The Evolution of Landscapes and Lineages in Pitcher Plants and Their Moths.

Margaret Ellen Oard
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THE EVOLUTION OF LANDSCAPES AND LINEAGES IN PITCHER PLANTS AND THEIR MOTHs

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Entomology

by

Margaret Ellen Oard
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M.S., Iowa State University, 1977
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December 1997
DEDICATION

Dedicated
to
SAI RAM
in Whom
Vision and Creation
are One
from Whom
all Insight and Inspiration
come
ACKNOWLEDGMENTS

I would like to thank the many people who have contributed to the research described herein. I would like to thank my major professor Dr. Dorothy Prowell for being my mentor and for her guidance and support. Dr. Nina Lam, my minor professor, many thanks for interesting voyages of discovery into the world of geography. Dr. Lowell Urbatsch lent his expertise, enthusiasm for pitcher plants and lab and computer facilities. Dr. Riley, thanks so much for the beautiful moth photographs and for your encouragement. Dr. Overstreet, I very much appreciated your sincere interest and support.

Dr. Tom Kubisiak, very special thanks for keeping the sequencer and me up and running and for all your help. Special thanks to Dr. Rob Doudrick, Glen, Mary, Kaye and Andrea.

Andreas Wistuba, Hennig von Schmelling and Ron Gagliardo, my heartfelt thanks for your unstinting generosity in the gift of valuable plant materials. Thanks for sharing the plants and your understanding of Pitcher Plants. John Kutis, your intrepid fieldwork and special talent for sleuthing moths is greatly appreciated. I look forward to meeting all of you someday and expressing my thanks in person.

Vicky, Roxanne, Debbie and Kathe, thanks for your help with the fieldwork and for your special friendship.

Thanks Jim!
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LIST OF ACRONYMS

COI.....Cytochrome Oxidase I
COII.....Cytochrome Oxidase II
DNA.....Deoxyribonucleic Acid
IGS.......Intergenic Spacer
ITS.......Internal Transcribed Spacer
OG.......Outgroup
rbcL.....Ribulose bisphosphate carboxylase-oxygenase Large Subunit
RNA....Ribonucleic Acid
tmF.....Transfer RNA F
tmL.....Transfer RNA L
ABSTRACT

Pitcher plants and pitcher plant moths exhibit two prominent themes in the long evolutionary history between plants and insects. These themes are carnivory and herbivory. In pitcher plants, the leaf has been modified into an elegant pitfall trap enabling these plants to subsist at both the producer and consumer trophic levels. Of the numerous insect species intimately associated with pitcher plants, pitcher plant moths are the only inquilines which have evolved the capacity to disrupt both the carnivore and photosynthetic functions of the host plant.

The carnivorous habit, considered an adaptation for nutrient deficient environments, has contributed to species radiations on three continents in the pitcher plant lineage and the allied insect trapping South African flybush lineage. In addition, pitcher plant moths display incipient host species specialization in larval feeding preference so that speciation in the moths may be a response to radiation in the pitcher plants.

The focus of this study has been the elucidation of patterns of speciation in both the pitcher plant and moth lineages. In chapters two and four, molecular phylogenetic reconstructions based on DNA sequence data are derived for the pitcher plant and moth lineages respectively. The sequence of origination of genera in the pitcher plant lineage was also approached by cladistic analysis of morphological data (chapter three). Trends in the evolution of host plant use by the moths are examined in parallel with species radiation in the host in chapter four.

Insights regarding landscape evolution gleaned from the palynological literature were then combined with laboratory investigations of biological evolution at the molecular level in order to derive speciation
models for both the plant and moth lineages (chapter five). Levels of divergence were commiserate with Holocene age estimates of landscape evolution reported in the palynological literature.
CHAPTER 1
OVERVIEW OF LINEAGES, LANDSCAPES AND OBJECTIVES

Introduction

Pitcher plants and pitcher plant moths exhibit two prominent themes in the long evolutionary history between plants and insects (Farrell and Mitter 1994). These are plant carnivory and insect herbivory (Jones 1921).

Carnivory, also known as insectivory, is a series of elaborate stratagems by which plants attract insects, entrap them and then consume them as prey items (Jaffe et al. 1992). Experimental evidence suggests it is an adaptation for survival in nutrient limited environments (Chapin and Pastor 1995, Givnish et al. 1984).

In herbivory, by contrast, insects acquire the capability to use host plant tissues as a food substrate and to evade host plant defenses. The term phytophagy is a synonym for herbivory.

Pitcher plants are carnivorous plants in which the leaf has evolved a dual function. Not only is it a photosynthetic organ but it has been highly modified into an elegant pitfall trap mechanism. The hollow tubular upright funnelform silhouette is a striking departure from the typical laminar plant leaf. In addition, the interior is filled with a digestive liquor of proteolytic enzymes secreted by the plant and/or commensal bacteria. Hence the common name pitcher plants. Prey, predominantly insects, are lured within the trap by nectar baits and visual illusion. Once inside they are ensnared by the slippery interior with its coating of sloughing scales and fence of downward projecting hairs that make egress impossible. The digestive liquor then converts the insect biomass into

Pitcher plant moths, on the other hand, are the only insects able to disarm the pitfall trap mechanism. This is accomplished by a suite of behavioral adaptations in the larva in which it girdles the top of the pitcher, webs off the orifice and cuts a drain hole in the base thus rendering the pitcher functionless. The voracious larva then consumes all the nutritive photosynthetic tissue of the leaf. The association of the moth with its host plant is obligatory to the extent that the entire life cycle of the moth is carried out within the confines of the pitcher (Jones 1921).

Depriving its host of both photosynthetic capacity and supplemental nutrients from carnivory, the moth’s herbivory is doubly detrimental to its host. No coevolutionary countermeasures are evidenced by the host in response to this devastating herbivory (Strong et al. 1984).

Definition of Lineages

The common names pitcher plants and pitcher plant moths refer, respectively, to the plant genus *Sarracenia* L. in the family Sarraceniaceae and the moth genus *Exyra* Grt. 1875 in the family Noctuidae (Jones 1921). The Sarraceniaceae contains two additional genera of pitched insectivorous plants. They are the monotypic genus *Darlingtonia* Torr., the California pitcher plant or cobra lily, and the genus *Heliamphora* Benth., the sun or marsh pitcher plants (Lloyd 1942 and Slack 1979).

For the scope of this study, the lineage of pitcher plants is defined as the entire family Sarraceniaceae. The sister family of nonpitchered insect trappers, the Roridulaceae or South African flybush lineage, is the designated outgroup. The Roridulaceae is a montypic family containing
the single genus *Roridula* L. (Obermeyer 1970). At the ordinal level, the Sarraceniaceae and Roridulaceae are placed in the Ericales, the heaths and their relatives (Watson and Dallwitz 1997).

For the moths, the lineage is composed of the genus *Exyra* plus the two closely related taxa *Tarachidia semiflava* Gn. 1852 and *Thioptera nigrofimbria* Gn. 1852 as outgroups (Hodges et al. 1983). A list of the individual taxa included in the study by lineage is provided in Table 1.1.

Outgroups are included to provide information regarding ancestral states for the molecular and morphological characters used in studying the evolution of these lineages. They are also used to root the phylogenetic trees once they have been derived (Maddison and Maddison 1992).

**Description of Landscapes**

Landscape in this study denotes the interactive system composed of landform, soils, the indigenous flora and fauna, the regional climate, and time. It is in some respects synonymous with ecosystem but at a local to regional scale. The aspect of time is of particular relevance as the geologic, climatic and vegetational history as it is known for each of the landscapes in which members of the pitcher plant lineage are found will be used in conjunction with the phylogenetic evidence to inform our understanding of the interplay between biological and landscape evolution in this lineage. This biogeographic synthesis will be the subject of chapter five.

The landscapes in which members of the pitcher plant-flybush lineages are found encompass ecosystems ranging from longleaf pine flatwoods in the southeastern U.S. to the fynbos of South Africa. At the
Table 1.1. List of taxa by lineage.

**Pitcher Plant Lineage**  
Family Sarraceniaceae (In Group)  
Genus Sarracenia  
S. alabamensis  
S. alata  
S. flava  
S. jonesii  
S. leucophylla  
S. minor  
S. oreophila  
S. purpurea  
S. psittacina  
S. rubra  
Genus Heliamphora  
H. heterodoxa  
H. ionasii  
H. minor  
H. neblinae  
H. nutans  
H. tatei  
Genus Darlingtonia  
D. californica  
Family Roridulaceae (Out Group)  
Genus Roridula  
R. dentata  
R. gorgonia

**Pitcher Plant Moth Lineage**  
Family Noctuidae (In Group)  
Genus Exyra  
E. fax  
E. ridingsii  
E. semicrocea  
Genus Tarachidia (Out Group)  
Tarachidia semiflava  
Genus Thioptera (Out Group)  
Thioptera nigrofimbria
global level, these landscapes are united by their common affinity as heathlands (Specht 1979). Under this broad umbrella classification are those ecosystems that are edaphically controlled, severely nutrient limited, fire adapted and frequently dominated by members of the heath family Ericaceae or its relatives.

