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Phylogenetic Relationships in Sarraceniaceae Based on Conserved Sequences Generated from Sarracenia alata

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Phylogenetic Relationships in Sarraceniaceae Based on Conserved Sequences Generated from *Sarracenia alata*

by

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Introduction

Carnivorous plants capture the imagination of scientists and the general population alike. Carnivorous plants are capable of photosynthesis as well as limited mineral uptake through root systems (McPherson 2007). Because many carnivorous plants grow in the acidic environment of nitrate and phosphate deficient soil in wet climates, they must acquire these essential nutrients through carnivory (Poole 1963). These plants digest flies, ants, beetles, crickets, moths, butterflies and any other insects able to be captured in the pitchers (Poole 1983). The idea of plants that catch and eat animals seems unbelievable. Carl Linnaeus considered carnivorous plants as against the order of nature. The existence of carnivorous plants in scientific terms was not documented until Charles Darwin's 1875 work *Insectivorous Plants* (Barthlott et al. 2007). Pitcher plants were described within the pages of this work.

Two families containing five genera of pitcher plants are found across the Americas: Sarraceniaceae containin *Heliamphora, Sarracenia, Darlingtonia,* and Bromeliaceae containing *Brocchinia, Catopsis* (McPherson 2007). Sarraceniaceae are "true pitcher plants". True pitchers have tubular, funnel, or cup-shaped reservoirs formed by a single leaf (McPherson 2007). The largest traps of all carnivorous plants are produced by true pitcher plants, as several species of *Sarracenia* grow to more than 100 cm in height (McPherson 2007). Pitchers lure prey using nectar, scent and coloration. Prey, insects or other small animals, that fall into the pitchers drown in the water and are eventually digested by enzymes and bacterial action, enabling pitcher plants to absorb the nutrients released by digested prey (McPherson 2007). Pitcher plants are passive traps in that they automatically ensnare insects without the employment of movement (Slack 1988). This mechanism of carnivory acts in contrast to well-known active traps such as *Dionaea*

(Slack 1999). Despite widespread interest in the genus, a good estimate of intergeneric relationships has not been presented for Sarraceniaceae.

There is no fossil evidence or pollen record to provide clues about the evolution of American pitcher plants (McPherson 2007). Due to this lack of fossil evidence, evolutionary history must be estimated based on contemporary data such as the distribution of the plants, morphology, and genetics (McPherson 2007). Clarification of phylogenetic relationships among taxa may help determine conservation priorities (Godt and Hamrick 1998). Sequences of internal transcribed spacer regions of nuclear ribosomal DNA (ITS) and the chloroplast gene *rbcL* from samples of Sarraceniaceae suggest *Darlingtonia* was the earliest divergent lineage and that *Heliamphora* and *Sarracenia* are sister taxa (Bayer et al. 1996; Neyland and Merchant 2006). These data suggest *S. alata* diverges from the other species of *Sarracenia* to form its own subclade but do not provide strong support for the relationships between the other species of *Sarracenia*.

Sarracenia characteristics

Sarracenia pitchers are upright, or in the case of *S. psittacina* spreading to decline on the ground, with a tubular portion and a lid-like hood, the operculum. Surrounding the mouth of the pitcher is a rounded rim called the nectar roll or the peristome. A wing runs down one side of the pitcher from the bottom of the nectar roll to the base of the leaf stem, forming the "seam." The entire exterior of the leaf is dotted with nectar glands. These glands are also abundant on the wing and the nectar roll (Slack 1988). Most *Sarracenia* species employ the same method of trapping insect prey (McPherson 2007). A heavy concentration of nectar beneath the hood and often colorful patterning attracts insects. Insects fail to maintain balance on the slippery, waxy

inner surface of the pitcher opening and fall into the digestive liquid within the pitcher. The prey is unable to escape due to a combination of inverted hairs lining the pitcher and the waxy interior surface of the leaf (Slack 1988, McPherson 2007).

