *atpH*; and *3'rps16* – *5'trnK*, and nine microsatellite loci to examine *S. oreophila*, *S. alabamensis*, and *S. jonesii*. We found the lowest microsatellite variation in the most widespread of the three – *S. oreophila*. The critically endangered *S. alabamensis* exhibited the greatest microsatellite diversity. Genetic effects of small population size were observed in all three study species. Strikingly, there was less genetic variation across the entire range of the three study species than at a single field site consisting of three more common species. Our study provides both conservation guidelines and insight into the population dynamics and evolutionary history of these species.

Introduction

The southeastern United States harbors some of the most diverse plant and animal communities in North America as well as one of the greatest concentrations of endangered species (Reid 1998). Many of these biodiversity “hotspots” are subject to major threats from habitat loss due to urbanization and agriculture (Ricketts and Imhoff 2003). Wetlands are particularly at risk, containing one-third of the threatened and endangered species in the U.S. (Murdock 1994). While the Southeast has more area of wetlands than any other region of the U.S., it has also suffered the greatest loss of wetlands of any region (Gutzwiller and Flather 2011; Herrington 2004). Reasons include agriculture, which has traditionally been the leading cause, as well as clearing for development, which has become an ever-increasing source of wetland loss (Brady and Flather 1994; Gutzwiller and Flather 2011). In one striking example, southeastern longleaf pine (*Pinus palustris*) savannas have been reduced by more than 95% since settlement, down from 90 million virtually contiguous acres to fewer than three million fragmented acres (Longleaf Alliance 2011). Even wetland habitats that are not completely lost can be drastically altered by changes in fire regimes, altered hydrology, and introduction of non-native species (Folkerts 1982; Platt et al. 1988; Schnell 2002).

As members of these wetland communities, carnivorous plants, such as members of the genus *Sarracenia*, have suffered from habitat destruction and alteration (U.S. Fish & Wildlife Service 1990, 1992, 1994). Carnivorous plant species, however, suffer additionally due to greater susceptibility to pollutants and from illegal collection and over collection (Jennings and Rohr 2011). As

efforts are made to conserve species affected by these and other anthropogenic forces, we are faced with conservation decisions that may have great impacts on the future of the species in question. In doing so, we must not only examine ecological and external threats, we must also consider levels of genetic diversity within and among remaining populations. Genetic diversity is important for both short-term adaptation to microhabitat differentiation and disease outbreaks (Huenneke 1991), as well as long-term survival through evolutionary adaptation to changing environments (Ellstrand and Elam 1993; Frankham et al. 2002; Holsinger and Gottlieb 1991). In addition, inbreeding depression resulting from reductions in population size may also be a threat (Frankham et al., 2002). Population genetic studies are a valuable way to address these issues, providing data that can be used to set conservation priorities and help identify populations suitable for ex situ conservation efforts. Here we examine genetic diversity in three narrowly-distributed, Federally Endangered species of North American Pitcher Plants.

Sarracenia oreophila (Kearney) Wherry, the green pitcher plant, is the most widespread of the three study species, found in five counties of the Ridge and Valley province of northeastern Alabama and two sites on the Blue Ridge near Lake Chatuge along the Georgia-North Carolina border (U.S. Fish & Wildlife Service 2011; U.S. Fish & Wildlife Service 1994). While historical populations have been said to occur in central Alabama, it is likely that any reference to such populations originates from Wherry's (Wherry 1935) collections, which were later determined to be *S. alabamensis* (Case and Case 1973). Less than 40 populations are now known to occur and nearly all are found on private land

(U.S. Fish & Wildlife Service 1994). It was listed by the U.S. Fish & Wildlife Service (FWS) as an endangered species in 1980.

Sarracenia alabamensis Case & Case, the Alabama canebreak pitcher plant, is likely the rarest of any taxon in the genus, known from only three counties along the fall-line of central Alabama. Out of 30 populations that were originally known, only twelve remained at the time it was added to the Endangered Species list in 1989 (U.S. Fish & Wildlife Service 1992). As many as half of these, however, may now be extirpated (Alabama Forever Wild Program, personal communication).

Sarracenia jonesii Wherry, the mountain sweet pitcher plant, occurs in four counties along the North Carolina/South Carolina border. Only ten populations are known to exist, and at least sixteen have been extirpated, leading to Federal listing as Endangered in 1988 (U.S. Fish & Wildlife Service 1990). Along with *S. oreophila* and *S. purpurea*, it is one of only three taxa in the genus found outside the coastal plain.

The Sarracenia rubra 'Complex'

While now generally accepted as separate species, there has been a long-standing debate over whether *S. alabamensis* and *S. jonesii* should be recognized at the level of species or simply represent regional variants or subspecies of *Sarracenia rubra*. Some authors, noting the overall similarities in leaf and flower morphology, have included these taxa as part of the “*Sarracenia rubra* complex” as subspecies or varieties (Bell 1949; McDaniel 1971; Schnell 2002). Others, noting morphological differences such as pitcher size, shape, and

venation, capsule size, and the presence or absence of phyllodia, are consistent and maintained under greenhouse conditions, have argued for species status (Case and Case 1976; Case and Case 1973). Furthermore, the issue was not resolved by recent phylogenetic work in the genus (Neyland and Merchant 2006). While the primary goal of the study is to provide a genetic framework for conservation in these taxa, it also presents an excellent opportunity to address this long-standing taxonomic question.

Study goals

This study examines genetic diversity in three Federally Endangered members of the North American Pitcher Plants, *Sarracenia*. We surveyed nine polymorphic microsatellite loci and sequenced three non-coding chloroplast regions in *S. alabamensis*, *S. jonesii*, and *S. oreophila*. For comparative purposes, we examined variation in those same microsatellite loci at 'Collier Pines', a single field site consisting of three more common *Sarracenia* species – *S. leucophylla*, *S. alata*, and *S. rubra* ssp *wherryi*. This is clearly a gross underestimate of species-wide genetic diversity in the three species at Collier Pines, and our analysis is not intended as a species-to-species comparison. We were guided by the following questions: (1) what is the distribution of genetic variation within and among populations of these three endangered species? (2) is there genetic evidence of population bottlenecks in any or all of the study species?, (3) how does genetic variation compare among study species and to the Collier Pines site consisting of three more common *Sarracenia* species?, and (4) Do *S. jonesii* and *S. alabamensis* exhibit sharing of microsatellite alleles and

chloroplast haplotypes? While these results will provide a basis for conservation strategies for these taxa, we also hope to provide insight into the evolutionary history and population dynamics of these species.

Materials and Methods

Study species

Members of the genus *Sarracenia* (Sarraceniaceae) are carnivorous, rhizomatous, long-lived, perennial herbs found primarily along the southeastern coastal plain of the United States, with a few populations in the extreme southern Appalachians and a single species (*S. purpurea*) extending north into New England and Canada (Schnell 2002). They are primarily found in the low-nutrient, acidic soils of sphagnum bogs, mountain seeps, and longleaf pine savannas (McDaniel 1971). Known pollinators include bees of the genera *Bombus* and *Augochlorella* and the fly *Fletcherimyia fletcheri* (Mandossian 1965; Ne'eman et al. 2006; Schnell 1983). There are no obvious seed dispersal mechanisms, and while migrating birds have been suggested as a possible vector (Harper 1918), to our knowledge this hypothesis has not been addressed empirically. Members of the genus are primarily outcrossing due to the unique morphology of flowers that makes natural selfing unlikely (Schnell 2002), and artificial selfing has been shown to produce fewer and less viable seeds (Sheridan and Karowe 2000). All species are capable of reproducing vegetatively through rhizomes (McDaniel 1971).

Plant collection

All necessary permits and/or landowner permission were obtained in advance of collection of plant material. A total of 190 individuals were sampled from nineteen populations of the three study species. Due to the habitat specificity of the genus, populations have relatively well-defined boundaries. The minimum distance between any two populations was approximately two kilometers. Sampling localities are shown in Table 2.1. Only approximate (i.e. county) locations are provided to protect plants from disturbance or illegal collection. Fifteen individuals from each population were sampled haphazardly with an emphasis on maximizing distance between samples. In populations of fewer than 15 clumps, all clumps were sampled. To avoid taking two samples from the same “clump” (and possibly the same genetic individual), all samples were separated by a minimum of one meter, well over the largest documented rhizome lengths measured in the genus (Schnell, 2002).

We also sampled 192 individuals from a single field site in Washington County, Alabama (“Collier Pines”), consisting of three *Sarracenia* species – *S. leucophylla*, *S. alata*, and *S. rubra* ssp *wherryi*. Samples were taken on two 10 X 10 meter grids and a 100 meter transect, sampling one plant every meter. If no plant existed within one half meter, then no sample was taken.

Identification of species was based on pitcher (i.e. leaf) morphology (Schnell 2002; Schnell 1978; Schnell 1977). This was generally not an issue at study sites of the three focal species, as *S. oreophila* and *S. alabamensis* exist completely allopatrically and *S. jonesii* only exists in sympatry with *S. purpurea*. Due to the endangered status of the study species, only a single pitcher was

taken as a voucher specimen from each species in each location and deposited at the University of Tennessee Herbarium (TENN).

Leaf material for DNA extraction was obtained by removing 1-2 cm² from the pitcher hood or ala (the seam-like structure running from the pitcher opening to the base; (Schnell 1998) and storing on ice for up to two days before transfer to -80° C storage. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen®, Valencia, California, USA).

Non-coding chloroplast DNA amplification

Three non-coding chloroplast regions were selected based on use in a previous study of all *Sarracenia* species (Furches and Small, in review). These regions – 3'*rps16* – 5'*trnK*, *atpI* – *atpH*, and *trnS* – *trnG*, were amplified on all 190 focal species individuals using the following protocol: 95°, 1 min; 35 X (95°, 1 min; 55°, 1 min; 72°, 1 min); 72°, 10 min. PCR products were then visualized by running on a 1.5% agarose gel for approximately 30 minutes at 96 mV.

Sequencing was performed on an Avant 3600 Genetic Analyzer (Applied BioSystems, Foster City, CA) at the University of Tennessee Molecular Biology Resource Facility (MBRF).

Alignment and processing

Initial examination of sequences, forming of contigs, and examination of ambiguous base calls were performed with SEQUENCHER v. 4.9 (Gene Codes Corporation, Ann Arbor, MI). Automatic alignment and further manual alignment was made using MESQUITE v. 2.72 (Maddison and Maddison 2009). In addition

to data generated in this study, we included eight sequences from our previous study (Furches and Small, in review). This includes one sequence from *Heliamphora nutans* (outgroup) and seven *Sarracenia* sequences representing major haplotypes from that study. All major haplotypes from the previous study were shared among species and were found in relatively contiguous geographic areas (Furches and Small, in review). In the current study, therefore, these haplotypes are identified by geographic origin, rather than species, as this will provide a more informative context in which to examine the study species. Those include: Haplotype 13 (North and South Carolina coast), Haplotype 20 (southern Alabama), Haplotype 50 (western Louisiana and eastern Texas), Haplotype 63 (west-central Georgia), Haplotype 52 (Florida panhandle), Haplotype 48 (Mississippi Gulf Coast), and Haplotype 62 (southeastern Georgia). Sequences from the three non-coding chloroplast regions were concatenated to produce a cpDNA dataset with a total aligned length of 3,507 base pairs [3'*rps15*-5'*trnK* (930 bp), *atpI*-*atpH* (1,069 bp), *trnS*-*trnG* (1,508 bp)]. All identical sequences from each population were then condensed to produce 17 haplotypes (*S. alabamensis* = 2, *S. jonesii* = 1, *S. oreophila* = 14), using the program COLLAPSE v1.2 (Posada 2006). Sequence gaps ("indels") were coded manually using the guidelines of Ochoterena (Ochoterena 2009). This produced an additional 28 multi-state characters for a total of 3,535 characters.

Analyses

To examine patterns of divergence in chloroplast haplotypes, we first independently analyzed each of the three sequence regions with the FindModel

(Los Alamos National Laboratory 2011) implementation of ModelTest (Posada 2008). This program provides an assessment of 28 models of evolution based on Akaike Information Criterion (AIC) scores. The complete partitioned dataset was then analyzed in MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) under several parameter scenarios to determine the most appropriate model for each partition. Models chosen were as follows: *3'rps16 – 5'trnK* (nst = 6, rates = propinv), *atpI – atpH* (nst = 6, rates = gamma), *trnS – trnG* (nst = 6, rates = gamma), indels with two states (coding = variable, rates = equal), indels with multiple states (coding = nopresence, rates = propinv). Finally, the analysis was run for 5×10^6 generations under the following conditions: burnin fraction = 0.25, temp = 0.08, swapfreq = 7, nswaps = 3. to illustrate relationships of haplotypes, we performed a haplotype network analysis using TCS v1.21 (Clement et al., 2000).