Pitcher plant heathlands rim the North American continent in the Atlantic Coastal Plain from Nova Scotia to Florida (Little 1979 and Whittaker 1979) and continue along the Gulf Coast into Texas (Christensen 1979). In the Pacific Northwest they occupy coastal plain and sites in the coast ranges of northern California and southern Oregon (Westman 1979). They span the continent's northern margin in the taiga-tundra boundary reaching to within five hundred miles of the Arctic Circle. They also occur sporadically inland as isolated bogs (Whittaker 1979).

Both the African and South American continents harbor heathlands containing members of the pitcher plant-flybush lineages. Species of *Heliamphora* are found in the heathlands of the tepuis, tabletop mountains of the Guayana Shield of northern South America (Cooper 1979 and Steyermark et. al. 1995). Fynbos is a type of heathland indigenous to the coastal mountains and forelands bordering the Cape Province, South Africa. The family Roridulaceae is endemic to this plant formation (Goldblatt 1978).

The vegetation of these heathlands is wet savanna or low shrubland. Pitcher plants dominate the herbaceous understory, often forming dense swards.

These are fire prone ecosystems due to their situation in regions of with high lightning strike frequencies and/or rock fall. As a result, natural
fires are prevalent with periodicities of 2-20 years on most sites. Fire inhibits encroachment by woody species, enhances litter decomposition and hastens nutrient cycling. Community aspect is one of open vistas with scattered overstory trees and sunlight penetrating to the herbaceous layer. Pitcher plants are an integral part of these fire adapted climaxes (Folkerts 1991, Frost et al. 1986 and Walker and Peet 1983).

Maritime influences are pervasive in the climates of these heathlands because of their coastal proximities. Rainfall is abundant, falling mainly in the winter or evenly split between winter and summer. Temperatures are equable with long growing seasons. Growing conditions would be nearly optimal if it were not for the severe nutrient limitations of the underlying substrate (Specht 1979).

*Sphagnum* spp. are a ubiquitous component of heathlands, playing a keystone role in engineering site hydrology, substrate pH and nutrient deficit (van Breemen 1995). Pitcher plants are obligately sphagnophilous except in a few isolated populations of *Darlingtonia californica* which occur on serpentine soils where *Sphagnum* spp. cannot survive. In these situations, nutrients are similarly unavailable to plants but due to the high alkalinity of the substrate rather than the acidic pH engendered in part by the presence of sphagnum.

As a general rule, heathlands are communities of low floral and faunal diversity. Those inhabited by members of the pitcher plant-flybush lineages are exceptional in this regard, harboring some of the world’s richest floras and faunas on some of its smallest land areas (Goldblatt 1978 and Whittaker 1979). What is also paradoxical is that this species richness tends to be concentrated in the least favorable sites (Woodwell...
Nutrient limitation seems to be positively correlated with floral diversity. In addition, much of this diversity is endemic, strongly suggesting that speciation has occurred in situ.

**Objectives**

The pitcher plant and moth lineages offer a unique investigative window into the process of speciation in these species-rich heathlands as patterns in these lineages mirror larger trends evident at the ecosystem level. The three genera of the family Sarraceniaceae plus the family Roridulaceae represent four separate radiations within heathlands on three continents. Thus in these lineages we have the speciation process replicated in both time and space.

A single radiation is represented by the moth lineage but it is indicative of the keystone role pitcher plants play in fostering faunal endemism in heathlands. The liquor of the pitcher supports an entire aquatic ecosystem, the phytotelm, albeit microcosmic in scale. A number of insect species are endemic to this miniature ecosystem. Other arthropod species are inquilines like the moths using the pitcher for hunting or nesting habitat or larval feeding. Of these numerous obligate commensals only the moths appear to have radiated in response to speciation in the host.

Pitcher plants are especially valuable for looking at speciation in heathlands because the lineage is known to be monophyletic and all members of the lineage are heathland endemics. By monophyly it is meant that the lineage as defined contains all the lineal descendant species of a single most recent ancestral species. Thus it is possible to retrace the path of descent from the common ancestral species and
reconstruct the sequence of origination of taxa. The evidence supporting monophyly will be presented in chapters two and three. The focal objective of this study has been the derivation of molecular phylogenies from DNA sequence data to reconstruct the pattern of speciation in both the pitcher plant and moth lineages.

Literature Cited


CHAPTER 2

MOLECULAR EVOLUTION IN PITCHER PLANTS (SARRACENIACEAE)

Introduction

Pitcher plants of the family Sarraceniaceae exemplify the phenomenon of plant carnivory, one of the more intriguing role reversals in the long evolutionary history between plants and insects (Farrell and Mitter 1994). In this scenario, plants have acquired the unusual capacity to prey on insects in contrast to the much more common insect-plant evolutionary outcome of herbivory in which insects consume their plant hosts (Jones 1921 and Juniper 1989).

Adaptation to the carnivorous habit has come about through a dramatic revamping of the architecture of the leaf for a dual role as a photosynthetic organ and insect trap (Lloyd 1942). The attendant morphological specialization has meant that many of the characters used in defining species are under strong selective constraints and are thus likely to be highly correlated. Molecular phylogenies derived using selectively neutral markers provide independent assessments of evolutionary trajectories without the added complication of character correlation. Under the assumptions of the molecular clock hypothesis, they also afford a means of estimating relative rates of divergence between taxa (Li 1997).

The Sarraceniaceae is a small New World family consisting of seventeen species in three widely disjunct genera (Watson and Dallwitz 1997). At the generic level, floral and pitcher morphologies are quite distinct. Flower form within genera is highly conserved such that species are delineated primarily on the basis of pitcher modifications (McDaniel
1971 and Maguire 1978). Though substantial information about the family exists in the taxonomic literature and the genera have been monographed, intrafamilial relationships were not investigated in a systematic attempt to derive the phylogeny until the recent $\textit{rbcL}$ and ITS rDNA phytogenies published by Bayer et al. (1996).

The carnivorous habit occurs in four other distantly related plant families: Nepenthaceae, Cephalotaceae, Lentibulariaceae and Droseraceae. Pitchered traps are found in two of the four families but these traps differ in morphology from one another and from the Sarraceniaceae (Albert et al. 1992, Arber 1941 and Franck 1976). Multiple origins for the carnivorous habit and for the pitched form of trap are implied by these observations. Multiple radiations have also taken place within familial lineages. Taken together, these trends are thought to be indicative of great antiquity for such lineages.

In the study outlined below, molecular phylogenies were constructed for the Sarraceniaceae using DNA sequences from spacer regions assumed to be selectively neutral from the nuclear and chloroplast genomes. The target regions were Internal Transcribed Spacers 1 and 2 (ITS 1 and 2) from the nuclear ribosomal genes and the $\textit{trnL-trnF}$ Intergenic Spacer (IGS) from the chloroplast genome. These sequence data were used for phylogeny reconstruction and the phylogenies so derived are used to: 1) trace the pattern of speciation in the Sarraceniaceae, 2) provide an independent assessment of the sequence of morphological changes involved in the evolution of carnivory in this group and 3) test the hypothesis of antiquity for the Sarraceniaceae.
The study, though similar in approach to that of Bayer et al. (1996), differs substantially in several significant aspects. For this study both species of the sister clade Roridulaceae were used as the outgroup rather than the more distant relative *Actinidia delicosa* used by Bayer et al. (1996). In addition, both nuclear and chloroplast sequences are used in species level phylogeny construction, providing two independent estimates of the phylogeny. The database contains a full representation of all described species and subspecies and selected taxa are duplicated as a control for within species polymorphism. The data set in Bayer et al. (1996) was incomplete in containing three of the six described species for *Heliamphora* and lacks representation for one species and two recognized subspecies of *Sarracenia*. Cycle sequencing with fluorescent dye chemistry and automated laser reading of the gels was performed in this study yielding full length reads of the target sequence in both directions. Finally, both parsimony based and maximum likelihood algorithms were used for phylogeny construction in this study increasing the probability of recovering the actual phylogeny.

**Materials and Methods**

**Taxa**

Taxa used in the study included DNA isolates from all ten described species of *Sarracenia* and all three subspecies of *S. rubra*, all six described species of *Heliamphora*, the single species of the monotypic genus *Darlingtonia* and both species in *Roridula*. These accessions, their places of origin and the names of the collectors are shown in Table 2.1.
Table 2.1 List of plant accessions used for DNA isolation.