Sarracenia is the most widespread genus of true pitcher plants. Also known as "Trumpet Pitchers", eight to eleven species of *Sarracenia* are typically recognized, *S. oreophila, S. minor, S. alata, S. flava, S. leucophylla, S. purpurea, S. rubra, S. psittacina*, *S. alabamensis* Case & R.B. Case, *S. jonesii* Wherry, and *S. rosea* Naczi, Case & R.B. Case (Slack 1988; McPherson and Schnell 2011). In this study, data are collected from the following *Sarracenia* species.

S. alata

The pale pitcher plant has a divided range. Eastern populations occur in southwestern Alabama, southern Mississippi, and southeastern Louisiana. Western populations extend from western Louisiana into Texas (McPherson 2007). *Sarracenia alata* is derived from the Latin word meaning "wing", referring to seam that extends down the front of the pitcher leaf (McPherson 2007). The leaves of *S. alata* are predominantly yellowish greened and lined with varying red or purple veins (McPherson 2007). The flowers are easily distinguishable from other *Sarracenia* species in coloration and shape. The petals are creamy white or pale green and oval shaped. Other *Sarracenia* species flower petals are strap-shaped (Slack 1988).

S. flava

Sarracenia flava is named for the Latin word meaning "yellow" (McPherson 2007). Differential exposure to sunlight causes an assortment of leaf colorations from pale green

to golden yellow (Slack 1988). The range of *S. flava* spans across southwestern Virginia, North Carolina, South Carolina, Georgia, western Florida, and southeastern Alabama in an arc (McPherson 2007). In some areas, the plants are considered useful wasp traps (Slack 1988).

S. leucophylla

The white trumpet pitcher plant leaf is often variegated in white, crimson, and pink (Slack 1988). The coloration of the leaves often makes them desirable in floral arrangements. The range of *S.leucophylla* extends from extreme southwestern Georgia, the Florida Panhandle, southern Alabama and southeastern corner of Mississippi (McPherson 2007). This species produces non-carnivorous leaves during early summer.

S. purpurea

Sarracenia purpurea, the "purple pitcher plant", is the most widespread species in the genus, ranging from southern Canada, all of the New England states, the Great Lakes states, Iowa, West Virginia, Maryland, New Jersey, Delaware, Virginia, North Carolina, southern Mississippi, Alabama, and the panhandle of Florida (McPherson 2007). Also known as Huntsman's cup, this species is notably more suited to survival in cold weather (McPherson 2007).

S. rubra

The smallest of the upright *Sarracenia* pitchers is named for the red coloration of its leaves and flowers (McPherson 2007). *S. rubra* is also called the "sweet trumpet" for the

rose scent of its flowers (Slack 1988). Distinct populations exist in North Carolina through South Carolina, Georgia, Florida, and Alabama. The small ranges leave the species vulnerable to habitat loss and extinction (McPherson 2007).

S. psittacina

The "parrot pitcher" is a small plant with tapering horizontal leaves similar in appearance to the beak of a parrot (Slack 1988). Populations can be found from southeastern Georgia to the Florida Panhandle, then west to extreme southeastern Louisiana (McPherson 2007). This species grows semi-aquatically when its habitat is flooded. *Sarracenia psittacina* employs a trapping process similar to other species in the genus, even though its rosette of pitchers rest horizontally and directly on the ground (McPherson 2007). The scent of nectar encourages insects to enter the small, inward-protruding funnel shaped hole at the center of the dome-shaped hole. The modified hood has small fenestrations that simulate sunlit exits out of the trap. The lighting of the fenestrations causes confusion, making it very difficult for insects to escape. Eventually they die of exhaustion or drown in the digestive fluid contained within the leaf (McPherson 2007). The orientation of the leaves allows *S. psittacina* to trap small aquatic animals as well as insects (Slack 1988).

In order to better understand the pattern of species divergence within the genus, we collected DNA sequence data from 10 loci. We present phylogenetic analyses examining the position of *Sarracenia* species within the genus. We also tested a set of three loci including *Darlingtonia californica* as a means of examining monophyly and species divergence of *Sarracenia*.