Microsatellite amplification and fragment analysis

A total of nine microsatellite loci were used in this study. Seven loci developed by Koopman et al (2009) for analysis in *Sarracenia alata* were found to cross-amplify in all three study species and shown to be polymorphic in one or more. Additionally, two loci developed by Rogers et al. (2010) met these criteria. Microsatellites were amplified using the method of Schuelke (2000). PCR products were genotyped by electrophoresing fluorescently labeled products through an Avant 3600 Genetic Analyzer (Applied Biosystems, Foster City, CA) at MBRF. Fragment sizes were determined by comparison to the ROX500 size standard in PEAKSCANNER software (Applied Biosystems). The prospect of

null alleles is always a concern when using microsatellites on multiple species. However, all loci used in this study amplified in 100% of the individuals examined, indicating that null alleles, if present, are so at a very low frequency.

Microsatellite data analysis

Standard measures of genetic diversity – alleles per locus (A), percent polymorphic loci (P), effective number of alleles per locus (A_e), observed heterozygosity (H_o), and expected heterozygosity (H_e) – were calculated for each of the three focal species (i.e. *S. alabamensis*, *S. oreophila*, and *S. jonesii*) as well as the Collier Pines site using GenAlEx v6.41 (Peakall and Smouse 2006). For the focal species, we also calculated Weir & Cockerham's (Weir and Cockerham 1984) F-statistics using FSTAT v2.9.3 (Goudet 2001) to examine hierarchical genetic variation among populations. This program was also used to test for genotypic disequilibrium among loci for each species, as well as test for population differentiation (Goudet et al. 1996) and calculate allelic richness (Leberg 2002) of populations and of each species. To examine pairwise population differentiation and differentiation among species, we used SMOGD (Crawford 2010) to calculate Jost's D (Jost 2008). This parameter has been shown to provide a more robust measure of population differentiation than pairwise F_{ST} and similar measures, particularly when many private alleles are present (Jost 2008). to test for evidence of recent bottlenecks we used Bottleneck v1.2 (Cornuet 1996), a program that tests for an excess of heterozygosity by comparing H_e , the measured expected heterozygosity, to H_{eq} , the equilibrium heterozygosity [*not* a test for $H_o > H_e$, (Piry et al. 1999)]. We used

the Two Phase Model (a combination of the Stepwise Mutation Model and the Infinite Alleles Model), as this has been shown to be most appropriate for microsatellites (Di Rienzo et al. 1994). We also used the Wilcoxon's test for significance because fewer than 20 loci were used (Cornuet 1996; Piry et al. 1999). We performed tests for Isolation By Distance in each species and an additional test using only the Alabama populations of *S. oreophila* using IBD Web Service (Jensen et al., 2005).

To examine underlying patterns of diversity within each species, we used the program STRUCTURE v2.3.1 (Pritchard et al. 2000). After performing 20 trial runs for 250 000 generations (with the first 50 000 generations discarded as 'burn-in') under each K value, we used both the original method described by Pritchard et al. (2000) as well as the method described by Evanno et al. (2005) to determine the most appropriate K value. Once this was determined, we then performed the final analysis for 1 000 000 generations with a burn-in of 250 000. Individuals were then assigned to clusters ("Ks") based on posterior probability estimates for inclusion with a particular group. Based on initial results for *S. oreophila*, we ran an additional analysis including only Alabama populations. We considered individuals with an admixture coefficient ≥ 0.9 as being confidently assigned to a given cluster. Individuals with lower admixture coefficients were considered "admixed", and therefore divided among clusters.

Results

Non-coding chloroplast DNA

We found a total of fourteen haplotypes in *Sarracenia oreophila*, one in *S. jonesii*, and two in *S. alabamensis*. The phylogram produced from the Bayesian analysis is shown in Figure 2.1. All sequences from the current study form a single clade with strong posterior probability (0.87). Within this group, there is a polytomy consisting of four sub-groups. These include 1) the lone *S. jonesii* sequence, 2) the single haplotype from OR-1 and OR-2, 3) a clade consisting of all *S. alabamensis* sequences, and 4) a clade consisting of all other *S. oreophila* sequences. Six haplotypes were found in OR-6, more than any population, while four were found in OR-4 and two in OR-3, OR-5, and OR-7. All other populations had a single haplotype. No chloroplast haplotypes were shared among the three study species.

Results from the haplotype network analysis are shown in Figure 2.2. Those from *S. alabamensis* and *S. oreophila* showed the greatest similarity, while the lone *S. jonesii* haplotype was seven changes from the nearest neighbor. The distribution of haplotypes among populations is illustrated in Figure 2.3. The only sharing of haplotypes occurred among populations of *S. oreophila*. Both Lake Chatuge populations were fixed for the same haplotype. In Alabama, three haplotypes were found in multiple populations.

Microsatellite diversity among species

For the nine polymorphic microsatellite loci surveyed, a total of 62 alleles were observed among 28 individuals of *Sarracenia alabamensis* (1-16 per

locus), 51 alleles were observed among 60 individuals of *S. jonesii* (1-16 per locus), and 46 alleles were observed among 102 individuals of *S. oreophila* (1-18 per locus). No significant linkage disequilibrium was found for any loci in any species. Microsatellite summary statistics are shown in Table 2.1. *Sarracenia alabamensis* had the greatest number of alleles per locus, allelic richness, and expected and observed heterozygosities overall, while *S. oreophila* had the least. Both *S. alabamensis* and *S. jonesii* were polymorphic at eight loci ($P = 88.9\%$), while *S. oreophila* was polymorphic at seven ($P = 77.8\%$). Effective alleles per locus were lowest in *S. oreophila* and highest in *S. jonesii*. Weir & Cockerham's (1984) F statistics are shown for each species in Table 2.2, along with results from tests for population differentiation. All three species showed significant population differentiation ($P < 0.001$), with *S. alabamensis* showing the lowest values overall.

To compare genetic diversity in the focal species to that of more widespread congeners, data from all three focal species were combined into a single dataset and compared to a similar size sample from the Collier Pines field site. Table 2.3 shows the number of alleles (A), effective number of alleles (A_e), and observed heterozygosity (H_o) for each locus and the mean across all loci for both the Collier Pines site and the combined focal species dataset. The effective number of alleles per locus and observed heterozygosity across all loci differed significantly between the two data sets, with the Collier Pines site greater in both.

Microsatellite diversity within species

In *S. alabamensis*, one locus was monomorphic in all accessions, while

the remaining eight were polymorphic in all populations (Table 2.1). Private alleles (those confined to a single population) ranged from 13 of 44 total alleles in AL-3 to 7 of 34 in AL-1. Population AL-3 had the greatest alleles per locus, allelic richness, and observed heterozygosity, while AL-1 had the greatest effective number of alleles per locus and expected heterozygosity. Significant deviations from Hardy-Weinberg Equilibrium, as indicated by F_{IS} values, were indicated in both AL-1 and AL-2. Pairwise differentiation measurements were all low (Table 2.4). However, AL-2 and AL-3 were much more similar than either was to AL-1. Based on BOTTLENECK analyses, none of the three *S. alabamensis* populations showed significant evidence of a recent bottleneck. Due to the small number of populations, a test for Isolation-By-Distance could not be performed on *S. alabamensis*.

In *S. jonesii*, one locus was monomorphic in all accessions, while the remaining loci were polymorphic in one to six populations (Table 2.1). Private alleles ranged from only 1 of 24 in JZ-5, to 6 of 18 in JZ-1 and 6 of 30 in JZ-3. Population JZ-3 had the greatest diversity by all measures – alleles per locus, percent polymorphic loci, effective number of alleles per locus, observed and expected heterozygosities, and allelic richness. Conversely, population JZ-4 had the lowest percent polymorphic loci, effective number of alleles per locus, observed and expected heterozygosities, and allelic richness, as well as the second lowest alleles per locus. Populations JZ-3 and JZ-5 had significant deviations from Hardy-Weinberg Equilibrium. Pairwise differentiation measurements ranged from a low of 0.004 (JZ-5 and JZ-6) to a high of 0.371 (JZ-1 and JZ-3; Table 2.5). Notably, the four highest values involved population

JZ-4. Significant evidence of a recent bottleneck was found in JZ-2 ($P = 0.03$).

The test for isolation-by-distance was not significant ($r^2 = 0.197$; $P = 0.118$).

Two loci in *S. oreophila* were monomorphic in all accessions (Table 2.1). The remaining seven loci were polymorphic in two to eight populations. Very few private alleles were found in *S. oreophila*. Three populations had none – OR-1, OR-5, and OR-7 (21, 13, and 15 total alleles, respectively), while another three had only one – OR-2, OR-6, and OR-8 (19, 18, and 15 total alleles, respectively). Population OR-3 had 3 of 23 total alleles, while OR-4 had 6 of 33 total alleles that were private. Population OR-4 had the greatest diversity by all measures. The lowest values were found in OR-5, OR-7, and OR-8. Significant deviation from Hardy-Weinberg Equilibrium was found in OR-1, OR-2, OR-3, OR-4, and OR-8. Pairwise differentiation measures ranged from virtually no differentiation ($D = 0.001$, OR-6 and OR-7), to 0.246 between OR-1 and OR-5 (Table 2.6). Population OR-1 had four values of $D > 0.200$, while no other population pair had a value greater than 0.111. No populations showed evidence of a recent bottleneck. A test for Isolation-By-Distance performed on *S. oreophila* was significant when all data were included ($r^2 = 0.285$; $P = 0.033$). When the Lake Chatuge populations (OR-1 and OR-2) were excluded only populations in Alabama were considered, the test was also significant ($r^2 = 0.266$; $P = 0.034$).

Pairwise estimates of differentiation between the three study species (Table 2.7) showed much lower differentiation between *S. jonesii* and *S. oreophila* ($D = 0.536$) than between *S. jonesii* and *S. alabamensis* ($D = 0.839$) or *S. oreophila* and *S. alabamensis* ($D = 0.836$).

Structure analyses of microsatellites

Using both the method of Pritchard et al. (2000) and Evanno et al. (2005), no clear choice for the most appropriate K value for *Sarracenia alabamensis* could be determined (i.e. likelihood values were not different). We therefore ran the entire analysis (1 000 000 iterations with 250 000 burn-in) under K values of 2, 3, 4, and 5. In each analysis, all individuals were admixed nearly evenly among all clusters, indicating that the likely choice for K should actually be one. This is consistent with the relatively low differentiation among the three populations sampled for this study.

The analyses for *Sarracenia jonesii* provided a clearer choice, with $K = 4$ indicated as the most appropriate value (Figure 2.4). The first cluster consists of all North Carolina individuals, populations JZ-1 and JZ-2. While the second cluster consists of some admixed individuals (five with less than 0.90 admixture coefficients), it includes all JZ-3 individuals. The third cluster forms the most cohesive group, with all individuals from population JZ-4, all with admixture coefficients >0.95 . The last group consists of all of populations JZ-5 and JZ-6, with five admixed individuals (<0.90).

Two STRUCTURE analyses were performed on *Sarracenia oreophila* data. Using the complete *S. oreophila* dataset, the most appropriate K value was determined to be two (Figure 2.5A). The results of this analysis showed a strong division between Alabama populations and Lake Chatuge populations. Only two out of 30 Lake Chatuge individuals were admixed (i.e. posterior probabilities <0.90). Eleven of 72 samples from Alabama were admixed, and four of these were more closely aligned with the Lake Chatuge accessions. To examine

population structure within Alabama, a second analysis was performed with the two non-Alabama populations excluded. In this analysis the most appropriate K value was four (Figure 2.5B). While some admixture was indicated, most individuals from a given population were found in the same cluster. The first consisted of all OR-3 individuals, along with some accessions from populations OR-6 and OR-7. The second cluster included all OR-5 individuals and the remaining OR-6 and OR-7 samples. The third cluster included all OR-4 samples, with some admixture from OR-3 and OR-6. The fourth and strongest cluster within Alabama consisted of all OR-8 accessions, all with posterior probabilities of >0.90 . While the authors of STRUCTURE warn that erroneous results may occur when examining systems with isolation-by-distance, our STRUCTURE results in *S. oreophila* show a pattern consistent both with geography and our non-coding chloroplast data.

Discussion

A central question in conservation genetics is: How does genetic variation compare among rare species versus their widespread relatives? While some studies have shown reduced variation in narrowly-distributed species, results have been mixed (see Gitzendanner and Soltis 2000 for a review). As outcrossing, long-lived perennials, *Sarracenia* exhibits some life history traits associated with higher levels of genetic diversity (Hamrick and Godt 1996). In our study, we found greater microsatellite diversity in a single field site consisting of three widely-distributed *Sarracenia* species than in the entire range of all three focal species combined. A possible contributing factor may be that, in addition to

the broader geographic ranges three species at Collier Pines, they are also often found in very large populations of thousands of clumps and covering many hectares (personal observation). This is in stark contrast to the three narrowly-distributed species we examined here. *Sarracenia oreophila*, the most common of the three species, is still found in fewer than three dozen small populations (U.S. Fish & Wildlife Service 1994). The three focal species were not devoid of genetic diversity, however. In fact, *Sarracenia alabamensis*, the rarest of the three, exhibited the highest levels of overall diversity.