<table>
<thead>
<tr>
<th>Taxon Name</th>
<th>Collection Locality</th>
<th>Collector or Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarracenia alabamensis 1</td>
<td>Chilton, Ga</td>
<td>Atlanta Bot. Gard.</td>
</tr>
<tr>
<td>S. alabamensis 2</td>
<td>Autauga, AL</td>
<td>Atlanta Bot. Gard.</td>
</tr>
<tr>
<td>S. alata 1</td>
<td>Kisatchie N. F., LA</td>
<td>D. Prowell</td>
</tr>
<tr>
<td>S. alata 2</td>
<td>Stennis Space Center, MS</td>
<td>M. Oard</td>
</tr>
<tr>
<td>S. flava 1</td>
<td>Colquitt, GA</td>
<td>Atlanta Bot. Gard.</td>
</tr>
<tr>
<td>S. flava 2</td>
<td>Croatan N. F., NC</td>
<td>T. Lamb</td>
</tr>
<tr>
<td>S. jonesii</td>
<td>Transylvania, NC</td>
<td>Atlanta Bot. Gard.</td>
</tr>
<tr>
<td>S. leucophylla</td>
<td>Baldwin, GA</td>
<td>Atlanta Bot. Gard.</td>
</tr>
<tr>
<td>S. minor</td>
<td>Colquitt, GA</td>
<td>Atlanta Bot. Gard.</td>
</tr>
<tr>
<td>S. oreophila</td>
<td>Towns, GA</td>
<td>Atlanta Bot. Gard.</td>
</tr>
<tr>
<td>S. psittacina</td>
<td>Lake Ramsey W. M.A., LA</td>
<td>M. Oard</td>
</tr>
<tr>
<td>S. purpurea 1</td>
<td>Green Swamp Preserve, NC</td>
<td>R. Rutledge</td>
</tr>
<tr>
<td>S. purpurea 2</td>
<td>McClean Bog Preserve, NY</td>
<td>K. Sharpe</td>
</tr>
<tr>
<td>S. rubra subsp. gulfensis</td>
<td>Escambia, FL</td>
<td>Atlanta Bot. Gard.</td>
</tr>
<tr>
<td>S. rubra subsp. rubra</td>
<td>Muscogee, GA</td>
<td>Atlanta Bot. Gard.</td>
</tr>
<tr>
<td>S. rubra subsp. wherryi</td>
<td>Baldwin, Al</td>
<td>Atlanta Bot. Gard.</td>
</tr>
<tr>
<td>Heliamphora heterodoxa</td>
<td>Kavanager, Gran Sabana, Ven.</td>
<td>H. vonSchmelling</td>
</tr>
<tr>
<td>H. ionasii</td>
<td>Ilu Tepui</td>
<td>A. Wistuba</td>
</tr>
<tr>
<td>H. minor</td>
<td>Cimanta Tepui</td>
<td>H. vonSchmelling</td>
</tr>
<tr>
<td>H. neblinae</td>
<td>Cerra de la Neblina</td>
<td>H. vonSchmelling</td>
</tr>
<tr>
<td>H. nutans</td>
<td>Traumen tepui</td>
<td>A. Wistuba</td>
</tr>
<tr>
<td>H. tatei var. tatei</td>
<td>Huachamacari Tepui</td>
<td>H. vonSchmelling</td>
</tr>
<tr>
<td>Darlingtonia californica</td>
<td>unknown</td>
<td>Mellinger's</td>
</tr>
<tr>
<td>Roridula dentata</td>
<td>Cape Province, South Africa</td>
<td>H. vonSchmelling</td>
</tr>
<tr>
<td>Roridula gorgonia 1 and 2</td>
<td>Cape Province, South Africa</td>
<td>H. vonSchmelling</td>
</tr>
</tbody>
</table>
Primer Sequences for Target Genes:

The sequences of all primers used in the study are given by target gene together with references in Table 2.2. For direct sequencing of the ribosomal spacers, the entire ITS region was first amplified with primers 18Z and 26S-350, then internal primers were used for sequencing. In the case of the tmL-tmF IGS, the amplifying and sequencing primers were the same except where the PCR product was cloned prior to sequencing. Cloned target sequences were sequenced with M13 Forward and Reverse primers supplied by Perkin Elmer in their AmpliTaq Cycle Sequencing Kit (Perkin Elmer, Foster City, CA).

DNA Isolation:

Leaf tissue from unopened pitchers or phyllodia was used for DNA isolation to avoid the possibility of contamination from DNA of prey species or bacterial commensals inhabiting mature pitchers. Plant materials were prepared for DNA isolation by grinding in liquid nitrogen or sterilized acid-washed sand in a mortar and pestle. DNA was isolated by the protocol of Doyle and Doyle (1990) using a 2% formulation of the hexadecyltrimethylammonium bromide (CTAB) isolation buffer.

Polymerase Chain Reaction (PCR) Amplification of Target Sequences:

Symmetric PCR was used to amplify target gene sequences. The reaction mixture contained 30-50 ng of total genomic DNA, 30 pmoles of each primer, 100 nmoles MgCl₂, 7.5 nmoles each dinucleotide triphosphate, 2 units of Tfi DNA Polymerase (Epicentre Technologies, Madison, WI) in a 100 ul volume. The reaction buffer was supplied by Epicentre Technologies. The thermocycle profile consisted of 2 minutes at 95°C, 25 cycles of denaturation at 95°C plus annealing at 58°C plus
<table>
<thead>
<tr>
<th>Genome</th>
<th>Target Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear</td>
<td>ITS rDNA</td>
<td>18Z</td>
<td>5'-GTAAGCGCGAGTCATCAGCTCG-3'</td>
<td>Urbatsch 1995</td>
</tr>
<tr>
<td>Nuclear</td>
<td>ITS rDNA</td>
<td>26S350</td>
<td>5'-CATCTTTCCCTCGCGGTACTTG-3'</td>
<td>Urbatsch 1995</td>
</tr>
<tr>
<td>Nuclear</td>
<td>ITS1 rDNA</td>
<td>pr2</td>
<td>5'-GCTGCCGTTCCTCATCGATGC-3'</td>
<td>White et al. 1990</td>
</tr>
<tr>
<td>Nuclear</td>
<td>ITS2 rDNA</td>
<td>pr3</td>
<td>5'-GCATCGATGAAGAAGCAGCAG-3'</td>
<td>White et al. 1990</td>
</tr>
<tr>
<td>Nuclear</td>
<td>ITS2 rDNA</td>
<td>pr4</td>
<td>5'-TCCTCCGCTTATTGATATGC-3'</td>
<td>White et al. 1990</td>
</tr>
<tr>
<td>Nuclear</td>
<td>ITS1 rDNA</td>
<td>pr5</td>
<td>5'-GGAAGTAAAAGTCGTAACAAGG-3'</td>
<td>White et al. 1990</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>tmL-tmF IGS</td>
<td>CP5</td>
<td>5'-GGTTCAAGTCCCTCTATCCC-3'</td>
<td>Taberlet et al. 1991</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>tmL-tmF IGS</td>
<td>CP6</td>
<td>5'-ATTTGAACTGGTGACAGCAG-3'</td>
<td>Taberlet et al. 1991</td>
</tr>
<tr>
<td>Plasmid</td>
<td>cloned PCR</td>
<td>M13F</td>
<td>5'-GCCAGGGTTTCCTCCAGTCACGA-3'</td>
<td>Perkin Elmer 1994</td>
</tr>
<tr>
<td>Plasmid</td>
<td>cloned PCR</td>
<td>M13R</td>
<td>5'-GAGCGGATAACAATTTCACACAGG-3'</td>
<td>Perkin Elmer 1994</td>
</tr>
</tbody>
</table>
extension at 72°C for 30 seconds each step, followed by a final 10 minutes of extension at 72°C. PCR products were either used directly for sequencing or cloned into the TA cloning vector pXCM1Kn (Kovalic et al. 1991). All PCR products whether used directly for sequencing or for cloning were cleaned by centrifugation through Quiagen spin columns (Quiagen Corporation, Chatsworth, CA) according to the protocol supplied by the manufacturer.

Cloning of PCR Products:

Cloning was done by digesting the pXcm1Kn vector with the restriction enzyme Xcm1 (New England Biolabs, Beverly, MA) in buffer supplied by the manufacturer for three hours at 37°C. Linearized vector was isolated on a 1% agarose gel and cleaned over a Quiagen spin column. Purified PCR products were ligated into the vector using the Fast-Link DNA Ligation Kit (Epicentre Technologies, Madison, WI) according to manufacturer's instructions for vectors with T overhangs and using a 3:1 insert to vector ratio in the ligation reaction followed by twelve hours of incubation at 14°C. Five nanograms of ligated vector was used to transform 50 ul aliquots of One Shot competent cells (Invitrogen Corporation, San Diego, CA) according to the protocol supplied by the manufacturer. Transformant colonies were detected as white colonies against a background of blue colonies on Luria Broth agar plates containing ampicillin and Xgal and transferred to overnight cultures of liquid Terrific Broth (Tartoff and Hobbs 1987). Plasmid DNA isolation was carried out using a modified alkaline lysis/PEG DNA precipitation protocol designed specifically to yield very pure intact plasmid DNA in high concentration suitable for automated fluorescent DNA sequencing (ABI
Identity of inserts was verified by PCR amplification from purified plasmid DNA with the same primers used to generate the original PCR product for cloning.

Automated Cycle Sequencing:

Cycle sequencing reactions were prepared using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit and AmpliTaq FS DNA polymerase (Perkin Elmer, Foster City, CA) according to manufacturer's directions. Thermal cycle parameters consisted of 25 cycles of denaturation at 96°C for 10 seconds, primer annealing at 50°C for 5 seconds and extension at 60°C for 4 minutes. Sequencing reaction products were purified on CentriSep spin columns (Princeton Separations, Adelphia, NJ), dried under vacuum and resuspended by vortexing in 3 ul of loading dye consisting of blue dextran and formamide in a 5:1 ratio. The 3 ul resuspension was subsequently loaded onto a 6% acrylamide gel prepared as described in the ABI Quick Reference Guide (ABI 1993). Gels were run for 14 hours on an ABI 373A Automated DNA Sequencer. The ABI Data Collection and Analysis software package (ABI 1992a) was used for automated tracking and gel reading. Sequence editing and alignment were done using the ABI SeqEd software program (ABI 1992b) and by visual inspection.