Materials & Methods

Sampling

Sarracenia alata individuals were collected from eastern and western populations across Mississippi, Louisiana, and Texas. Leaf tissue was collected from five other *Sarracenia* species and *Darlingtonia californica* from ex situ populations (Carnivorous Plants Nursery, Derwood MD).

DNA Extraction, Amplification, and Sequencing

DNA was extracted from leaf tissue using the DNeasy plant extraction kit (Quiagen, Valencia, CA). A set of 76 loci throughout the *S. alata* genome was previously generated via next generation sequencing technologies using the 454 platform (Zellmer et al. 2012). A single representative sequence from each locus was drawn and used for primer development using default settings in Primer^{3}Plus (Untergasser et al. 2007). The majority of primers designed were developed without ambiguous sites, although a select number contained between one and no more than two ambiguous sites within each primer. Primer combinations for all 76 loci were tested in *S. alata*. Primer combinations generating PCR products were further tested with standard PCR protocols in the remaining *Sarracenia* species (*Sarracenia leucophylla, S. psittacina, S. purpurea, S. flava,* and *S. rubra)*.

Optimization of PCR thermoprofile occurred on a locus by locus basis to maximize PCR product. Loci were amplified under the following PCR conditions in 40 uL volume reactions: 1X Standard PCR buffer with $MgCl_2$, 0.125mM each dNTP, 1 unit *Taq* DNA polymerase (New England Biolabs), 0.25mM PCR primers (forward and reverse), and 1-10ng genomic DNA. Thermoprofile conditions were as follows: 94°C for 2 min; N cycles (Table 1) of 94°C for 30

sec, T_a (Table 1) for 30 sec, 72°C for 30 sec; followed by a final extension at 72°C for 2 min. PCR products around 500 bp in length were cleaned using the Qiagen Quick PCR Clean Up kit.

PCR products of loci in which all *Sarracenia* species amplified were sequenced using BigDye Terminator v.31 (Applied Biosystems, Foster City, CA). Sequences were cleaned using Sephadex and visualized an ABI PRISM 3130XL. Sequences were edited in Sequencher v4.6 (Gene Codes, Ann Arbor, MI). Sequences were aligned visually using MacClade v4.08a (Maddison and Maddison 2000). The number of variable sites for each locus was computed using PAUP* (Swofford 2003).

Phylogenetic Analysis

Models of DNA sequence evolution were estimated in jModelTest 0.1.1 (Posada 2008) using AIC criteria. Gene trees were generated using Garli 2.0 (Zwickl 2006). Species trees were generated using *BEAST (Heled & Drummond 2010). Analyses were conducted using a strict clock. Locus 131 was fixed at a clock rate of 1.0, and all other loci were assigned gamma distribution. Analyses were run for 50 million generations, sampling every 5,000 generations, the first 4,000 of which were discarded as burn-in. Each locus was allowed to evolve under its own best fit model. Convergence was assessed in Tracer 1.5 (Rambaut & Drummond 2007).

Results

Ten of the 76 loci tested were amplified in *Sarracenia alata* as well as a single individual of five additional species in the genus (*S. leucophylla, S. psittacina, S. purpurea, S. flava,* and *S. rubra*). A single individual from possible sister taxon *Darlingtonia* was also cleanly amplified in three of these loci. The length of each locus alignment, number of variable sites, and models for AIC nucleotide substitution are provided in Table 2.

The species tree estimate of all ten loci (Fig. 1) suggests a basal split dividing *Sarracenia alata* from its congeners. The strong posterior probability supporting a subclade containing only *S. alata* is mirrored within most of the gene trees generated. The addition of *Darlingtonia californica* to the species estimate tree using three loci (Fig. 2) generates high posterior probability for two monophyletic groups within the subclade sister to *S.alata*. The group including *S. leucophylla* and *S. purpurea* has stronger support than the group including *S. psitticina, S. flava,* and *S. rubra*, though the low posterior probabilities support all of these relationships.