Sarracenia alabamensis

Sarracenia alabamensis is by far the rarest and most narrowly-distributed of any species in the genus. The populations we examined were of similar habitat types (mountain seeps), although they differed in level of disturbance (personal observation). We found two chloroplast haplotypes in *S. alabamensis*, and no population exhibiting both haplotypes. This is consistent with our previous work on the genus, where isolated, allopatric populations have generally been monomorphic for chloroplast sequences (Furches and Small, in review). Our microsatellite data was very different, however. Despite the small sample size, in *S. alabamensis* we found nearly seven alleles per locus, far more than *S. jonesii* or *S. oreophila*. In fact, within-population microsatellite diversity in *S. alabamensis* is comparable to that found in *S. alata*, a much more widespread species (Koopman and Carstens 2010). This is consistent with the allozyme study by Godt and Hamrick (1998), which found higher levels of diversity in *S. alabamensis* than the more widespread *S. rubra* ssp. *rubra*. Population

differentiation was significant in *S. alabamensis*, but at far lower levels than other study species ($\theta = 0.075$; Table 2.2). This is likely due to their close proximity to each other (< 4 km), which is only slightly farther than documented foraging distances for bumblebees, a primary pollinator (Osborne et al. 2008).

While *S. alabamensis* does maintain substantial variation, there are several reasons for concern over the future of this species in the wild. Significant reductions in heterozygosity in AL-1 and AL-2 suggest that populations are being impacted by environmental changes. While AL-3 is found in a relatively undisturbed and inaccessible woodland, AL-1 and AL-2 are found on former pasture land and a utility right-of-way. Population AL-3, the largest of the three study sites, was the only population with seedlings present, exhibited the greatest microsatellite diversity, and was the only population without a significant inbreeding coefficient (Table 2.1). The number of populations had already dropped from thirty to just twelve at the time of Federal listing (U.S. Fish & Wildlife Service 1992). Just three years later, Murphy and Boyd (Murphy and Boyd 1999) found only ten of those populations, plus a previously unknown site. At that time they estimated that no more than three of these were sustainable. As of 2011, only about half of these remain (Alabama Forever Wild Program, personal communication). Clearly, *Sarracenia alabamensis* is a species in serious danger of extinction. With so few populations in existence and even fewer of a potentially sustainable size, a single stochastic event such as a prolonged drought or fire, could be catastrophic. Fortunately, relatively high levels of diversity still remain, meaning that if populations are protected, the potential for long-term survival still exists.

Sarracenia jonesii

Sarracenia jonesii is only slightly more common than *S. alabamensis*. Habitats in which *S. jonesii* populations are found vary considerably, however, from the edge of dammed streams (JZ-1 and JZ-4), to the understory of mesic forests (JZ-2 and JZ-5), to patches of soil on steep granite outcrops covered by flowing water (JZ-3 and JZ-6; personal observation). We found less overall variation than in *S. alabamensis*. All three non-coding chloroplast regions were monomorphic across all accessions – the only taxon in the genus that we have failed to find variation in either of the three regions sequenced (current study; Furches and Small, in review). Measures of microsatellite diversity were lower by nearly all measures. In fact, the most diverse *S. jonesii* population had fewer alleles per locus and lower heterozygosities than the least diverse *S. alabamensis* population. Our results are again consistent with the work of Godt and Hamrick (1996), who found low levels of allozyme diversity in this species. Clustering analysis showed JZ-4 – one of the largest, yet the least diverse, populations – forms a strong cluster with very little admixture (Figure 2.4). Divergence measurements were also highest for this population. This may be partly explained by a history of disturbance at this site. *Sarracenia jonesii* was formerly found only along the edge of the small stream that ran through this site, which was later dammed. While some plants were possibly destroyed in the process, the population has now spread along the edge of the resulting reservoir (Mary Bunch, South Carolina Heritage Program, personal communication). The apparent lack of diversity for a population of this size may be due to recent population growth, which would make this one of the rare cases where

Sarracenia may have actually *benefited* from anthropogenic changes in hydrology.

While *S. jonesii* is somewhat more common than *S. alabamensis*, it remains extremely rare. Moreover, the lower levels of genetic diversity present in *S. jonesii* may present an even greater concern for long-term survival. However, there are several factors from which this species may benefit. *Sarracenia jonesii* is unlikely to suffer from the effects of suppression of the natural fire regime that plagues many species in the genus. While *Sarracenia* generally require frequent, natural fires that serve to reduce competition and prevent closing of the canopy (Brewer 2001; Folkerts 1982; Platt et al. 1988), *Sarracenia jonesii* inhabits an area with a much lower natural fire frequency (Delcourt and Delcourt 2000). Instead, *S. jonesii* tends to inhabit areas where soil conditions and hydrology assure high levels of sunlight and reduced competition, such as steep granite outcrops with thin patches of soil and continuously flowing water. *Sarracenia jonesii* was unique among the three study species in that we found seedlings in several populations (JZ-3, JZ-4, and JZ-5). In addition, existing populations are provided greater protection than most *Sarracenia* populations. All four South Carolina study sites are within state park or designated wilderness areas. One of the two North Carolina study sites is protected by the Nature Conservancy, while the other is on private commercial property whose owner has taken an active role in protecting the site.

Sarracenia oreophila

Sarracenia oreophila had by far the most chloroplast haplotypes of the three study species (14), compared to only two in *S. alabamensis* and one in *S. jonesii*. In fact, we found six haplotypes in a single population (OR-6; Figure 2.2). This is a surprisingly high level of chloroplast diversity for a *Sarracenia* species, given our previous work in the genus. In contrast, we found relatively low levels of microsatellite variation in *S. oreophila*. In fact, despite being the most widespread of the three study species, *S. oreophila* had the lowest overall and within-population microsatellite diversity. The patterns of diversity, however, were similar to that of non-coding chloroplast haplotypes, with the greatest diversity in the area around Little River Canyon in extreme northeastern Alabama, as well as strong differentiation among the more geographically isolated populations. When all populations were considered, there was a strong delineation between Alabama populations (OR-3 - OR-8) and 'Lake Chatuge' populations (OR-1 and OR-2; Figure 2.5A). These two populations possessed no chloroplast variation, and the lone chloroplast haplotype was not found in any other population. Our data clearly show the Lake Chatuge populations are unique and an important part of any conservation plan for this species. There is a heightened cause for concern due to the loss of other populations in the area, encroachment by residential development, altered hydrology, and agricultural runoff (U.S. Fish & Wildlife Service 1994, 2004).

Within Alabama, there was strong clustering of all individuals from OR-8, the most geographically isolated of the Alabama populations, lying west of Lookout Mountain (Figures 2.3 & 2.5b). Like the Lake Chatuge populations, OR-

8 also possessed a single, unique chloroplast haplotype (Figure 2.3). At the time of our visit, the site was covered with dense undergrowth characteristic of an altered fire regime, and only 11 clumps and no seedlings could be located. Of the remaining five Alabama sites, one is located along a utility right-of-way on private land and another is within a privately-owned campground. The smallest population, OR-5, is entirely located on a river sandbar. All but two Alabama populations from this study, OR-4 and OR-5, are located on private land.

The Sarracenia rubra 'Complex'

Both *Sarracenia jonesii* and *S. alabamensis* were once considered subspecies of *Sarracenia rubra* (Schnell 1977, 2002). This '*Sarracenia rubra* Complex', as it came to be known, has been the source of the greatest taxonomic debate within the genus (Case and Case 1973, 1976). While not the primary focus of our study, we used the data obtained to compare the two taxa and perhaps provide additional insight into the debate. As shown in Table 2.7, measurements of microsatellite differentiation between *S. alabamensis* and *S. jonesii* are similar to those between *S. alabamensis* and *S. oreophila*, and even greater than that between *S. jonesii* and *S. oreophila*. As shown in Figure 2.2, no chloroplast haplotype was shared between the two taxa, and the two *S. alabamensis* haplotypes are more similar to many haplotypes present in *S. oreophila* than the lone *S. jonesii* haplotype. Our data show greater genetic differentiation at both microsatellite loci and non-coding chloroplast regions between *S. jonesii* and *S. alabamensis* than between *S. alabamensis* and the

morphologically and ecologically distinct *S. oreophila*. Our study further supports the consideration of these taxa as separate species.

Conservation implications and future work

The current state of the three species examined here exemplifies the threats facing many wetland species in the southeastern United States. The greatest threat remains habitat destruction and alteration, although illegal collection continues to plague *Sarracenia* and other carnivorous plant genera. Stabilization and protection of remaining populations should form the basis of any conservation efforts. Our study will help guide future conservation efforts in these three taxa.

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Appendix 1 – Figures and Tables

Table 2.1. Sampling sites and microsatellite diversity in the three focal species.

	Location	N	A (SE)	P (%)	A _E (SE)	H _O (SE)	H _E (SE)	A _R	A _P	F _{IS}
<i>Sarracenia alabamensis</i>										
AL-1	Autauga Co., AL	8	3.778 (0.662)	88.9%	2.721 (0.387)	0.389 (0.113)	0.550 (0.081)	3.77	7	0.353*
AL-2	Autauga Co., AL	8	3.667 (0.624)	88.9%	2.258 (0.352)	0.319 (0.078)	0.460 (0.086)	3.66	8	0.365*
AL-3	Chilton Co., AL	12	4.889 (1.020)	88.9%	2.582 (0.402)	0.444 (0.098)	0.511 (0.090)	4.16	13	0.172
	All populations	28	6.889 (1.541)	88.9%	3.080 (0.683)	0.393 (0.086)	0.561 (0.083)	6.88	37	
<i>Sarracenia jonesii</i>										
JZ-1	Henderson Co., NC	5	2.000 (0.373)	55.6%	1.611 (0.289)	0.200 (0.067)	0.253 (0.094)	1.92	6	0.314
JZ-2	Transylvania Co., NC	4	1.556 (0.242)	44.4%	1.483 (0.198)	0.167 (0.110)	0.229 (0.091)	1.56	3	0.400
JZ-3	Greenville Co., SC	15	3.333 (0.667)	88.9%	2.158 (0.362)	0.274 (0.074)	0.432 (0.087)	2.40	6	0.395*
JZ-4	Greenville Co., SC	15	1.778 (0.465)	33.3%	1.180 (0.105)	0.111 (0.060)	0.110 (0.060)	1.40	5	0.026
JZ-5	Greenville Co., SC	15	2.667 (0.601)	66.7%	1.915 (0.381)	0.215 (0.083)	0.327 (0.102)	2.14	1	0.373*
JZ-6	Greenville Co., SC	6	2.333 (0.408)	77.8%	1.800 (0.303)	0.204 (0.072)	0.336 (0.087)	2.16	2	0.469
	All populations	60	5.667 (1.772)	88.9%	3.224 (0.808)	0.198 (0.059)	0.495 (0.112)	5.05	30	
<i>Sarracenia oreophila</i>										
OR-1	Clay Co., NC	15	2.333 (0.471)	66.7%	1.713 (0.270)	0.126 (0.064)	0.302 (0.094)	2.00	0	0.606*
OR-2	Towns Co., GA	15	2.111 (0.423)	66.7%	1.500 (0.224)	0.133 (0.066)	0.237 (0.084)	1.77	1	0.464*
OR-3	Cherokee Co., AL	15	2.556 (0.626)	66.7%	1.624 (0.202)	0.185 (0.070)	0.298 (0.088)	2.07	3	0.408*
OR-4	De Kalb Co., AL	15	3.667 (1.179)	66.7%	2.200 (0.536)	0.259 (0.089)	0.368 (0.106)	2.61	6	0.327*
OR-5	De Kalb Co., AL	5	1.444 (0.242)	33.3%	1.286 (0.181)	0.111 (0.075)	0.140 (0.076)	1.44	0	0.310
OR-6	De Kalb Co., AL	15	2.000 (0.441)	55.6%	1.317 (0.135)	0.126 (0.054)	0.186 (0.068)	1.73	1	0.352
OR-7	De Kalb Co., AL	11	1.667 (0.236)	55.6%	1.214 (0.085)	0.101 (0.044)	0.146 (0.054)	1.49	0	0.351
OR-8	De Kalb Co., AL	11	1.667 (0.441)	33.3%	1.298 (0.159)	0.061 (0.040)	0.154 (0.078)	1.52	1	
	All populations	102	5.000 (1.724)	77.8%	1.962 (0.293)	0.145 (0.048)	0.380 (0.097)	3.89	25	

AL = *Sarracenia alabamensis*, JZ = *Sarracenia jonesii*, OR = *Sarracenia oreophila*. N = clumps sampled, A = alleles per locus, P = percent polymorphic loci, A_E = effective alleles per locus, H_O = observed heterozygosity, H_E = expected heterozygosity, A_R = allelic richness, A_P = private alleles, and F_{IS} = inbreeding coefficient. F_{IS} values marked with an asterisk (*) indicated statistically significant values (p<0.05).

Table 2.2. F-Statistics (Weir & Cockerham 1984) across loci and test for population differentiation.