Data Analysis and Phylogeny Construction:

Aligned sequences were entered as MacClade version 3.05 (Maddison and Maddison 1992) data matrices for all subsequent database manipulations and cladistic analyses. A subset of the total sequence data encoded all the informative indels (insertion/deletion sites) so that the phylogenetic signal from indels alone could be
partioned out and analyzed separately. Cladistic analyses were performed with PAUP version 3.1.1 (Swofford 1993) using parsimony based algorithms for phylogeny construction. The PAUP analyses consisted of heuristic searches for the most parsimonious trees using both branch swapping with tree bisection/reconstruction and stepwise random addition methodologies. This insured that a full range of possible trees was derived and evaluated to yield maximal parsimony. Both heuristic search methodologies gave the same results in all cases so that only one tree is presented for each data set. For data sets containing less than 20 taxa, heuristic searches were followed by branch and bound search routines to test the stability of tree topologies under repeated resampling and rearrangement. This gave a quantitative measure of the support in the data for each branch in the resultant tree. One hundred replicates of bootstrapping were used for data sets containing more than 20 taxa to make the computer time and memory requirements more tractable with the tradeoff that such routines are less exhaustive than branch and bound searching. For comparative purposes, phylogenies were generated by the method of maximum likelihood for a combined database that included DNA sequences for all three target genes. This served as a test for algorithm dependence in the generation of tree topologies. Nine replicate runs were made on a SUN workstation using a UNIX1 version of the fastDNAml software (Olsen et al. 1993).

Results
Format of the Data

Variation in the length of ITS 1, ITS 2 and trnL-trnF IGS were observed in the different taxa. The results are summarized in Table 2.3.
Table 2.4 contains a side by side comparison of the different data sets regarding the gene sequences included in each data set, the number of taxa, number of total characters and number of phylogenetically informative characters and the distribution of informative characters between coding and noncoding spacer regions. Several indices are used to evaluate the goodness of fit between the data set and tree topology. These indices are the consistency index or C.I., the homoplasy index or H.I. and the retention index or R.I. Values for these indices are given in Table 2.5 along with the tree lengths and the number of most parsimonious trees found by each PAUP run.

Pairwise intertaxa genetic distances were computed with the distance algorithm available in PAUP. The resulting distance matrix is presented in Table 2.6.

Descriptions of the individual data sets are given below. The topology of the output consensus tree associated with each data set is also summarized.

Description of the Data Sets

The sequence data from each target gene region was first analyzed separately. In the case of ITS 1, the data was analyzed using three different outgroups. One outgroup consisted of *R. gorgonia* and *R. dentata*, one contained *Actinidia deliciosa* which was the outgroup used by Bayer et al. (1996) and the last outgroup contained all three species. The sequences of ITS 1 and ITS 2 were also pooled and analyzed jointly. A combined data set with sequences from ITS 1 and 2 and the trnL-trnF IGS was analyzed using maximum parsimony and maximum likelihood algorithms for phylogeny reconstruction. Finally, the insertions and
Table 2.3. Spacer lengths in basepairs of DNA for tmL-tmF IGS, ITS 1 and ITS 2 rDNA in Sarraceniaceae and Roridulaceae.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>tmL-tmF IGS</th>
<th>ITS 1 rDNA</th>
<th>ITS 2 rDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. alata</td>
<td>341</td>
<td>243</td>
<td>224</td>
</tr>
<tr>
<td>S. leucophylla</td>
<td>341</td>
<td>243</td>
<td>224</td>
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<tr>
<td>S. flava</td>
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<td>224</td>
</tr>
<tr>
<td>S. minor</td>
<td>333</td>
<td>243</td>
<td>224</td>
</tr>
<tr>
<td>S. oreophila</td>
<td>341</td>
<td>243</td>
<td>223</td>
</tr>
<tr>
<td>S. psittacina</td>
<td>333</td>
<td>243</td>
<td>224</td>
</tr>
<tr>
<td>S. purpurea 1</td>
<td>341</td>
<td>243</td>
<td>223</td>
</tr>
<tr>
<td>S. purpurea 2</td>
<td>341</td>
<td>243</td>
<td>223</td>
</tr>
<tr>
<td>S. rubra subsp. wherryi</td>
<td>341</td>
<td>243</td>
<td>224</td>
</tr>
<tr>
<td>H. neblinae</td>
<td>345</td>
<td>244</td>
<td>214</td>
</tr>
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<td>H. nutans</td>
<td>345</td>
<td>246</td>
<td>216</td>
</tr>
<tr>
<td>H. ionasii</td>
<td>345</td>
<td>244</td>
<td>216</td>
</tr>
<tr>
<td>H. heterodoxa</td>
<td>345</td>
<td>244</td>
<td>214</td>
</tr>
<tr>
<td>H. tatei</td>
<td>345</td>
<td>244</td>
<td>215</td>
</tr>
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<td>H. minor</td>
<td>337</td>
<td>240</td>
<td>214</td>
</tr>
<tr>
<td>D. californica</td>
<td>343</td>
<td>238</td>
<td>218</td>
</tr>
<tr>
<td>R. gorgonia</td>
<td>308</td>
<td>251</td>
<td>212</td>
</tr>
<tr>
<td>R. dentata</td>
<td>308</td>
<td>251</td>
<td>212</td>
</tr>
</tbody>
</table>

S.=Sarracenia, H.=Heliamphora, D.=Darlingtonia, R.=Roridula

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Table 2.4. Composition of data sets and numbers of total and informative characters in coding and spacer regions.

<table>
<thead>
<tr>
<th>Data Set</th>
<th># Taxa</th>
<th># Char Total</th>
<th># Char Coding</th>
<th># Char Spacer</th>
<th># Inf Char Total</th>
<th># Inf Char Coding</th>
<th># Inf Char Spacer</th>
</tr>
</thead>
<tbody>
<tr>
<td>trnL-trnF IGS</td>
<td>19</td>
<td>450</td>
<td>100</td>
<td>350</td>
<td>24</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>ITS1(R+A=OG)</td>
<td>24</td>
<td>364</td>
<td>107</td>
<td>257</td>
<td>120</td>
<td>3</td>
<td>117</td>
</tr>
<tr>
<td>ITS1(A=OG)</td>
<td>22</td>
<td>364</td>
<td>107</td>
<td>257</td>
<td>42</td>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>ITS1(R=OG)</td>
<td>23</td>
<td>364</td>
<td>107</td>
<td>257</td>
<td>118</td>
<td>3</td>
<td>115</td>
</tr>
<tr>
<td>ITS2</td>
<td>25</td>
<td>427</td>
<td>198</td>
<td>229</td>
<td>112</td>
<td>13</td>
<td>99</td>
</tr>
<tr>
<td>ITS1+ITS2</td>
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<td>771</td>
<td>285</td>
<td>486</td>
<td>221</td>
<td>12</td>
<td>209</td>
</tr>
<tr>
<td>ITS1+ITS2+IGS</td>
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<td>1221</td>
<td>385</td>
<td>836</td>
<td>240</td>
<td>12</td>
<td>228</td>
</tr>
<tr>
<td>ITS1+ITS2+tmL-trnF IGS/ML</td>
<td>18</td>
<td>1221</td>
<td>385</td>
<td>836</td>
<td>240</td>
<td>12</td>
<td>228</td>
</tr>
</tbody>
</table>

Indels                     | 18     | 57           | NA            | NA            | 57               | NA                | NA                |

Char=Characters, Inf Char=Informative Characters, ML=Maximum Likelihood, NA=Not Applicable
Table 2.5. Consensus tree lengths, number of most parsimonious trees, consistency index, homoplasy index and retention index.

<table>
<thead>
<tr>
<th>Data Set</th>
<th>Tree Length</th>
<th># MP Trees</th>
<th>C. I.</th>
<th>H. I.</th>
<th>R. I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>tmL-tmF IGS</td>
<td>28</td>
<td>4</td>
<td>.89</td>
<td>.11</td>
<td>.96</td>
</tr>
<tr>
<td>ITS1(R+A=OG)</td>
<td>163</td>
<td>4</td>
<td>.93</td>
<td>.07</td>
<td>.97</td>
</tr>
<tr>
<td>ITS1(A=OG)</td>
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<td>4</td>
<td>.93</td>
<td>.07</td>
<td>.98</td>
</tr>
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<td>ITS1(R=OG)</td>
<td>135</td>
<td>7</td>
<td>.97</td>
<td>.04</td>
<td>.98</td>
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<td>167</td>
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<td>ITS1+ITS2</td>
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<td>93</td>
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</table>

MP=Most Parsimonious, C. I.=Consistency Index, H. I.=Homoplasy Index, R. I.=Retention Index
ML=Maximum Likelihood, NA=Not Applicable
Table 2.6. Matrix of pairwise intertaxa relative genetic distances calculated across ITS 1, ITS 2, and \( m_{\text{ml}}-m_{\text{m}} \) F IGS DNA sequence data for Sarraceniaceae and Roridulaceae.