Discussion

The singular composition of *S. alata* within one subclade of *Sarracenia* proposed by rbcL and ITS sequences (Bayer et al. 1996; Neyland and Merchant 2006) is confirmed in the initial species tree estimate. The placement of *S. leucophylla* and *S. purpurea* as sister species within the other subclade of *Sarracenia* is consistent with previous studies (Bayer et al. 1996, Schnell and Krider 1976).

The strong support for monophyletic subclade of *S. alata* could be an artifact of the number of individuals represented in the *Sarracenia* species tree estimate (Table 3). The same phylogenetic analyses were run using single random individuals from eastern and western *S. alata* populations. The resulting species estimate tree (Fig. 3) confirms the basal subclade consisting of *S. alata*. The phylogeny also suggests evolution of species based on geographic distribution. The phylogeny may suggest that the ancestral *Sarracenia* species arose in the Gulf coast and speciated north along the Atlantic coast in a fashion similar to the speciation within eastern and western populations of *S. alata* (Zellmer et al. 2012).

The loci represented within this study are possible conserved regions within Sarraceniaceae. These regions may not evolve rapidly enough to provide phylogenetic resolution between the other species of *Sarracenia*. The addition of more loci within each species may help clarify the relationships between these species. Natural populations may provide more phylogenetic elucidation because hybridization is known between species of *Sarracenia* (Bell 1949, McDaniel 1971). This study could be improved by the addition of more individuals from natural populations of *Sarracenia leucophylla, S. psittacina, S. purpurea, S. flava, S. rubra* and *Darlingtonia californica* in isolation from one another. Further PCR optimization is necessary to amplify additional loci across these individuals. Additional species of *Sarracenia,* species of *Heliamphora*, and outgroups for family Sarraceniaceae may also provide more resolution and a more complete phylogenetic evaluation of the ingroup taxa.

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Table 1. List of Loci, Species Amplified, Primer Sequences, Number of Cycles, and Annealing Temperatures for PCR Amplification.

Locus		Primer Sequence (5'-3')	N	T_a
4	F	ACAACATATACCATTGTCAAGAGG	35	54
	R	CAACTAATCAGAGCCGAGTGC		
131	F	ACGAAAGCGGTGGAGATG	35	54
	R	CCAACTTGAAGTATTTTCCTCGT		
146	F	TGATGTATGTATGCATTGTCCAG	35	54
	R	CGTGCAATACCTGATAATGTAGGA		
173	F	YGAATATAGATGAAAGGGATTCT	26	48
	R	GCCCGTYTGTCCTTCTACTG		
220	F	GATACTCCACCGGCATAGGA	26	48
	R	GAGATACCATTGGGYGACAT		
230	F	TGAGATGTTATGCRTATAGGGATTC	26	48
	R	ATCCTACYGATCCCGCTCAA		
297	F	ACACCGGGAGGGTTTTAGAG	35	54
	R	AGATTTCGATCCGTCTGCAA		
314	F	ACTCCTCGCTYCARACGTAA	26	48
	R	TTCATGGACCTYGTGAACAG		
323	F	AGACGACGTAACCGGATCAA	35	54
	R	CGTCGATGGGTTCTGTATGA		
408	F	GACGTGGTCGACAATACGAA	35	48
	R	AAGACAATGCGGAAGAAAC		

Table 2. List of Loci, Length of Consensus Sequences, Number of Variable Sites for All Samples, Number of Variable Sites Resulting from *S. alata* Sequences, and DNA Sequence Evolution Model Estimates.

Locus	Length	Variable Sites (All Samples)	Variable Sites (*S.alata)*	Proposed Model
4	266	8	4	GTR+I
131	399	101	5	HKY+G
146	372	22	6	HKY
173	447	42	26	GTR+G
220	442	60	22	HKY+G
230	389	33	14	GTR+I
297	326	14	4	F81+I
314	430	34	15	K80+I
323	511	5	2	F81
408	483	42	9	GTR

Table 3. Number of Individuals Amplified at Each Locus.