	<i>F</i>	<i>θ</i>	<i>f</i>	Pop. Diff.
<i>S. alabamensis</i>	0.333	0.075	0.279	p < 0.001
<i>S. jonesii</i>	0.639	0.438	0.357	p < 0.001
<i>S. oreophila</i>	0.639	0.356	0.439	p < 0.001

F = a measure of the variation observed at the individual level relative to the total amount of variation across all individuals

θ = a measure of the amount of variation residing within subpopulations relative to the total variation observed in the species

f = a measure of the variation in individuals relative to the subpopulation in which the individual resides

Table 2.3. Microsatellite diversity across the entire range of the three species examined in this study (“Focal Species” - *S. alabamensis*, *S. jonesii*, *S. oreophila*) and a single population of three widespread species (“Collier Pines” - *S. alata*, *S. leucophylla*, *S. rubra* ssp *wherryi*)

Focal Species					Collier Pines				
Locus	N	A	H _o	A _E	Locus	N	A	H _o	A _E
S-07	190	42	0.332	7.788	S-07	190	35	0.495	10.679
S-18	190	10	0.211	4.255	S-18	192	17	0.786	5.975
S-19	190	21	0.205	5.119	S-19	192	36	0.771	11.677
S-21	190	15	0.237	3.126	S-21	192	27	0.760	9.883
S-27	190	6	0.084	2.142	S-27	192	10	0.318	3.237
S-44	190	3	0.026	1.376	S-44	192	6	0.547	3.569
S-47	190	9	0.353	4.300	S-47	192	12	0.406	4.601
R-53	190	11	0.200	3.913	R-53	192	1	0.000	1.000
R-27	190	6	0.137	3.058	R-27	192	11	0.630	3.167
Mean	190	13.667	0.198*	3.897*	Mean	191.8	17.222	0.524*	5.976*
SE	0	3.965	0.035	0.620	SE	0.222	4.202	0.086	1.279

N = clumps sampled, A = alleles per locus, H_o = observed heterozygosity, A_E = effective alleles per locus. Mean values marked with an asterisk (*) indicated statistically significant values (p<0.05).

Table 2.4. Population differentiation (Jost 2008) for *Sarracenia alabamensis*.

	AL-2	AL-3
AL-1	0.0711	0.0701
AL-2	--	0.0396

Table 2.5. Population differentiation (Jost 2008) for *Sarracenia jonesii*.

	JZ-2	JZ-3	JZ-4	JZ-5	JZ-6
JZ-1	0.237	0.371	0.315	0.250	0.206
JZ-2	--	0.178	0.351	0.147	0.127
JZ-3	--	--	0.269	0.201	0.205
JZ-4	--	--	--	0.231	0.234
JZ-5	--	--	--	--	0.004

Table 2.6. Population differentiation (Jost 2008) for *Sarracenia oreophila*.

	OR-2	OR-3	OR-4	OR-5	OR-6	OR-7	OR-8
OR-1	0.035	0.107	0.146	0.246	0.231	0.211	0.233
OR-2	--	0.025	0.080	0.065	0.053	0.048	0.098
OR-3	--	--	0.081	0.092	0.048	0.042	0.094
OR-4	--	--	--	0.043	0.029	0.036	0.094
OR-5	--	--	--	--	0.003	0.007	0.110
OR-6	--	--	--	--	--	0.001	0.059
OR-7	--	--	--	--	--	--	0.055

Table 2.7. Species differentiation (Jost 2008) for *Sarracenia alabamensis* (AL), *S. jonesii* (JZ), and *S. oreophila* (OR).

	JZ	OR
AL	0.839	0.836
JZ	--	0.536

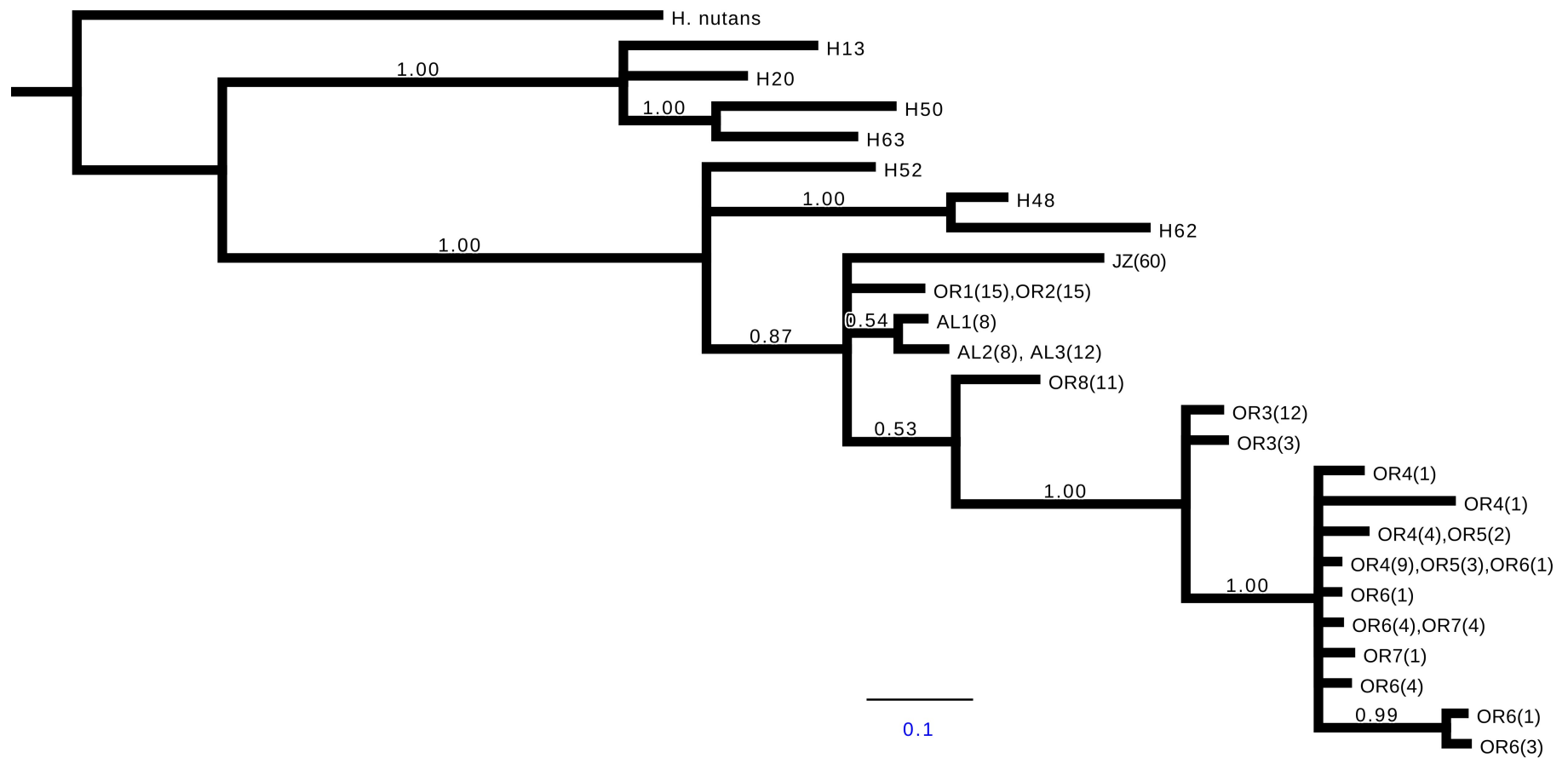


Figure 2.1. Non-coding chloroplast DNA phylogram of three study species with *Heliamphora nutans* as the outgroup. Posterior probabilities of less than 1.00 are shown. Branch labels with “H” represent haplotype numbers from Furches and Small (in review). Branch labels with 'AL', 'JZ', and 'OR' represent *Sarracenia alabamensis*, *S. jonesii*, and *S. oreophila*, respectively, followed by the population number and number of individuals in parentheses. For example, 'OR4(9)' represents nine accessions from population OR-4.

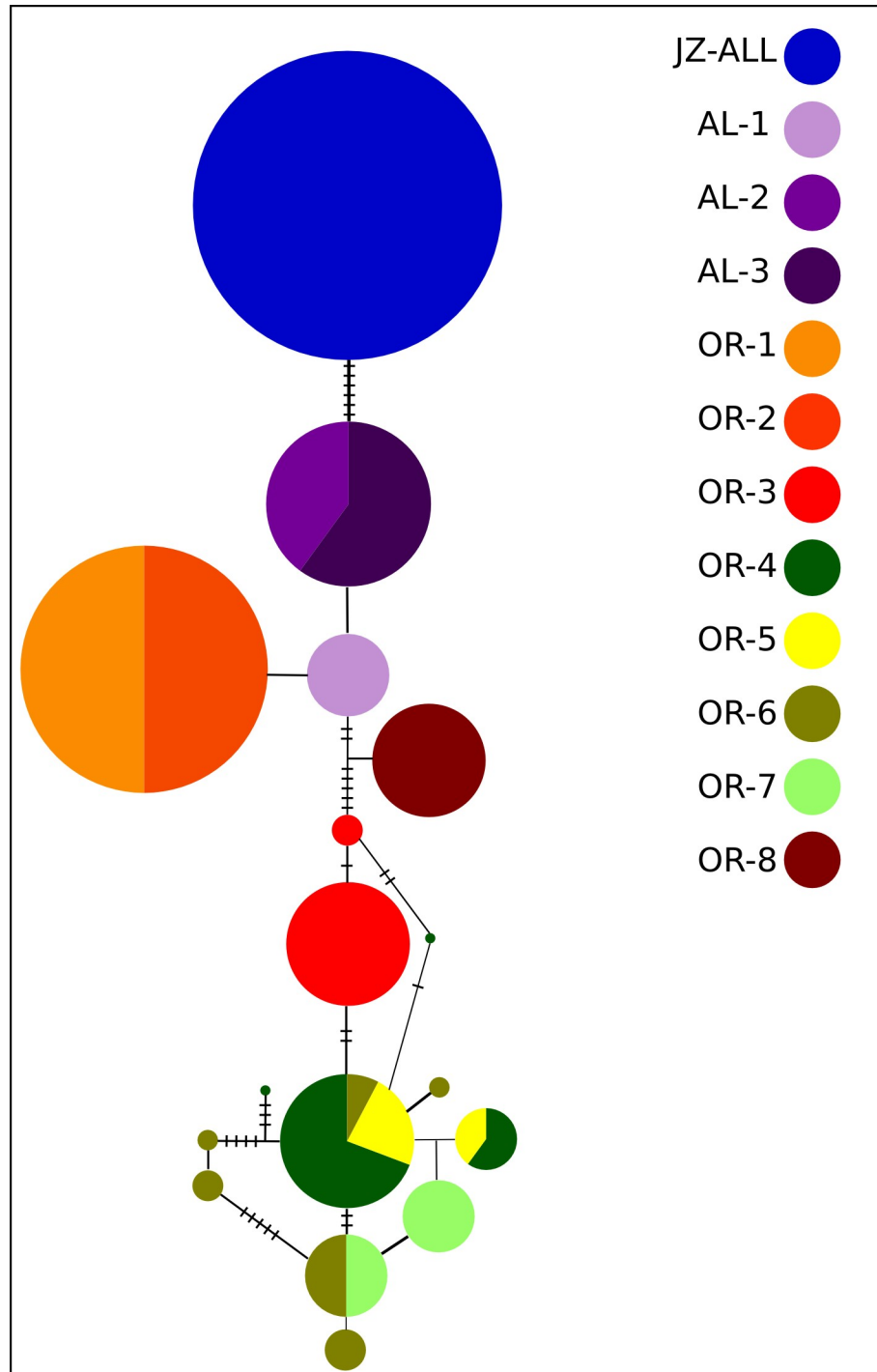


Figure 2.2. Haplotype network analysis of the 17 haplotypes found in the three focal species.