<table>
<thead>
<tr>
<th></th>
<th>Sa</th>
<th>Si</th>
<th>Sf</th>
<th>Sm</th>
<th>So</th>
<th>Sp</th>
<th>Su1</th>
<th>Su2</th>
<th>Sr</th>
<th>Hn</th>
<th>Hu</th>
<th>Hi</th>
<th>Hh</th>
<th>Ht</th>
<th>Hm</th>
<th>Dc</th>
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<tr>
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<td>0.008</td>
<td>0.025</td>
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<td>0.025</td>
<td>0.034</td>
<td>0.021</td>
<td>0.017</td>
<td>0.004</td>
<td>0.393</td>
<td>0.389</td>
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<td>0.358</td>
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<td>0.381</td>
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<td>0.794</td>
<td>0.789</td>
</tr>
<tr>
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<td>0.030</td>
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<td>0.025</td>
<td>0.021</td>
<td>0.017</td>
<td>0.013</td>
<td>0.021</td>
<td>0.393</td>
<td>0.389</td>
<td>0.371</td>
<td>0.358</td>
<td>0.384</td>
<td>0.381</td>
<td>0.226</td>
<td>0.794</td>
<td>0.789</td>
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<td>0.380</td>
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<td>0.789</td>
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<tr>
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<td>0.038</td>
<td>0.043</td>
<td>0.021</td>
<td>0.393</td>
<td>0.389</td>
<td>0.371</td>
<td>0.358</td>
<td>0.384</td>
<td>0.381</td>
<td>0.226</td>
<td>0.794</td>
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<td>0.389</td>
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<td>0.358</td>
<td>0.384</td>
<td>0.381</td>
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<tr>
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</tr>
<tr>
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<tr>
<td>Sr</td>
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<td>0.039</td>
<td>0.043</td>
<td>0.030</td>
<td>0.060</td>
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<td>0.082</td>
<td>0.061</td>
<td>0.399</td>
<td>0.391</td>
<td>0.222</td>
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Sa=Sarracenia alata, Sl=S. leucophylla, Sf=S. flava, Sm=S. minor, So=S. oreophila, Sp=S. psittacina, Su=S. purpurea, Sr=S. rubra, Hn=Heliamphora neblinae, Hu=H. nutans, Hi=H. ionasii, Hh=H. heterodoxa, Ht=H. tatei, Hm=H. minor, Dc=Darlingtonia californica, Rd=Roridula dentata, Rg=R. gorgonia
deletions or indels were encoded and analyzed separately. Phylogenies reconstructed on the basis of indels alone sometimes give different topologies because indels are thought to be less prone to reversion than single base substitutions.

Description of Consensus Tree Topologies

For each data set analyzed, a set of most parsimonious trees was generated and then a fifty percent majority rule consensus tree was computed from all the most parsimonious trees found by the search algorithm. The heuristic support values are found above the branch nodes and the bootstrap (or branch/bound) support values are found below each node. The support values are the percentages of most parsimonious trees in which the given branch configuration occurred. The consensus trees can be found in Figs. 2.1-2.6.

tmL-tmF IGS

The topology for the tmL-tmF IGS consensus tree is seen in Fig. 2.1. Darlingtonia is shown as the basal member of the Sarraceniaceae. Within Sarracenia, there is a polytomy (S. minor, S. oreophila, S. psittacina) but no further resolution of the rest of the genus. The situation is analogous for Heliamphora. There is a polytomy (H. neblina, H. tatei, H. minor) but this is the extent of resolution within the genus.

ITS1

Three consensus trees were computed for this data set; one for each of the different outgroups used in the analysis as explained above. Only the topology of the consensus tree for the phylogeny reconstruction where Roridula dentata and R. gorgonia were used alone as the outgroup is shown in Fig. 2.2 because it gave better resolution than either the
Fig. 2.1. Fifty percent majority rule consensus tree for tmL-tmF IGS. Heuristic search branch support values above nodes and branch/ bound values below nodes.
Fig. 2.2. Fifty percent majority rule consensus tree for ITS 1. Heuristic search branch support values above nodes and bootstrap support values below nodes.
Fig. 2.3. Fifty percent majority rule consensus tree for ITS 2. Heuristic search branch support values above nodes and bootstrap support values below nodes.
Fig. 2.4. Fifty percent majority rule consensus tree for ITS 1+ITS 2. Heuristics search branch support values above nodes and bootstrap values below nodes.
Fig. 2.5. Fifty percent majority rule consensus tree for tml-tmf IGS+ITS 1+ITS 2. Heuristic search branch support values above nodes and bootstrap support values below nodes.
Fig. 2.6. Fifty percent majority rule consensus tree for indels. Heuristic search branch support values above nodes and bootstrap support values below nodes.
analysis using *Actinidia deliciosa* sequence alone or the analysis using *Roridula dentata* and *R. gorgonia* plus *Actinidia deliciosa* sequences together. In all three analyses, *Darlingtonia* and *Sarracenia* are lumped together as one large polytomy. The three analyses also found the polytomy (*H. neblina*, *H. tatei*, *H. minor*) as in the *tmL-tmF* IGS topology above. The analysis with *Roridula dentata* and *R. gorgonia* as the outgroup recognized (*H. nutans*, *H. ionasii* ) as sister taxa.

**ITS 2**

Resolution is more complete in the consensus tree for the ITS 2 reconstruction (Fig. 2.3). There is resolution of (*H. neblina*, *H. tatei*, *H. minor*) and of (*H. nutans*, *H. ionasii*) but not of any of these clades with respect to (*H. heterodoxa*). For *Sarracenia* there is also some internal resolution. The two accessions of *S. flava* and of *S. purpurea* are each seen to be sister taxa. The two *S. alata* accessions are not, however, found to be sister taxa and one is designated as the basal member of the genus though this branch did not receive any bootstrap support. A nested series of polytomies is seen but there is very little resolution of *Sarracenia* in this topology.

**ITS 1+ITS 2**

In the consensus tree for the joint analysis of ITS 1 and ITS 2 (Fig. 2.4), *Heliamphora* is fully resolved. Working down through the *Sarracenia* clade, *S. flava* is shown to be sister to *S. psittacina* with *S. minor* as basal to this clade. There is no bootstrap support for this branching pattern. The basal segregation of *S. alata* 1 is fully supported but there is no additional resolution within *Sarracenia* other than recognition of the sister pairing (*S. purpurea* 1, *S. purpurea* 2).
ITS 1+ITS 2+tmL-tmF IGS

When sequences from all three target genes are analyzed together there is substantial improvement in the resolution within Sarracenia (Fig. 2.5). Five species are fully resolved and the three species (S. minor, S. psittacina, S. oreophila) encountered as a polytomy in previous analyses are still unresolved but this polytomy is now shown to be basal in the genus. Full resolution for Heliamphora is confirmed with moderate to strong branch/bound support.

The maximum likelihood analysis for this data set gave the same tree for all nine replicate runs. The topology is identical to that in Fig. 2.5 obtained for the maximum parsimony analysis.

Indels

The consensus tree topology reconstructed for the indel data set can be seen in Fig. 2.6. Within Heliamphora, only the sister clade (H. nutans, H. ionasii) is recognized. The rest of the genus is unresolved. For Sarracenia, S. minor and S. psittacina are sister taxa. Arising above this clade is a second clade ((S. flava, S. rubra subsp. wherryii), S. oreophila) with moderate to full support. The remaining species are joined to this clade as a polytomy though the sister pairing (S. purpurea 1, S. purpurea 2) is recognized.

Discussion

Variation in Spacer Length

The largest length variation was found in the tmL-tmF IGS with a size range of 308-345 bp (Table 2.3). R. gorgonia and R. dentata share two deletion synapomorphies, one of 30 bp and one of 3 bp, which account for the low value of 308 bp. S. minor and S. psittacina are united
by an 8 bp deletion, otherwise the length is constant at 341 bp over the rest of the genus. In Heliamphora, the length is 345 bp with H. minor autapomorphic in having an 8 bp deletion.

The length of ITS 1 is invariant in Sarracenia at 243 bp. H. minor and H. nutans are autapomorphic, having a 4 bp deletion and a 2 bp insertion, whereas the base number in Heliamphora is 244 bp.

Only minor variation in length is seen in ITS 2. It is 223-224 bp in Sarracenia and 214-216 bp in Heliamphora. Darlingtonia and Roridula are similar at 218 bp and 212 bp respectively.

Effect of Combining Data Sets

Significant improvement in resolving power was realized by combining molecular data sets. Data sets containing sequence from a single target gene were primarily useful in defining relationships at the generic level and in reinforcing the monophyly of genera as they are currently circumscribed. Except in the case of ITS1 where the phylogeny placed Darlingtonia in the same genus with Sarracenia, existing generic concepts were supported at the 100% level in all trees.

The increase in sample size with merging data sets substantially increased the strength of the phylogenetic signal in the data with no detectable increase in homoplastic noise. An example of this improvement in phylogenetic signal to noise ratio is seen in the comparison between the resolution in the tml-trnF IGS data set and the combined three gene data set. The number of informative characters increase ten-fold when the tml-trnF IGS data with 24 informative characters was merged with the ITS 1 and ITS 2 data sets, giving a total 240 informative characters. Whereas the tml-trnF IGS data set was only
able to differentiate *Sarracenia* and *Heliamphora* into two clades each, the combined data set resolved five of the eight *Sarracenia* species at the 100% support level in all five cases. Species relationships within *Heliamphora* were fully resolved albeit at differing levels of support. The number of taxa was essentially the same in both data sets. The only difference was that the outgroup member *R. gorgonia* was duplicated in the tmL-tmF IGS data set but was represented by a single accession in the combined data set.