		Number of Individuals Amplified of Each Species							
		***S.alata* East**	***S.alata* West**	***S. leucophylla***	***S. purpurea***	***S. psittacina***	***S. rubra***	***S. flava***	***D. californica***
Loci	**4**	16	28	1	1	1	1	1	1
	131	14	24	1	1	1	1	1	1
	146	14	20	1	1	1	1	1	1
	173	8	12	1	1	1	1	1	-
	220	42	40	1	1	1	1	1	-
	230	34	38	1	1	1	1	1	-
	297	12	12	1	1	1	1	1	-
	314	18	26	1	1	1	1	1	-
	323	8	8	1	1	1	1	1	-
	408	14	10	1	1	1	1	1	-

Figure 1. Species Tree Estimate of Six *Sarracenia* Species Based on Ten Loci.

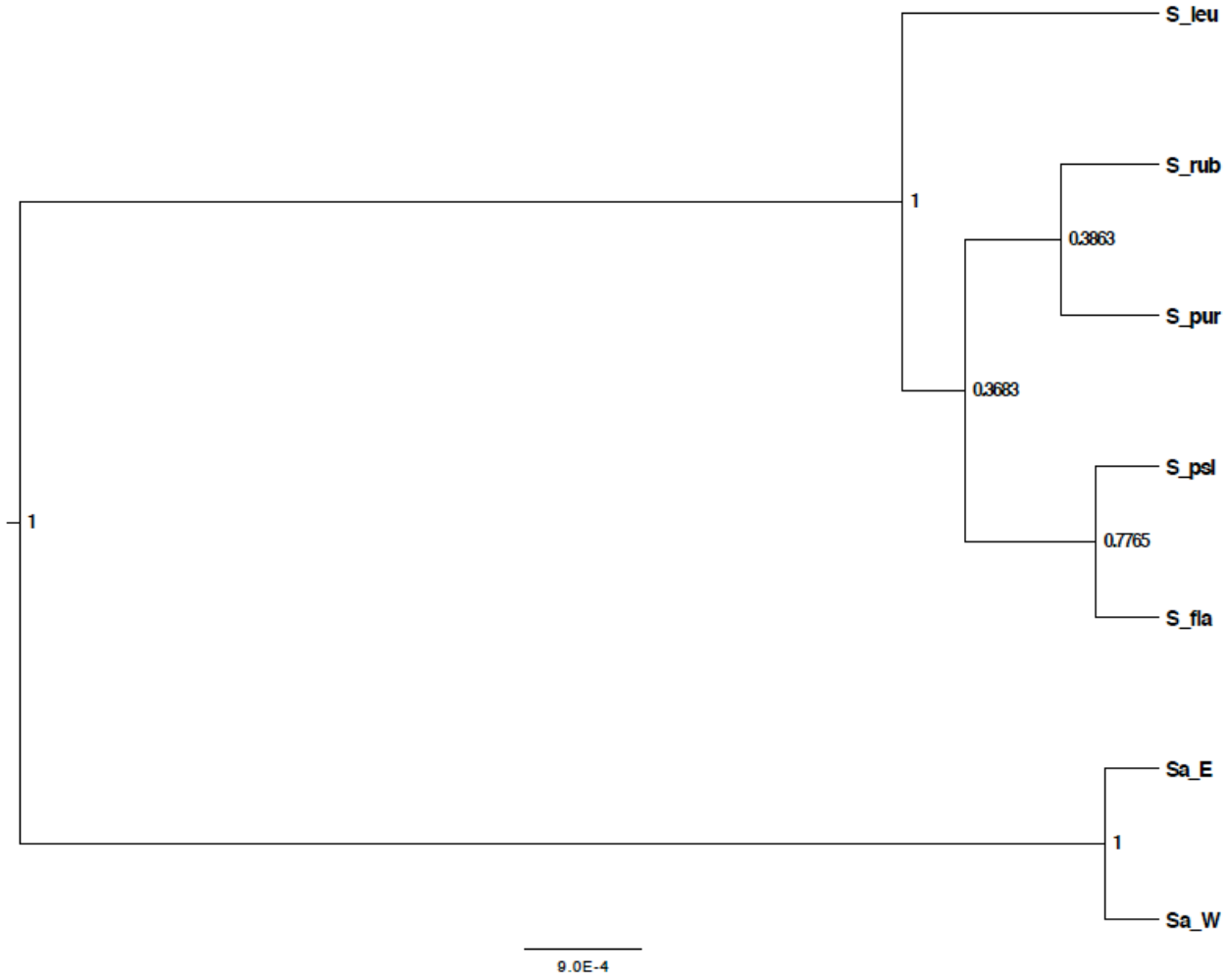

Figure 2. Species Tree Estimate of Six *Sarracenia* Species and *Darlingtonia californica* Based on Three Loci.

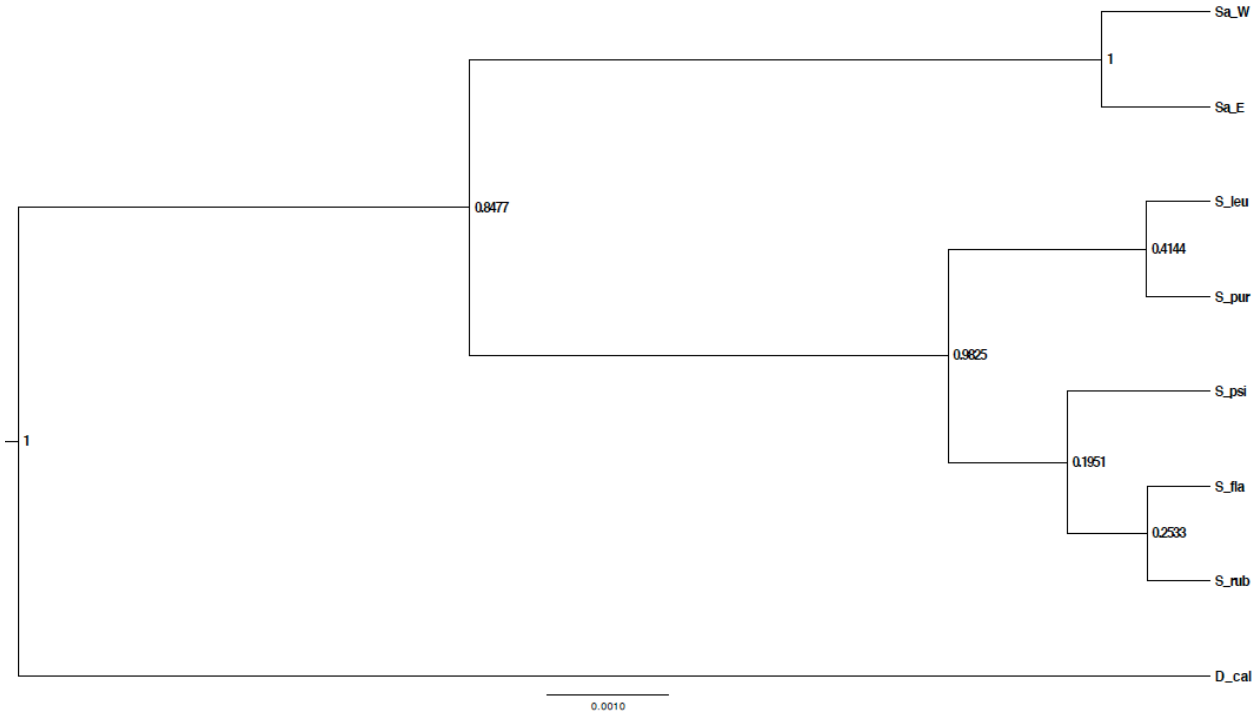

Figure 3. Species Estimate Tree with Singular Individuals from Each Species.