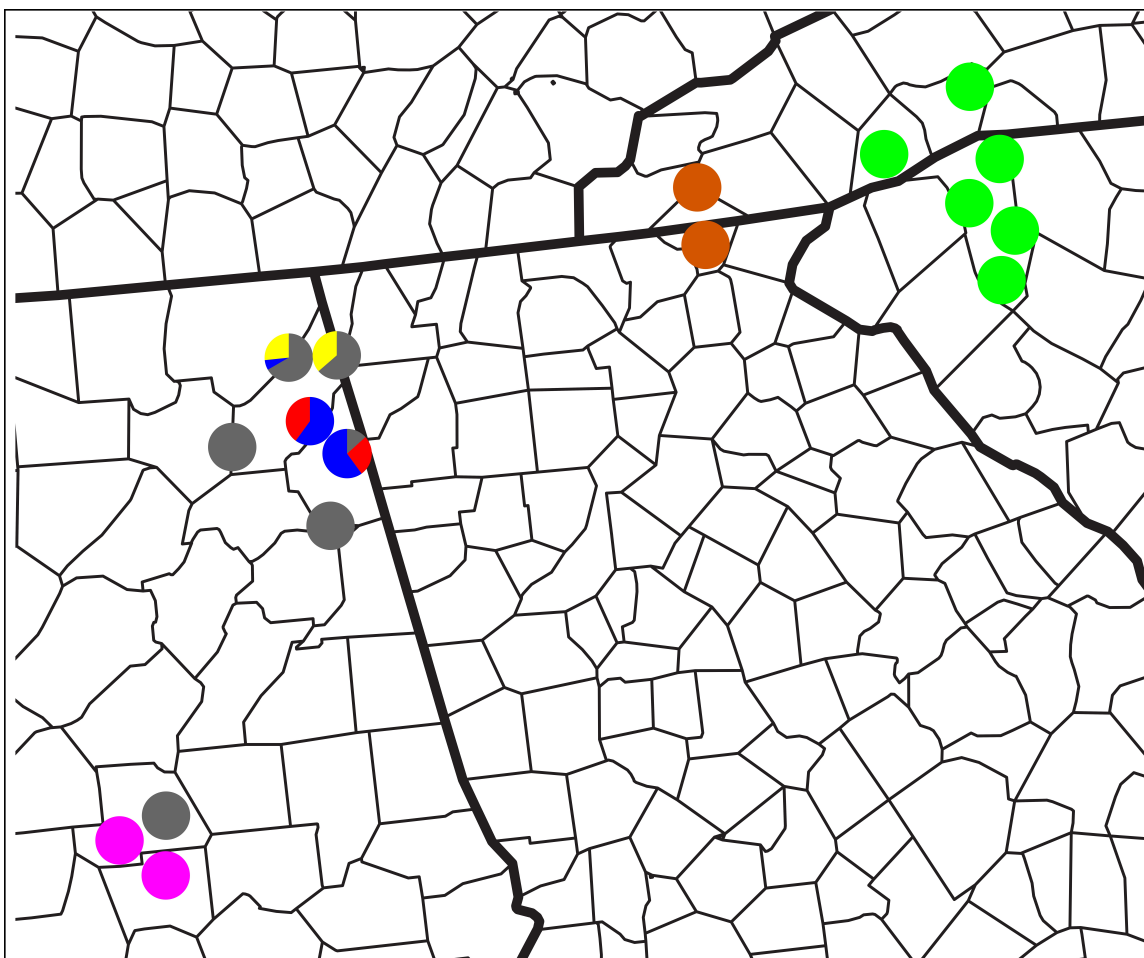


Figure 2.3. Haplotype sharing among populations of the three focal species. Gray indicates haplotypes private to a given population.

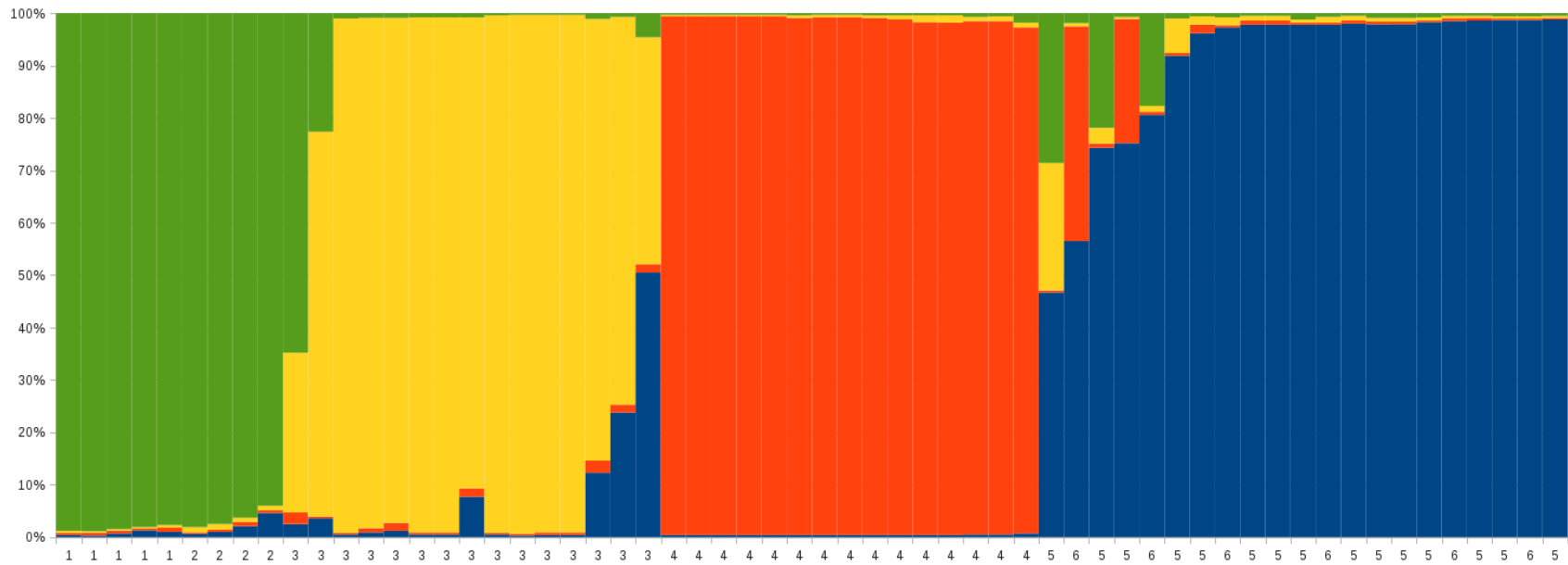


Figure 2.4. Structure analysis of *Sarracenia jonesii* microsatellite data. The y-axis indicates the proportion of an individual's genome assigned to a given group. The x-axis indicates the individual's population number (i.e. “1” = “JZ-1”)

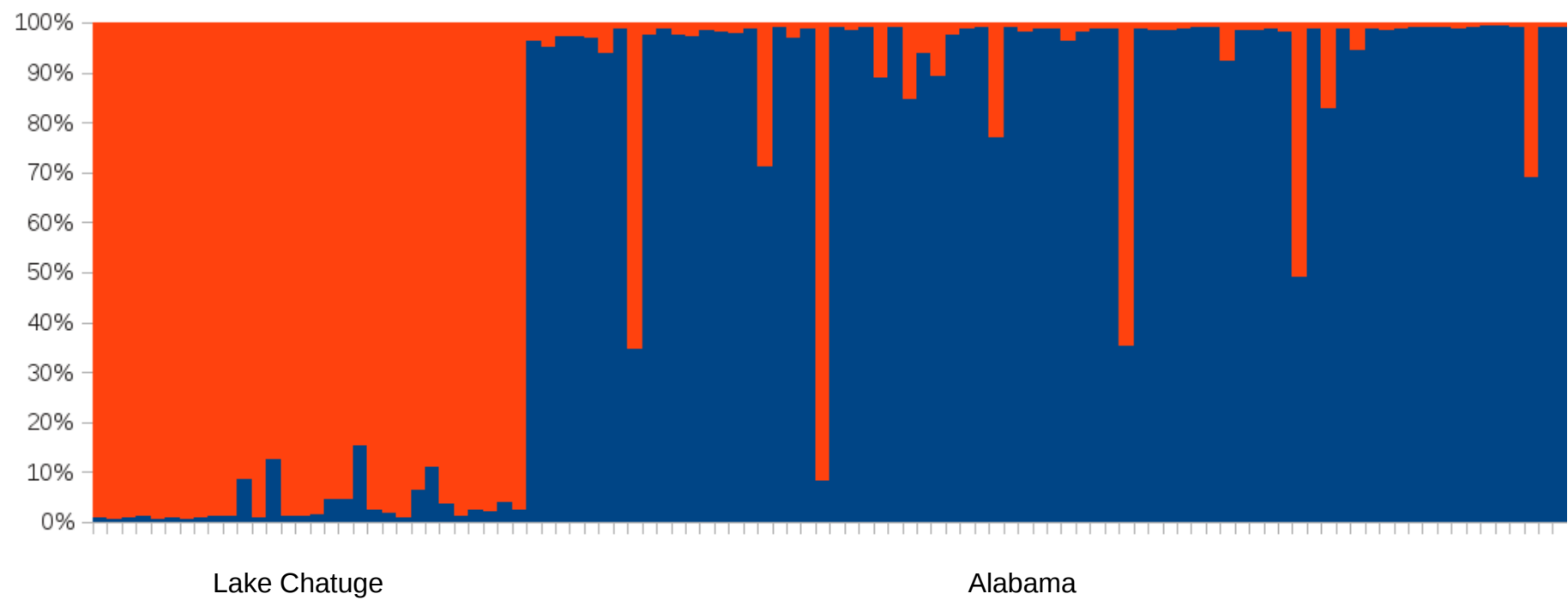


Figure 2.5A. Structure analysis of *Sarracenia oreophila* microsatellite data.

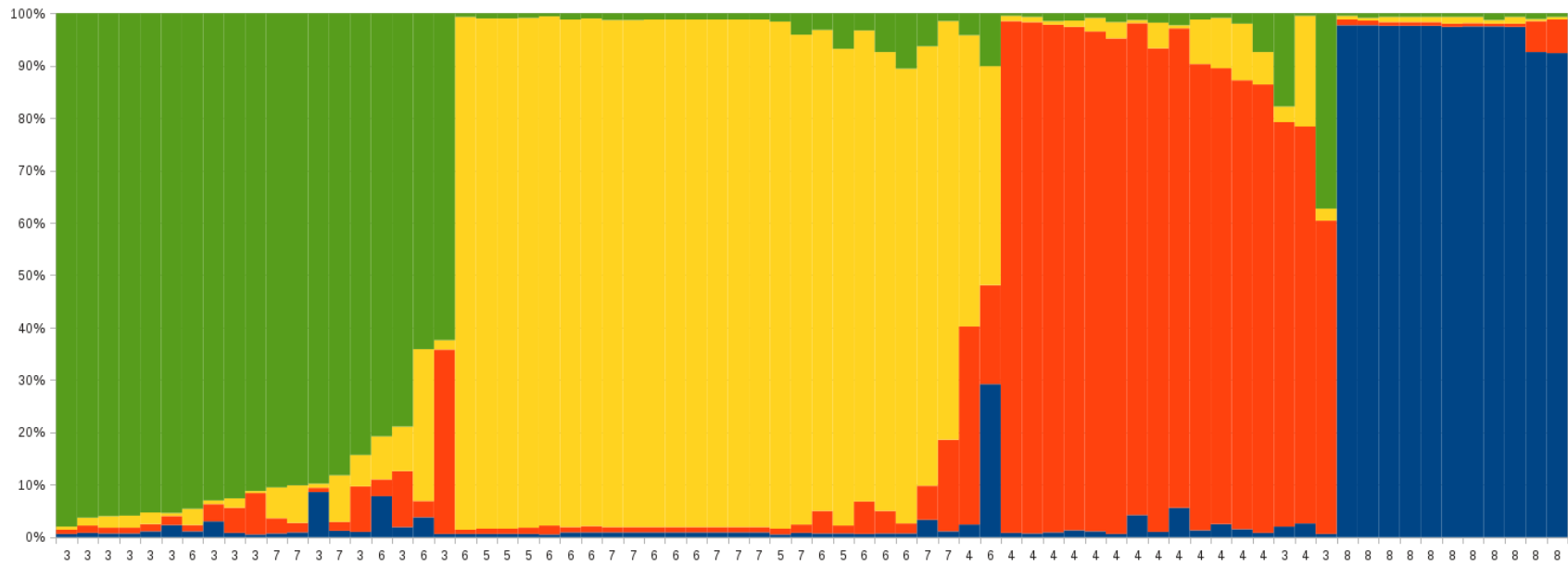


Figure 2.5B. Structure analysis of *Sarracenia oreophila* microsatellite data with only Alabama populations included. The y-axis indicates the proportion of an individual's genome assigned to a given group. The x-axis indicates the individual's population number (i.e. "3" = "OR-3").

CHAPTER 3

HYBRIDIZATION LEADS TO INTERSPECIFIC GENE FLOW IN *SARRACENIA*

Abstract

The role of hybridization in plant evolution remains a source of intense debate. Potential consequences range from genetic dead-ends to species fusion or hybrid speciation. While much has been learned from model systems such as *Populus*, *Iris*, and *Helianthus*, many questions remain. Consisting of eleven species, all of which are capable of hybridizing, *Sarracenia* presents an excellent system in which to study hybridization. We examined a single field site consisting of three species – *S. leucophylla*, *S. alata*, and *S. rubra* ssp *wherryi* to determine the level of genetic admixture and how this compared to allopatric sites of the same taxa. In contrast to the well-defined clusters formed when we examined the allopatric sites, the sympatric field site exhibited a wide range of admixture. Additionally, when the relative genetic makeup of 'pure' species at the site was compared to the makeup of hybrids, we found that *Sarracenia alata* contributed disproportionately to the hybrid genomes. Our study provides further evidence that hybridization is contributing to interspecies gene flow in the genus.

Introduction

The extent to which hybridization contributes to morphological and genetic diversity within populations has long been a source of debate (Mayr, 1992; Arnold, 2006). Often, the important question is not simply whether hybridization *occurs*, but whether hybridization substantially affects the gene pools of the species involved, and thus their evolution. Formation of initial F_1 hybrids may be common, yet never contribute to genetic exchange among species if backcrosses never occur (Uthicke et al., 2005). Even rare F_1 hybrids, however, can contribute greatly to interspecific gene flow if backcrosses then form (Mallet, 2007). Evolutionary consequences of introgressive hybridization vary widely, and range from species fusion (Seehausen et al., 1997), gene flow between species (Cruzan and Arnold, 1994), or even formation of new species (Rieseberg, 1991).