In this study, ITS1 provided no resolution whatsoever within *Sarracenia* or between *Sarracenia* and *Darlingtonia*. This lack of resolution persisted despite the fact that the replacement of *Actinidia delicosa* by *Roridula* spp. as the outgroup resulted in a three-fold increase in the number of informative characters, from 42 to 118.

ITS2 sequence data is noticeably better than ITS1 data in sorting out relationships within genera though the number of informative characters is about the same for both, 112 and 118 respectively. The full resolution of species relationships within *Heliamphora* seen in this data set is strongly supported by bootstrap values of 86% and greater. This resolution is upheld in the combined data sets as well though at a lower level of support.

Use of *S. rubra* subsp. *wherryi* As a Surrogate for *S. jonesii*-*S. rubra*- *S. alabamensis* Alliance in Data Analyses

In the three gene combined data set *S. rubra* subsp. *wherryi* is used as a surrogate for the entire *S. jonesii*- *S. alabamensis*- *S. rubra* alliance, essentially collapsing the alliance into a single branch. Justification for this stems from the near genetic identity found for all
members of the alliance at the level of ITS 1 and ITS 2. The pertinent portion of the pairwise intertaxa genetic distance matrix (Table 2.6) has been extracted and can be viewed in Table 2.7. The greatest distance across the alliance is 0.009. By comparison, the intrataxon distance for S. alata is 0.014 and for S. purpurea it is 0.005. Genetic distances between the different species of the alliance are equal to or less than distances within other Sarracenia taxa. The alliance may be, in actuality, a single species genetically. In any case, other genes capable of resolving relationships at the population level would be needed to clarify the situation within the alliance so that treating them as a single species for purposes of this study seems warranted.

Phylogenetic Resolution Within Heliamphora

The molecular phylogeny for Heliamphora is in accord with morphology and geography. The sister clade (H. tatei+H. neblinae) is so close morphologically that Steyermark (1984) considers H. neblinae to be a variety of H. tatei, H. tatei var. neblinae, rather than a separate species as described in Maguire (1978). Maguire commented on the strong similarity between H. nutans and H. ionasii, saying that H. ionasii was the ultimate development of the H. nutans type. These two sister species in conjunction with H. heterodoxa are a clade that is restricted to the eastern Guayana. H. minor is found in the eastern Guayana but on the western edge.

There is considerable morphological overlap between species and considerable plasticity within species in this genus (Maguire 1978 and Steyermark 1984). No morphological synapomorphies have been identified that distinguish clades within the genus. It would appear that
Table 2.7. Matrix of pairwise intertaxa relative genetic distances for selected taxa in Sarraceniaceae calculated from ITS 1 and ITS 2 DNA sequence data.

<table>
<thead>
<tr>
<th></th>
<th>S. alab</th>
<th>S. jone</th>
<th>S. ru-gu</th>
<th>S. ru-ru</th>
<th>S. ru-wh</th>
<th>S. alata1</th>
<th>S. alata2</th>
<th>S. pur1</th>
<th>S. pur2</th>
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<td>.005</td>
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<td>.005</td>
<td>.009</td>
<td>.014</td>
<td>.018</td>
<td>.028</td>
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<td>.000</td>
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<td>.032</td>
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S. alab=Sarracenia alabamensis, S. jone=S. jonesii, S. ru-gu=S. rubra subsp. gulfensis, S. ru-ru=S. rubra subsp. rubra, S. alata=S. alata, S. pur=S. purpurea
species have been distinguished in this group largely on geographic
criteria. The values for genetic distances between taxa in Table 2.6
parallel geographic trends except in the case of *H. minor* which is closely
allied genetically with *H. neblinae* and *H. tatei* in Amazonian Guayana to
the west while its own geographic situation is that of eastern Venezuelan
Guayana.

These results are compatible with the *Heliamphora* clade
published by Bayer et al. 1996. Only three *Heliamphora* accessions were
used in that study but *H. minor* is more closely associated with *H. tatei*
than with *H. nutans* as reported in this study.

**Phylogenetic Resolution within Sarracenia**

The most significant result in this study is the resolution achieved
within *Sarracenia*. Five of the ten species are fully resolved. The inability
to resolve the *S. jonesii*-S. *rubra*-S. *alabamensis* species alliance stems
from the low levels of differentiation found between and within these taxa
and suggests these are nascent species.

Corroboration for the molecular phylogeny is seen in the fit
between molecular and morphological evolution within *Sarracenia*. The
synapomorphy of aerolation of the hood unites *S. minor* and *S. psittacina*
and affirms their basal position in the genus because it is a
synapomorphy shared with *Darlingtonia*. The synapomorphy of lateral
placement of the orifice in both *S. psittacina* and *Darlingtonia* further
strengthens the case for basal positioning of *S. psittacina*. *S. leucophylla*
and *S. purpurea* share the synapomorphy of undulate margins on the
hood, adding credence to their adjacent placement in the molecular
phylogeny (Lloyd 1942 and McDaniel 1971).
There is little correlation between these results and that of Bayer et al. (1996). Salient features of the Sarracenia phylogeny in Bayer et al. (1996) are: 1) basal placement for S. alata, 2) sister taxa status for S. leucophylla and S. purpurea and 3) assignment of S. flava, S. psittacina and S. minor to their own polytomy. In this study, S. alata is placed at the tip of the phylogeny, S. leucophylla is positioned below S. purpurea and S. psittacina and S. minor are more closely allied to S. oreophila than to S. flava.

Literature Cited


____ 1992b. ABI 373A SeqEd software program, Version 1.0.3. Applied Biosystems Division, Perkin Elmer. Foster City, CA.


CHAPTER 3
MORPHOLOGICAL EVOLUTION IN PITCHER PLANTS

Introduction

The Sarraceniaceae are a small New World family comprising three widely disjunct genera and seventeen described species (Watson and Dallwitz 1997). Ever since their discovery in the seventeenth and eighteenth centuries, their curious leaf form and carnivorous habit have evoked constant conjecture and scientific inquiry (Lloyd 1942). Darwin devoted an entire book to the subject of plant carnivory in which the family figures prominently (Darwin 1875).

In the course of evolution, the leaf has been highly modified, resulting in an elegant funnel-form pitfall trap with, typically, a handle-like hood arching above the orifice, hence the common name pitcher plants (Lloyd 1942). The organography is so singular that Franck (1975) was led to comment upon the pitcher of one family member, the monotypic genus *Darlingtonia*, as being among the most striking of all angiosperm leaves.

The family is undeniably monophyletic with all members sharing the same unique leaf morphology and ontogeny (Franck 1976b). Independent corroboration at the molecular level has been provided by the *rbcL* phylogeny of the carnivorous plants in Albert et al. (1992).

What unites the pitcher plant lineage also serves to set it apart to the extent that Metcalfe and Chalk (1950) concluded,

the plants are so specialized in structure in correlation with their peculiar mode of life that it is rather difficult, on anatomical grounds, to decide which of the positions to which the family has been assigned in different systems of classification is most likely to be correct.
Cognizant of this uniqueness, the family has sometimes been given separate ordinal status though most current schemes place it within the Ericales (Watson and Dallwitz 1997).

Evolutionary affinities within the family itself remained equally unresolved even though two centuries of investigation amassed a wealth of information regarding morphology and biology. The question of intrafamilial relationships is amenable within a phylogenetic context but such an approach had not been applied in a systematic way until recently when Bayer et. al. (1996) derived a molecular phylogeny for the group using ITS rDNA sequence data.

Drawing upon the extensive literature detailing the family's unique morphology, cladistic analysis was used to construct the generic level morphological phylogeny for the family reported herein. Historical trends in the traits associated with carnivory and floral structure as well as specialized aspects of the ecology of the group are discussed in detail.

Materials and Methods

Taxa

Taxa included in the study are the three genera *Sarracenia*, *Darlingtonia* and *Heliamphora* comprising the ingroup Sarraceniaceae; the genus *Roridula* which forms the sister clade and monotypic family Roridulaceae; and the related genus *Actinidia* in the family Actinidiaceae. Outgroup designation was based on the molecular phylogeny of Albert et al. (1992).

Morphological Data

From a survey of the relevant taxonomic literature twenty informative characters were compiled. The traits and their character states are
defined in Table 3.1. Table 3.2 contains a data matrix of the coded characters for each genus with zero being assigned to the character state found in *Actinidia*. Because the primary objective was a delineation of relationships between genera within Sarraceniaceae, only traits that are diagnostic at the generic level and consistent within genera were used in this study.

Data Analysis and Phylogeny Construction:

The character codes were entered as a MacClade version 3.05 (Maddison and Maddison 1992) data matrix for all subsequent database manipulations and cladistic analyses. Cladistic analyses were performed with PAUP version 3.1.1 (Swofford 1993) using parsimony based algorithms for phylogeny construction. The PAUP analyses consisted of heuristic searches for the most parsimonious trees using the method of stepwise random. Heuristic searcheing was followed by branch and bound search routines to test the stability of tree topologies under repeated resampling and rearrangement. This gave a quantitative measure of support in the data for each branch in the resultant tree.

Results

Heuristic random stepwise addition and branch/bound searches each yielded a single most parsimonious tree of length 33 (Fig. 3.1). Several indices are used to evaluate the goodness of fit between the data set and tree topology. They are the consistency index or C.I., the homoplasy index or H.I. and the retention index or R.I. The C.I. value was 0.85, the H.I. was 0.15 and the R.I. was 0.75. These indices range between zero and one. Large values (>0.80) of the C.I. and R.I. and small values (<0.20) of the H.I. are considered indicators of very good fit.
Table 3.1. Morphological characters and character codes.

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</tr>
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<td>2. Phyllly</td>
<td>(1) Symbiont digestion</td>
</tr>
<tr>
<td>3. Leaf Form</td>
<td>(2) Plant digestion</td>
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<tr>
<td>4. Leaf Areolation</td>
<td>(0) Homophyllous</td>
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<td>5. Leaf Alation</td>
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<td>6. Mean Vessel Element Length</td>
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<td>7. Mean Vessel Element Diameter</td>
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<td>8. Inflorescence Type</td>
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<td>(1) Raceme</td>
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<td>(2) Scapose</td>
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(table cont’d)
9. Carpel Number 17
   (0) 6-30
   (1) 3
   (2) 5

10. Ovary Vestiture 7,10,11,12
    (0) Pubescent
    (1) Glabrous

11. Carpel Connation 17
    (0) Synovarious
    (1) Synstylovarious
    (2) Syncarpous

12. Stylar Form 17
    (0) Free and multiple
    (1) Simple
    (2) Lobed

13. Heteranthery 4,7,10,11
    (0) Absent
    (1) Present

14. Staminal Insertion 17
    (0) Oppositipetalous
    (1) Oppositisepalous

15. Anther Attachment 17
    (0) Dorsifixed
    (1) Basifixed

16. Anther Versatility 17
    (0) Versatile
    (1) Nonversatile

17. Exine Texture 12,18,
    (0) Psilate
    (1) Verruculate
    (2) Scrobiculate

18. Embryogeny 17,19, R
    (0) Solanad
    (1) Caryophyllad

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<td>(1) Well differentiated</td>
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<td>20. Perianth Nectaries</td>
<td>4,7,10,18</td>
<td>(0) None</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1) Sepals nectiferous</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2) Sepals and petals</td>
<td></td>
</tr>
</tbody>
</table>

\[\text{nectiferous}\]

\[1^{1} (\text{Ellis and Midgley 1996}),^{2} (\text{Jaffe et al. 1992}),^{3} (\text{Juniper et al. 1989}),^{4} (\text{Lloyd 1942}),^{5} (\text{Slack 1979}),^{6} (\text{Franck 1976a}),^{7} (\text{McDaniel 1971}),^{8} (\text{Schnell 1976}),^{9} (\text{Franck 1976b}),^{10} (\text{Li 1952}),^{11} (\text{Obermeyer 1970}),^{12} (\text{Maguire 1978}),^{13} (\text{Arber 1941}),^{14} (\text{DeBuhr 1977}),^{15} (\text{Carquist 1976}),^{16} (\text{Metcalfe and Chalk 1950}),^{17} (\text{Watson and Dallwitz 1997}),^{18} (\text{Schmid 1978}),^{19} (\text{Vijayaraghavan 1965})\]
Table 3.2. Data matrix of morphological character codes.

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<th>4</th>
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<td>1</td>
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</tr>
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</tbody>
</table>
Fig. 3.1. Fifty percent majority rule consensus tree for morphological data set. Heuristic search branch support values above nodes and branch/bound values below nodes.
between the data and the tree output. The topology shows *Heliamphora* to be the basal group in the family with *Sarracenia* and *Darlingtonia* as sister taxa. Pairwise intertaxa phenetic distances were computed with the distance metric available in PAUP (Table 3.3).

In the course of the study two previously unreported traits were discovered for *Roridula* and one for *Sarracenia*. A new interpretation for the function of an existing trait in *Roridula* and one in *Sarracenia* were also revealed.

**Discussion**

The Nature of Morphological Evidence as Opposed to Molecular Evidence

The elevation of *Darlingtonia* to sister status with *Sarracenia* and delegation of *Heliamphora* to a basal position in the morphology-based phylogeny is not unexpected. The strong resemblance between *Darlingtonia* and *Sarracenia* has been noted in previous studies (DeBuhr 1977). This likeness is especially apparent when *D. californica* is compared with *S. psittacina*. The two species are identical in the early stages of the development of the pitcher (Lloyd 1942). The mature pitchers differ only in the width of the alata, the addition of the cobra's tongue appendage to the hood in *D. californica* and the spiral rotation of its pitcher about the longitudinal axis. Otherwise the pitchers are indistinguishable though the floral morphology is very distinct in the two genera.

It is important to keep in mind that morphological traits are phenotypes and not genotypes as in the case of molecular sequence data. Major changes in phenotype can be mediated by small numbers of genes. Traits such as those dealing with floral or pitcher morphology are
<table>
<thead>
<tr>
<th></th>
<th>Sar</th>
<th>Dar</th>
<th>Hel</th>
<th>Ror</th>
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<td>0.200</td>
<td>0.700</td>
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<td>0.800</td>
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<tr>
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<td>0.800</td>
<td>0.950</td>
<td></td>
</tr>
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<td>0.000</td>
<td>0.400</td>
<td>0.850</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ror</td>
<td>0.000</td>
<td>0.500</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Act</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
</tr>
</tbody>
</table>

Sar=Sarracenia, Dar=Darlingtonia, Hel=Heliamphora, Ror=Roridula, Act=Actinidia
directly involved with fitness and are likely to be under strong selection. Therefore observed changes are not the result of stochastic accumulation of DNA base changes as is the case in selectively neutral spacer regions. Thus, when we look at morphological changes we are looking at a subset of the genome that is likely to be actively involved in the speciation process not passively tracking divergence as spacer regions are alleged to do. For this reason there is no a priori expectation that morphological evidence will give exactly the same portrait of evolution in a lineage as molecular evidence though congruence in the respective phylogenies would be strong corroboration. There is, however, the potential for complementarity in viewing evolution from both the morphological and molecular windows into the genome, giving in the end a more complete picture of the actual sequence by which evolution has proceeded in a lineage.

The Nature of the Support for the Morphological Phylogeny

The sister relationship between *Sarracenia* and *Darlingtonia* is supported by ten synapomorphies in the set of twenty informative characters. Synapomorphies are unique character states that are shared by the members of a putative clade and that set them apart from the rest of the lineage. Six of these traits are involved with floral morphology and the rest are split between leaf form, pollen surface texture and wood anatomy.

Trends in Character Evolution

When the synapomorphies are mapped back on the phylogeny, several broad trends are evident. In terms of wood anatomy, the trend is one of progressive reduction in vessel element length and diameter as
the character trace moves up through the phylogeny. This corresponds with a parallel trend in loss of woodiness or secondary xylem. *Actinidia*, at the base of the phylogeny, is strongly woody with a typical habit as tall shrubs and climbing lianas which produce abundant secondary xylem with elements that are long and large diametered. In *Roridula* the habit is one of suffrutescent subshrubs that are woody only at the base with a concomitant shortening and narrowing of the vessel elements. *Heliamphora* represents the inception of the herbaceous state in the lineage but with retention of a somewhat dendroid habit. At the top of the phylogeny, *Darlingtonia* and *Sarracenia* are fully herbaceous and produce very few vessel elements. In keeping with the established pattern that first formed vessel elements are small, the elements of *Darlingtonia* and *Sarracenia* are the smallest in this phylogenetic series because only a few initial elements are ever produced in these plants.

Areolation of the upper regions of the hood covering the opening of pitcher is a trait found only in *Darlingtonia* and *Sarracenia*. Areolae are localized areas of thinning in the leaf epidermis that are transparent to light. The effect is one of a glass ceiling or hundreds of skylights in the hood. Acquisition of areolation gives the pitchers in these genera the added capability of functioning as light traps as well as pitfall traps. Insects, particularly flying insects, are attracted to the lighted portion of the hood and upon entering the hood follow the light path as it arches downward into the pitcher itself where it deadends and an array of fencelike hairs and slippery scales prevent egress. The sequence on the tree is from noncarnivory in *Actinidia* to flypaper traps in *Roridula* to
simple pitfall traps in *Heliamphora* culminating in the sophisticated lighted pitfall traps of *Darlingtonia* and *Sarracenia*.

The floral and pollen synapomorphies are all part of a larger evolutionary syndrome in this clade and that is specialization for vibratile pollination. Obligate pollination by bees is symplesiomorphic or ancestral in this phylogeny as it is found in all five genera. In vibratile pollination, the bee grasps the stamens with its feet. The flower is pendant so the bee is upside down when in position for vibration. While grasping the stamens the bee vibrates its wings at high frequency. The vibration is transferred through the bee’s feet to the anther, ejecting the pollen in a cloud. Much of the pollen is retrieved by the bee for consumption as food. Before it exits the flower the bee grooms itself with special pollen combs on its legs. The pollen is then packed into pollen baskets on the legs. The bee is able to comb most of its body except the midline of the underside. Pollen deposited in this area is the pollen that effects pollination when this part of the bee’s body rubs across the stigma as it exits the flower. Vibratile pollination is typically effected by large bumblebees.