Study of the maintenance of species boundaries between hybridizing species is important for gaining insight into evolutionary processes surrounding speciation and secondary contact in closely related species. Under the genic view of speciation, the genome as a whole is porous, while a small number of loci under selection are responsible for species differentiation (Wu, 2001). This theory has been supported empirically (Emelianov et al., 2004; Minder and Widmer, 2008), and may explain why some taxa maintain species integrity in the presence of hybridization.

Systems involving hybridization among three or more species are inherently more complex, hence most studies have focused on species pairs (Carney et al., 1994; Burgarella et al., 2009). A few studies, however, have provided insight into this

phenomenon by examining hybridization in populations of three (Moran et al., 2011) or even four species (Lepais et al., 2009). The genus *Sarracenia* provides an opportunity to study one such system, in which 2-5 species, all capable of hybridizing, may be found at a single site.

Sarracenia

Sarracenia, the North American Pitcher Plants, is a genus eleven species of carnivorous, rhizomatous, perennial herbs found primarily along the southeastern coastal plain of the United States, with a few populations in the extreme southern Appalachians and a single species (*S. purpurea*) extending into New England and Canada (Schnell, 2002). They are found primarily in the low-nutrient, acidic soils of sphagnum bogs, mountain seeps, and longleaf pine savannas (McDaniel, 1971). The group has been popular in cultivation since the 19th century due to their unusual morphology, carnivorous habit, and the ability to form a variety of hybrids, including complex hybrids (i.e., multi-species) and backcrosses (Moore, 1874; Masters, 1881; Harper, 1918; Russell, 1918; Bell, 1952; Bell and Case, 1956).

Hybridization has long been of interest in *Sarracenia*. In an 1874 issue of *The Gardener's Chronicle*, Dr. David Moore described '*Sarracenia mooreii*', "the first hybrid *Sarracenia* which has yet flowered" (Moore, 1874). He described the plant as a cross between *S. flava* and *S. leucophylla*, stating it was "as nearly as possible intermediate between those two noble species of the genus" (Moore, 1874). An article in the same publication in 1881 described four different hybrids, including "*S. melanorhoda*" ([*S.*

purpurea X *S. flava*] X *S. purpurea*), a backcross hybrid (Masters, 1881). Russell (1918) made detailed anatomical comparisons of three hybrids and their parent species. He reported that “hybrid forms, in comparison with their parents, are intermediate in relation in almost all details” (Russell, 1918). However, without the advantages of genetic analyses, studies of hybrids in *natural* environments were necessarily limited to those individuals that could be readily identified morphologically as hybrids (i.e. those that *were* intermediate). Later Bell (1952) and Bell and Case (1956) described a total of 19 *Sarracenia* hybrids known at that time, many of which had been originally proposed or published as new species, and only later determined to be hybrids. Subsequently, however, the focus of study in the genus shifted to taxonomy, and despite intense interest in hybridization for nearly a century, little work has been done in the last 50 years. This is particularly surprising given that hybridization has often been cited as an obstacle to attempts at determining phylogenetic relationships in *Sarracenia* (McDaniel, 1971; Bayer et al., 1996; Neyland and Merchant, 2006).

Every pair of *Sarracenia* species has been found to be able to hybridize in cultivation (D’Amato, 1998; Schnell, 2002). Additionally, nearly all 'geographically possible' (i.e., two species having at least some portion of their ranges that overlap) simple hybrids of *Sarracenia* have now been recorded in nature as well (Russell, 1918; Bell, 1952; Bell and Case, 1956). However, no study has examined the role of hybridization in natural populations of *Sarracenia*. It is unknown whether F₁ hybrids act as a 'bridge' that allows for interspecific gene flow, or whether their formation is a genetic dead-end, contributing nothing to genetic and morphological variation. Here we

report the results from a single field site consisting of three *Sarracenia* species to investigate the extent and type of natural hybridization and gain a better understanding of its role in the genus.

Study goals

We used eight microsatellite loci to examine nearly three hundred *Sarracenia* individuals at a single field site where three species of *Sarracenia* coexist. In addition, we sampled twenty individuals from nearby allopatric sites that contained only one species each for comparison. Specifically, we sought to answer the following questions:

1) Is there evidence of introgressive hybridization at this field site? If little or no hybridization is occurring, we expect individuals at the sympatric site to be as well-differentiated (i.e. show little or no admixture) as those at the allopatric sites. If hybridization is occurring, but limited to F_1 s, we only expect to find individuals with admixture of approximately 0.50 of the two parent species. However, if introgressive hybridization is occurring, we expect to see a wide spectrum of admixture, representing potential later generation hybrids, backcrosses or complex crosses. 2) If hybrids are present, which species are involved and are they involved equally? If all species are equally likely to hybridize, then the contribution of each species to hybridization will be proportional to the pure species makeup of the field site. We therefore expect the overall average genetic makeup of the hybrid individuals to approximately equal that of pure species at the site. For example, if 50% of the pure species at the field site are *Sarracenia leucophylla*, then the average genetic makeup of all hybrids should be 50%

S. leucophylla. Deviation from this expectation will be indicative of differential hybridization. 3) What are the implications for species dynamics and conservation in the genus?

Materials and Methods

Study site and collection of plant material

The study site is located on a privately owned parcel of land in rural Washington County, Alabama. It consists of 2+ hectares of undisturbed longleaf pine (*Pinus palustris*) savanna. At the time of the study, the site appeared to have experienced a burn in the previous 3-5 years. During this and two previous visits to the site, *Sarracenia leucophylla*, *S. alata*, and *S. rubra* ssp *wherryi* were common, while no other *Sarracenia* species were observed at the site. A wide range of pitcher morphological variation was observed, including many individuals exhibiting characteristics intermediate between species ((Schnell, 1998)personal observation). Collections were made from three locations within the site. In two areas, individual samples were taken along a 10 X 10 meter grid. Samples were taken at one meter intervals, for a total of 100 sampling points within each grid. If no clump (i.e. cluster of pitchers possibly belonging to the same genetic individual) existed within ½ meter of the sampling point, then no sample was taken. In the third sampling area, individual samples were taken along a 100 meter transect, again sampling at one meter intervals. Photographs were taken of each of the 292 plants sampled. Sampling material consisted of 1-2 cm² of leaf tissue taken from the hood or ala. Leaf tissue was placed in 1.5 ml tubes and kept on

ice for up to 48 hours until it could be stored at -80° C. DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA). Individuals from allopatric populations of each of the three species were also included in the study to representative “pure” populations. These were chosen because the absence of other *Sarracenia* species at these sites means they are unlikely to have experienced recent hybridization. Included were nine individuals of *S. alata* (Stone County, Mississippi, approximately 80 km away), five individuals of *S. leucophylla* (Sumter County, Georgia; approximately 250 km away), and six individuals of *S. rubra* ssp *wherryi* (Washington County, Alabama; approximately 15 km away).

Microsatellite amplification and fragment analysis

A total of eight microsatellite loci were used in this study. Seven loci developed by Koopman et al (2009) for analysis in *Sarracenia alata* and used in previous work on other *Sarracenia* species (Furches and Small, In Review) were used in this study. An additional locus from Rogers et al (2010) was used as well. Loci were chosen on the basis of consistent amplification across all study species and variation in fragment size among study species as determined in preliminary studies. Fragment amplification was performed using the method of Schuelke (2000). PCR products were genotyped by electrophoresing fluorescently labeled products through an Avant 3600 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) at the University of Tennessee Molecular Biology Resource Facility. Fragment sizes were determined by

comparison to the ROX500 size standard in PEAKSCANNER software (Applied Biosystems).

Data analysis

To assess patterns of microsatellite variation, a Bayesian analysis was performed using the software STRUCTURE v2.2 (Pritchard et al., 2000). This program has been shown to perform comparably to other hybrid detection methods when used in two-species systems (Burgarella et al., 2009) and is the only software that can be used to examine hybridization among three or more species. The program works by using a coalescent model to assign individuals to clusters (K) in a manner that minimizes linkage disequilibrium. Under the admixture model, an individual may be 'admixed', meaning different portions of that individual's genome are assigned to different clusters. That portion is referred to as the 'admixture coefficient.' For example, an individual with no history of hybridization should therefore have an admixture coefficient of nearly 1.00 for one cluster and of nearly zero for other clusters. An individual that is the direct product of a hybridization event (an F_1), however, is expected to have admixture values of near 0.50 for two different clusters, representing the two parent species. Since only three species exist at the site, simulations were run at $K=3$ for 10^6 generations with the first 25% discarded as burn-in. As they are unlikely derived from hybridization events, individuals from allopatric populations of each of the three study species were used as standards (POPINFO=1 in STRUCTURE).

To assess the demographic makeup of the site, we divided individuals up into genotypic categories based on their assignment in STRUCTURE. The categories were delineated based on the proportion of an individual's genotype assigned to a given species and are shown in Table 3.1. While non-hybrid individuals are expected to have an admixture coefficient of 1.00, a conservative cut-off value of 0.85 was chosen for 'pure' species. Values for hybrids include ± 0.10 from the expected value (e.g., a backcross hybrid would be expected to have admixture values of 0.75 for one species and 0.25 for another). Rather than "F₁ hybrid", we refer to "F_x hybrids", as an individual in which approximately half of the genome is from one species and half from another does not necessarily represent an F₁ (e.g., it could represent an F₂). All remaining admixture coefficient combinations were placed in the 'Complex hybrid' category.

To assess the propensity of each species to hybridize, we examined the makeup of both pure species and hybrids within the site following the methods of Lepais et al (2009). We averaged the STRUCTURE admixture coefficients into two categories: pure species, as defined above, and hybrid individuals (all remaining individuals). For each species, we compared the average genetic makeup of hybrids to that of the pure individuals and performed Student's T-test. If all species are equally likely to hybridize, then the genetic makeup of hybrids should be based solely on the frequencies of the pure species, and these two values should be similar. However, if a given species is hybridizing more often than would be expected by chance, then the average admixture coefficient for that species in hybrid individuals will be higher than that among pure

individuals. Similarly, a significantly lower average coefficient among hybrids would indicate that species is less likely to hybridize (Lepais et al., 2009).

Results

Admixture and STRUCTURE analyses

The results of STRUCTURE analyses are shown in Figure 3.1. Figure 3.1a represents individuals from allopatric populations of *Sarracenia rubra* ssp *wherryi*, *S. leucophylla*, and *S. alata*. Each individual was assigned to a cluster with other members of the same species with an admixture coefficient of > 0.98 (mean > 0.99). This contrasts with Figure 3.1b, which represents 292 individuals from the study site. At this location, 183 individuals (62.7%) were assigned to a single species based on admixture coefficients above 0.85, while the remaining 109 (37.3%) were admixed among two or three groups.

Genotypic categories

Of the 183 individuals assigned to a single group, 96 (52.5%) were *Sarracenia leucophylla*, 74 (40.4%) were *S. rubra* ssp *wherryi*, and 13 (7.1%) were *S. alata* (Figure 3.2). The largest F_x group was between *S. leucophylla* and *S. rubra* ssp *wherryi*, with 26, compared to 7 *S. leucophylla* X *S. rubra* ssp *wherryi*, and 5 *S. rubra* ssp *wherryi* X *S. alata*. Possible backcrosses were also highest between *S. leucophylla* and *S. rubra* ssp *wherryi*. A total of 15 individuals were designated as 'complex' crosses.

Propensity to hybridize

When admixture coefficients of 'pure' versus hybrid individuals were examined, we found differing makeups between the two categories (Figure 3.3). *Sarracenia leucophylla* had an average admixture coefficient of 0.527 among individuals designated as pure species, but only 0.424 among hybrid individuals ($t = 2.720$, $df = 264.4$, $P = 0.007$), indicating that *S. leucophylla* is less likely to contribute to hybridization than would be expected if hybrids formed between random individuals. *Sarracenia alata* had an average coefficient of only 0.063 among pure individuals, but 0.194 among hybrids ($t = 5.147$, $df = 250.4$, $P < 0.001$), indicating that this species is more likely to contribute to hybridization than random. *Sarracenia rubra* ssp *wherryi* had an average of 0.410 among pure species and a similar value of 0.382 among hybrids ($t = 1.149$, $df = 266.4$, $P = 0.251$), showing no significant deviation from random hybridization. We obtained the similar levels of significance when analyses were performed using a range of 'pure species' thresholds (from 0.80 to 0.95).

Although the focus of the current study is genetic, a basic comparison between the genetic category assignments as determined by STRUCTURE and field observations is necessary. Individuals assigned to hybrid categories did show characteristics of both parent species, as shown in the examples in Figure 3.4.

Discussion

Our study shows that hybridization is leading to interspecific gene flow in *Sarracenia*. Allopatric populations containing single species are genetically

differentiated from each other and share little genetic variation. The sympatric population studied here, on the other hand, contained individuals with a wide range of genetic admixture between the three species. While it has long been widely-accepted among botanists that members of the genus can and do form hybrids, the question of whether this leads to interspecific gene flow has yet to be addressed empirically. Taken as a whole, the data indicate that hybridization is not a genetic dead-end at the F_1 generation, and interspecific gene flow is likely playing a role in the evolution of the genus.

Genetic admixture

The high level of admixture at the study site (Figure 3.1b) is in stark contrast to the samples from allopatric sites (Figure 3.1a). Samples from nearby allopatric populations were strongly delineated by species, with all individuals assigned to their respective group with admixture coefficients of ≥ 0.98 . At the study site, however, more than 37% of individuals sampled were admixed, even using our conservative 'pure species' threshold of 0.85. Members of the three study species are less genetically differentiated at the field site where they exist sympatrically than at nearby allopatric sites, indicating that introgressive hybridization is likely occurring.

Relative contributions of each species

When only individuals in the pure species category are considered, our data reveal a population made up of more than half *Sarracenia leucophylla* (52.5%), and

more than 40% *S. rubra* ssp *wherryi*, with the remaining 7% was made up by *S. alata*. Not surprisingly, the largest group of admixed individuals (i.e. likely hybrids) was made up of *S. leucophylla* and *S. rubra* ssp *wherryi*, while the smallest consisted of *S. rubra* ssp *wherryi* and *S. alata* (Figure 3.2). However, when relative abundance was taken into account, *S. alata* was shown to contribute a much greater proportion to hybridization than expected – 6.3% of the pure species genetic makeup compared to 19.4% of the hybrid genetic makeup ($P < 0.001$; Figure 3.4). In contrast, *S. leucophylla* contributed less to hybridization – 52.7% of the pure genetic makeup versus 42.4% of the hybrid genetic makeup ($P < 0.01$). *Sarracenia rubra* ssp *wherryi* showed no significant deviation from random (41.0% vs 38.2%; $P = 0.25$).

Demography and biology

The differential levels of hybridization may be explained by either biological and/or demographic mechanisms. The biological mechanisms include both potential pre-zygotic mating barriers such as timing of flowering and pollinator relationships, and post-zygotic barriers such as selection against hybrids. The former seem unlikely for several reasons. *Sarracenia leucophylla* has the longest flowering period of the three study species, flowering from early March through late April (Schnell, 2002). This provides ample opportunity for hybridization with *S. alata*, which flowers from early March into April, and *S. rubra* ssp *wherryi*, which begins flowering in April and lasts until May (Schnell, 2002). Timing of flowering often fluctuates from year-to-year and is influenced by factors such as rainfall and fire regime (McDaniel, 1971; Folkerts, 1982).

Overall flower morphology is quite similar among the three species. *Sarracenia alata*'s flowers are yellow-green, unlike *S. leucophylla* and *S. rubra* ssp *wherryi*, which are red or maroon. Previous work in the genus has indicated bumble bees (*Bombus*) as primary pollinators (Mandossian, 1965; McDaniel, 1971; Schnell, 1983), and has indicated no apparent pollinator preference among species or flower colors (Folkerts, 1982; Schnell, 2002).

Potential post-zygotic barriers also appear unlikely, at least among the three species studied here. Many authors have noted the awkwardness of pitchers produced by hybrids between species with decumbent, open pitchers such as *S. purpurea* or *S. rosea*, and those with erect, hooded pitchers such as *S. leucophylla*, *S. minor*, or *S. flava* (Folkerts, 1982; D'Amato, 1998). These hybrids produce tall, erect, open pitchers, which often fill with rainwater and fall to the ground. This would be a clear example of a post-zygotic barrier to interspecific gene flow. This is not a factor in the current study, however, as all three species produce erect, hooded pitchers, and F₁ hybrids show no obvious disadvantages in pitcher morphology. Some authors (Schnell and Krider, 1976) have noted that such hybrids show no apparent disadvantages and appear as vigorous as parental species, while others (Folkerts, 1982) have reported them to be more susceptible to water stress and drought than parental species. Currently, there appears to be no empirical study that has thoroughly addressed such issues.

The demographic explanation suggests that if one species is less abundant, its females will be overwhelmed by a large number of heterospecific gametes, leading to an increased likelihood of errors in mate-recognition (if any exists) and asymmetric

hybridization with the more common species (Mayr, 1963; Wirtz, 1999; Lepais et al., 2009). This could explain the increased contribution of *Sarracenia alata* to hybridization in this study. Onset of flowering in both *S. alata* and *S. leucophylla* occurs in early March. It is possible that the much rarer *S. alata* is overwhelmed by pollen from the more common *S. leucophylla* (or *S. rubra* ssp *wherryi* later on). The demographic and biological explanations are not mutually exclusive, however, and both may contribute to the varying levels of admixture seen in this study.

Maintenance of species integrity in the genus

The extent to which hybridization occurs across the range of these and other species in *Sarracenia* is not entirely known. However, our previous work in the genus (Furches and Small, in review) showed that most species of *Sarracenia* – particularly those whose center of diversity lay on the Atlantic coastal plain – formed relatively cohesive clusters when examined with six microsatellite loci. *Sarracenia* sites along the Gulf Coast tend to have multiple species – up to five in some sites in southern Alabama (personal observation; Schnell, 2002). While Atlantic Coast sites may also have at up four species in one location, most have fewer. There may also be additional factors that limit hybridization in this region. One factor is the pitcher shape that results from hybrids of certain species. Many of the multi-species sites in this region include the tall, hooded pitcher species *S. minor*, and *S. flava*, along with either *S. purpurea* or *S. psittacina*. As previously mentioned, hybrids between *S. purpurea* and species with tall, hooded pitchers are not efficient at catching insects because the tall, open pitchers tend

to fill with rainwater and fall over. A similar situation arises in hybrids with *S. psittacina*. While *S. flava* and *S. minor* both form tall, hooded pitchers and have some overlap in flowering times, Folkerts (1982) noted that hybrids between the two usually flower during a time when neither parent species is in peak bloom, reducing the opportunity for introgression.

Conservation implications

The effects of hybridization on conservation must be considered, as three of the nine species in the genus are Federally endangered (U.S. Fish & Wildlife Service, 1990, 1992, 1994) and all are suffering from habitat degradation and destruction (Folkerts, 1982; Schnell, 2002). Of the three endangered species, two (*S. oreophila* and *S. alabamensis*) exist entirely disjunct from other species in the genus. The third, *S. jonesii*, has only a small area of sympatry with *S. purpurea*. Genetic swamping through introgressive hybridization is therefore unlikely an issue with these species. Others, however, such as *S. rubra* ssp *wherryi* and *S. rubra* ssp *gulfensis*, are restricted to just a few counties and are commonly found with other species. An additional concern, as habitats continue to be lost, arises over whether or not reductions in population size affect hybridization rates. Folkerts (1982) noted that hybrids appeared to become more common in disturbed sites. At one location where hybrids had been rare or absent previously, he noted that hybrids began appearing along a footpath used by visitors to the site.

Conclusions

The role of hybridization in plant speciation and evolution and the maintenance of species boundaries in hybridizing taxa has long been a source of debate and has become an intense area of study in recent years. With nine species capable of hybridizing and existing in varying levels of sympatry, the genus *Sarracenia* presents an excellent opportunity to examine many of these questions.

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Appendix 1 – Figures and Tables

Table 3.1. Genetic categories of *Sarracenia* individuals based on admixture coefficients

Category	Admixture Value (Species 1)	Admixture Value (Species 2)	Admixture Value (Species 3)
'Pure' Species	≥ 0.85		
Backcross hybrid	0.65 – 0.85	0.15 – 0.35	< 0.20
F _x hybrid	0.40 – 0.60	0.40 – 0.60	< 0.20
Complex hybrid	≥ 0.34	≥ 0.20	≥ 0.20

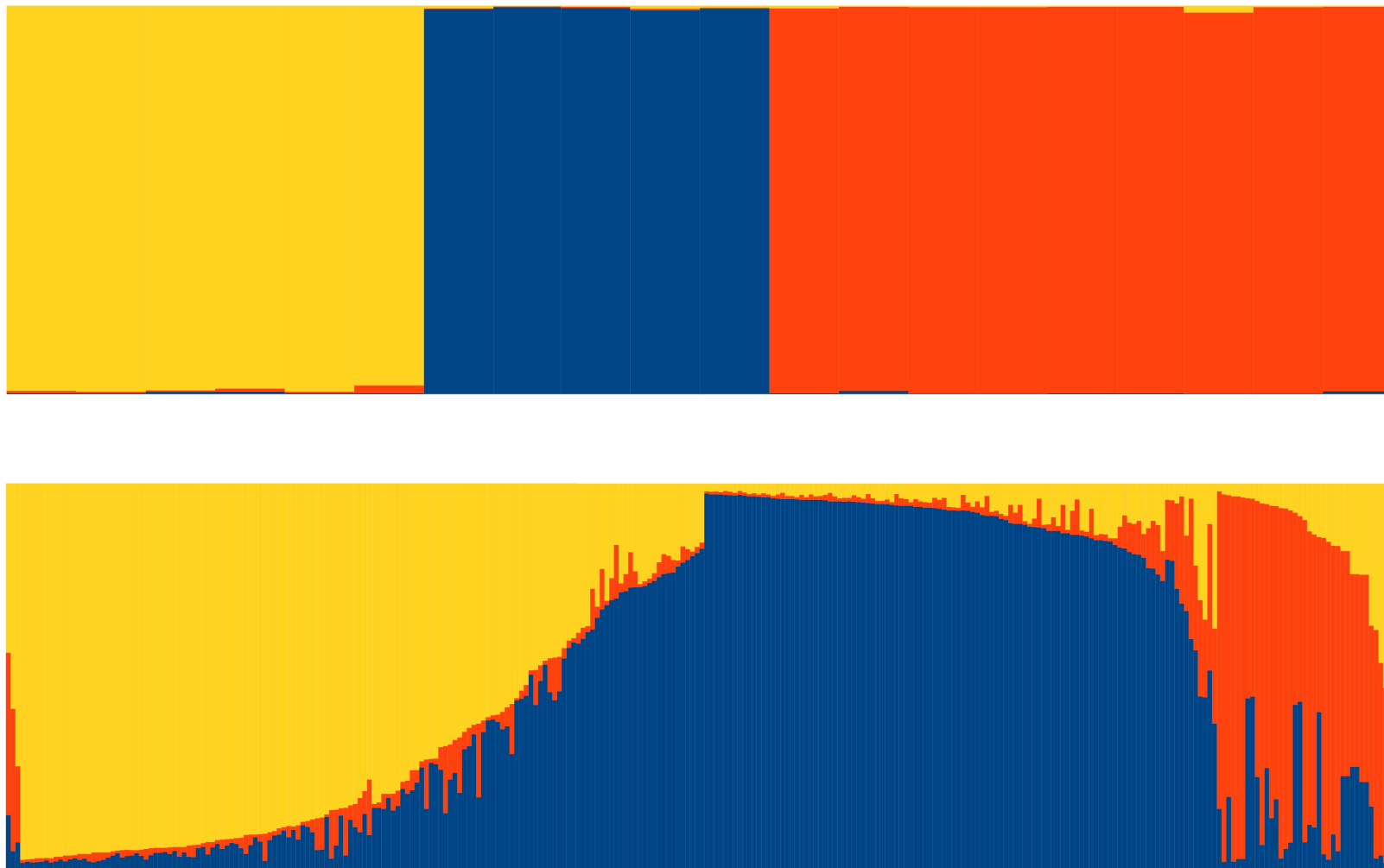


Figure 3.1 STRUCTURE analyses of microsatellite in three *Sarracenia* species – *S. alata* (orange), *S. leucophylla* (blue), and *S. rubra* ssp *wherryi* (yellow). Vertical bars represent the proportion of an individual's genome assigned to a particular group (“admixture coefficient”). Top - (A) Twenty individuals from nearby allopatric populations of the three study species. Bottom - (B) 294 individuals from the study site located in Washington County, Alabama.

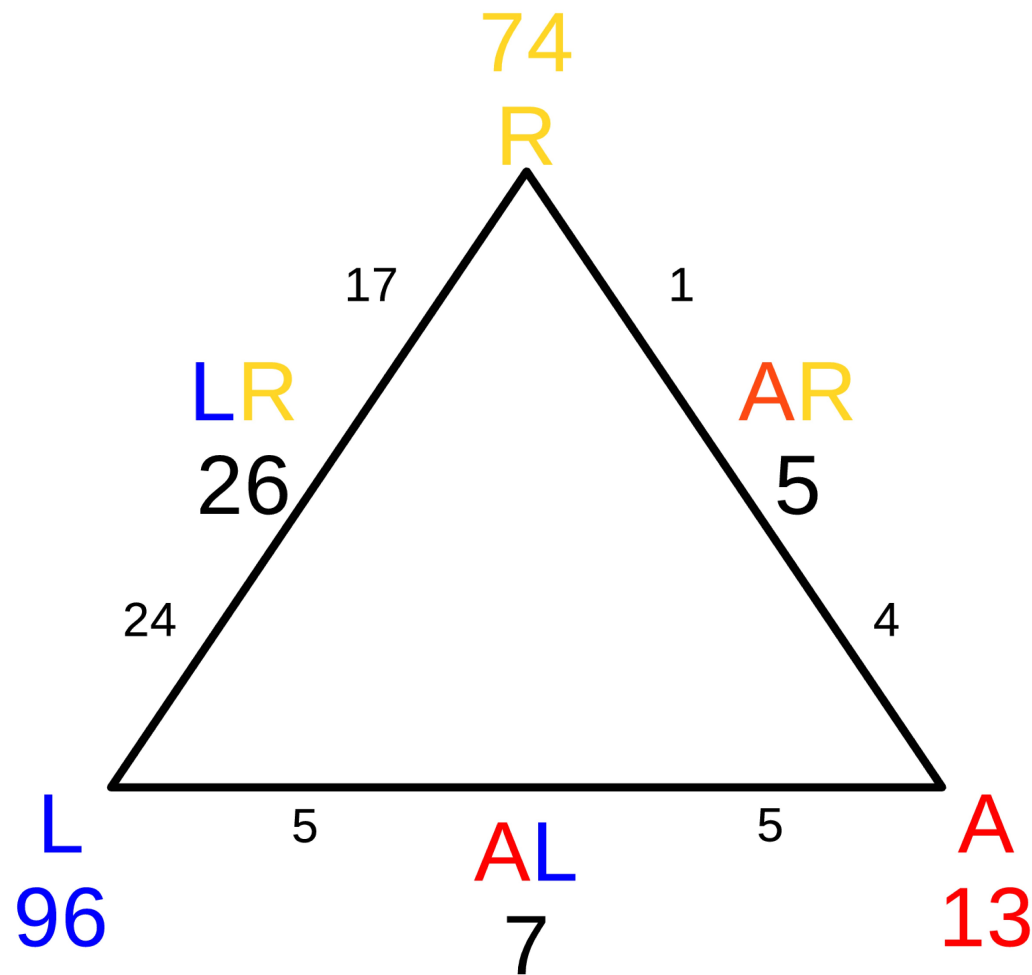


Figure 3.2 Genotypic classes of individuals at the study sight based on STRUCTURE admixture coefficients. The numbers at the three corners represent individuals assigned to the 'pure' category (L = *Sarracenia leucophylla*, A = *S. alata*, R = *S. rubra* ssp. *wherryi*). Two-letter combinations on each side represent individuals assigned to the F_x category. Smaller numbers with no letters represent those assigned to 'backcross' categories.

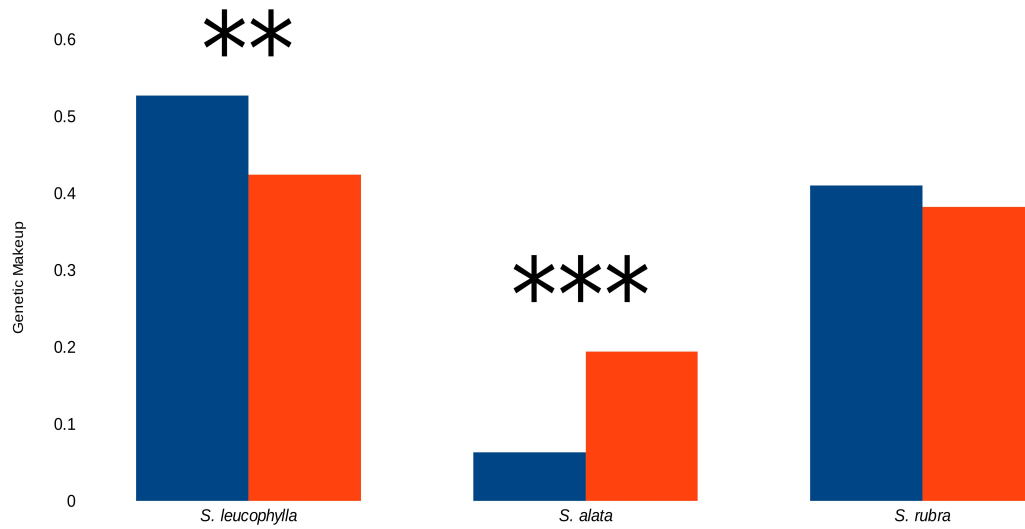


Fig. 3.3 Average admixture coefficients in 'pure' and 'hybrid' categories for each species. The blue bars represent the 'pure' category, while red bars represent the 'hybrid' category. ** indicates $P < 0.01$. *** indicates $P < 0.001$.

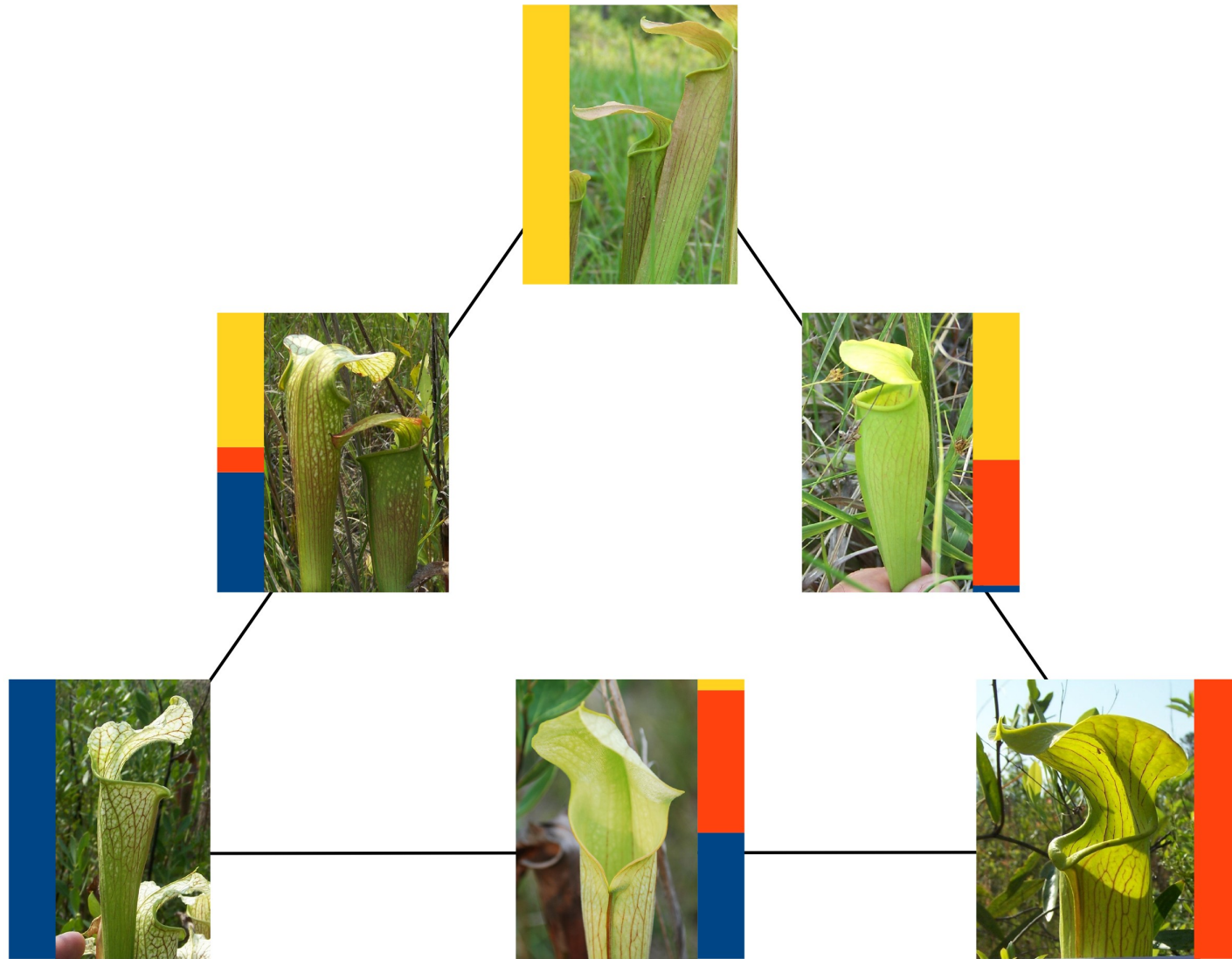


Fig. 3.4 Representatives of pure species and individuals assigned to F_x hybrid groups based on STRUcTURE admixture coefficients (shown alongside each photo). Top – *Sarracenia rubra* ssp. *wherryi*. Lower left – *S. leucophylla*. Lower right – *S. alata*.

CONCLUSION

Chapter 1: Nuclear and chloroplast genomes reveal contrasting patterns of diversity in North American Pitcher Plants (*Sarracenia*)

- *Premise of the study:* The North American Pitcher Plants (*Sarracenia*) have long fascinated naturalists and botanists for both their carnivorous habit and ability to easily hybridize in cultivation. While their ability to form hybrids has been widely recognized, the role hybridization plays in natural populations has been virtually ignored. We sought to examine the distribution of chloroplast and microsatellite variation in the genus, and to determine whether this variation was geographically or genetically structured.
- *Methods:* We examined a total of 265 individuals from eleven species in 34 field sites across the southeastern United States using three non-coding chloroplast regions totaling 3,903 bp. We also examined a subset (115 accessions) with six microsatellite loci. We used a permutation analysis to determine if sympatric populations of species pairs were more likely to share chloroplast haplotypes than allopatric populations of the same species.
- *Key results:* While extensive sharing of chloroplast haplotypes existed across species, analysis of microsatellites showed a strong species pattern for most taxa. The greatest admixture of nuclear genomes was present in species in the Gulf Coast region. Permutation analyses show that these some species are also more likely to share chloroplast haplotypes when they exist in the same populations.

- *Conclusion:* Differential rates of hybridization are occurring or has occurred across *Sarracenia* species. Introgression of chloroplast genomes appears to have occurred in some species in the genus) but not in others. Combined with microsatellite data, our study shows that introgressive hybridization has likely occurred between *S. alata* and *S. leucophylla*, and is likely unidirectional in *S. flava* and *S. psittacina*.

Chapter 2: Genetic diversity in three endangered pitcher plant species

(*Sarracenia*) is dramatically lower than widespread congeners

- *Premise of the study:* *Sarracenia oreophila*, *S. alabamensis*, and *S. jonesii* are three Federally endangered members of the North American Pitcher Plants. Members of the genus are known to hybridize in nature, and a previous study (Chapter 1) has shown that hybridization may be playing a role in generating genetic diversity in the genus. The three endangered taxa, however, exist almost entirely allopatrically throughout their respective ranges (only *S. jonesii* shares a small portion of its range with *S. purpurea*). We compared levels of genetic diversity across the range of these three endangered taxa as well as a single field site consisting of three more common species known to hybridize.
- *Methods:* We examined nine microsatellite loci in eight populations of *S. oreophila*, including the only two populations outside of Alabama and six populations in northeastern Alabama. We also examined six populations of *S. jonesii* and three populations of the critically endangered *S. alabamensis*. For comparative purposes, we also examined a single field site of three more

common species – *S. rubra* ssp *alabamensis*, *S. leucophylla*, and *S. alata*.

- *Key results:* The most widespread of the three taxa, *S. oreophila*, also had the greatest chloroplast diversity, while *S. jonesii* had only a single haplotype and *S. alabamensis* had two. However, *S. oreophila* had the lowest levels of microsatellite diversity, particularly peripheral populations. Surprisingly, *S. alabamensis* had the greatest microsatellite diversity of the three endangered taxa. When compared to a single field site of three more common species, there was less microsatellite diversity across the entire range of the three endangered taxa than a single field site of more common species.
- *Conclusion:* While not genetically depauperate, the three endangered taxa have much less variation than more common species in the same genus. Moreover, habitat degradation and destruction is leading to further erosion of genetic diversity in these three taxa

Chapter 3: Hybridization leads to interspecific gene flow in *Sarracenia*

- *Premise of the study:* Previous work (Chapter 1) has shown that introgressive hybridization has likely occurred or is occurring in members of the genus – particularly those found in the Gulf Coast region. We examined a single field site of three species – *S. leucophylla*, *S. alata*, and *S. rubra* ssp *wherryi* to determine the genetic makeup of the population and whether hybridization is proceeding beyond the F₁ generation. We also sought to determine if each of the three species were hybridizing equally, or if there were differential rates of

hybridization.

- *Methods:* We examined 292 individuals at a single field site consisting of three *Sarracenia* species. For comparative purposes, we also examined 20 individuals from three allopatric populations of the three study species. We used eight microsatellite loci and performed Bayesian clustering analysis with STRUCTURE
- *Key results:* While allopatric populations formed clearly delineated genetic clusters with virtually no admixture, the sympatric field site showed a wide range of admixture values.
- *Conclusion:* We found a wide range of admixture values at the sympatric field site, indicating that hybridization is progressing beyond the first generation and backcrosses are occurring. Introgressive hybridization is playing a role in the generation of genetic diversity in the three species examined. Additionally, we found that *Sarracenia alata* is hybridizing at a higher rate than expected based on its prevalence in the population.

VITA

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