There is considerable elaboration of this theme in the phylogeny but three general trends are illustrative. One is increasing floral coverage in terms of the number and distribution of nectaries. Nectaries serve as attractants and food rewards for bees. Floral nectaries are absent in *Actinidia*, localized to the sepals in *Roridula* and *Heliamphora* and profusely cover both sepals and petals in *Darlingtonia* and *Sarracenia*. In addition, in *Sarracenia* the swollen basal portion of the ovary is covered with copious nectaries to the extent that pools of nectar envelop the base.
A progression is seen in staminal arrangement. Clustering of the stamens allows the bee to eject pollen from several anthers simultaneously. Stamens are borne singly in *Roridula* and in *Heliamphora*, in a double whorl in *Darlingtonia* and fascicled in *Sarracenia*.

The most dramatic transition in floral morphology is seen in the enclosure of the flower by the perianth in conjunction with progressively elaborate lobing of the connate styles. The flowers open flat and the styles are free in *Actinidia*. In *Roridula* and *Heliamphora* the petals are inflexed creating an expanded flared tube. In *Darlingtonia* and *Sarracenia* the petals hang down fully enclosing the flower which is covered at the bottom by the projecting stylar lobes. This trend reaches its maximal expression in *Sarracenia* where the petals form a curtain around what appears to be an upturned umbrella stuck to the end of the flower, the spectacular umbraciform style that is found nowhere else in the flowering plants.

Completely enclosing the flower creates a paradox for the bee which must literally break into the flower in order to pollinate it. The mode of entrance and exit was observed (V. Moseley and M. Oard, pers. obs.) when a large *Bombus* spp. queen entered a *S. alata* flower. The mechanism is a one way turnstile affair because the notching of the petals lock into the upturned stylar lobes and prevent the petals from bending inward. The bee is forced to enter by pushing in on the petal itself. It exited over the stylar lobe which readily deflected outward like a miniature gangplank under the weight of the bee. When a flower was detached and the entrance/exit routine simulated with finger pressure, the
stylar lobe did not deflect from the outside even when considerable force was applied (V. Moseley and M. Oard, pers. obs.). The implication of this one way exit is that it is a means to insure selfing. The flowers are fully self compatible but do not self pollinate in the absence of bees (Schnell 1976). The inner tip of the stylar lobe contains the raised stigma which rakes the midline of the underside of the bee as it exits, picking up pollen deposited there when the bee buzzed the stamens. Even if it were possible for the bee to enter by deflecting the stylar lobe the stigma would be on the wrong surface of the lobe to effect pollination. This is the first report proposing the umbraciform style as an adaptation for self pollination.

Accompanying this transition in floral enclosure is a parallel switch in inflorescence type. Multiflowered cymes and racemes are found in *Actinidia*, *Roridula* and *Heliamphora*. In *Darlingtonia* and *Sarracenia*, the inflorescence is scapose consisting of a single large flower on a sturdy stalk. Perhaps this modification in inflorescence is in response to increasing reliance on large heavy bodied bees for pollination.

New traits were uncovered in the course of this study and an existing trait redefined in terms of its function. Heteranthery and white pollen were seen in *Roridula*. These are adaptations for vibratile pollination as described by Buchmann (1983). White colored pollen was observed in *S. alata* as well. The yellow swollen base of the anther in *Roridula* is not a nectary but a fake pollen mass (Buchmann 1983). It serves as an enticement to bees to repeatedly buzz anthers after dehiscence in order to insure that all the pollen is ejected.
Literature Cited


CHAPTER 4

MOLECULAR EVOLUTION OF PITCHER PLANT MOTHS
(EXYRA: NOCTUIDAE)

Introduction

Pitcher plant moths comprise the genus *Exyra* Grote in the Order Lepidoptera, Family Noctuidae. There are three described species, *E. fax*, *E. ridingsii* and *E. semicrocea*. All three species spend the entire life cycle within the confines of the pitchers of various species of the carnivorous pitcher plant genus *Sarracenia*.

Of the numerous insect species which are inquiline, or live habitually in pitcher plants, the three species of *Exyra* are the only ones which have evolved the capacity to disarm the pitfall trapping mechanism of the pitcher. They do so by tapping a hole in the base of the pitcher, and draining the digestive fluid contained therein (Hubbard 1894 and Jones 1921). A web is spun across the throat of the pitcher and the top is girdled so that it collapses in on itself, preventing other insects from entering the pitcher. The voracious larva feeds within the webbed off chamber, stripping away the epidermis and consuming all the photosynthetic tissue, leaving only the outer epidermis intact. In this manner the larva renders the pitcher completely functionless, depriving the pitcher of both the autotrophic nutrition of photosynthesis and the heterotrophic nutrition of its insect prey.

The plant has not evolved any counteroffensive to this highly efficient and destructive herbivory suggesting the intimate association between the moths and their hosts may be relatively recent in origin. Yet, of the many species of inquilines which are endemic within the confines
of the pitchers, *Exyra* is singular in that this endemism encompasses the entire genus and there is host specialization in larval feeding preference. Larvae of *E. fax* specialize on *S. purpurea* while those of *E. rideingsii* are found exclusively on *S. flava*. The third species, *E. semicrocea*, is a generalist and has been found in association with all *Sarracenia* species.

Retracing the sequence of speciation events in the *Exyra* lineage provides a means of elucidating how host plant specialization has proceeded in the course of evolution in this lineage. This can be especially informative when the phylogeny of moth speciation can be compared with the companion phylogeny of the pitcher plant hosts.

To this end, molecular phylogenies were generated for *Exyra* in like manner to those derived for *Sarracenia* as described in chapter two. In keeping with the rationale of using sequence data from more than one genome in order to have independent estimates of the actual evolutionary trajectory for the group, sequences were obtained from both the nuclear and mitochondrial genomes. The targeted regions were Internal Transcribed Spacer 1 (ITS 1) from the nuclear ribosomal DNA and Cytochrome Oxidase I and II (COI-COII) from the mitochondrial genome.

The COI-COII region is a coding region and has no spacer sequence. Under the Wobble Hypothesis, the third position in a codon can vary at the DNA level without effecting corresponding changes at the protein level. Because of this redundancy whereby multiple codons can specify the same protein when the differences are in the third position, the third position is considered to be under neutral selection. As a result third codon positions can accumulate mutations at rates comparable to spacer
regions. The COI-COII region has proven informative in a number of insect phylogenies as reviewed in Simon et al. (1994).

Materials and Methods

Taxa

Taxa used in the study included DNA isolates from two individuals of each described species of *Exyra* and the two outgroup species, *Tarachidia semiflava* and *Thioptera nigrofimbria*. These accessions, their places of origin and the names of the collectors are shown in Table 4.1. Species definitions for the three *Exyra* species are as circumscribed in Folkerts and Folkerts (1996) and Lafontaine and Poole (1991).

The monophylly of *Exyra* is generally accepted but the sister taxon is unknown. Outgroup selection was based largely on the fact that the three *Exyra* species and the outgroup species named above were initially placed together in the same genus *Xanthoptera* by Gueneé. Grote subsequently segregated the three species found only on pitcher plants into the separate genus *Exyra* (Grote 1879). There are strong morphological resemblances between these species in their yellow wings with black borders. In addition, *T. semiflava* also has a host range that includes *Sarracenia flava* and *S. minor*.

Primer Sequences for Target Genes

The sequences of all primers used in the study are given by target gene together with references in Table 4.2. The amplifying and sequencing primers were the same except where the PCR product was cloned prior to sequencing. Cloned target sequences were sequenced with the M13 Forward and Reverse primers supplied by Perkin Elmer in their Amplitaq Cycle Sequencing Kit (Perkin Elmer, Foster City, CA).
<table>
<thead>
<tr>
<th>Taxon Name</th>
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<th>Collector</th>
<th>Stage</th>
<th>Caught In</th>
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<td>MO</td>
<td>Adult</td>
<td>S. flava Pitcher</td>
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<td>Apalachicola NF, FL</td>
<td>MO</td>
<td>Adult</td>
<td>S. purpurea Pitcher</td>
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<td>S. flava Pitcher</td>
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<td>4. Exyra ridingsii 2</td>
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<td>MO</td>
<td>Adult</td>
<td>S. alata Pitcher</td>
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<tr>
<td>6. Exyra semicrocea 2</td>
<td>Kisatchie N, LA</td>
<td>MO</td>
<td>Adult</td>
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</tr>
<tr>
<td>7. Tarachidia semiflava 1</td>
<td>Sandy Hollow WMA</td>
<td>DL</td>
<td>Adult</td>
<td>Light Trap</td>
</tr>
<tr>
<td>8. Tarachidia semiflava 2</td>
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<td>DL</td>
<td>Adult</td>
<td>Light Trap</td>
</tr>
<tr>
<td>9. Tarachidia semiflava 3</td>
<td>Kisatchie NF, LA</td>
<td>DL</td>
<td>Adult</td>
<td>Light Trap</td>
</tr>
<tr>
<td>10. Thioptera nigrofimbria 1</td>
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<td>DL</td>
<td>Adult</td>
<td>Light Trap</td>
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<tr>
<td>11. Thioptera nigrofimbria 2</td>
<td>Tunica Hills, LA</td>
<td>DL</td>
<td>Adult</td>
<td>Light Trap</td>
</tr>
</tbody>
</table>

NF=National Forest, WMA=Wildlife Management Area  
MO=Margi Oard, DL=Deborah Landau, DP=Dorothy Prowell
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<th>Primer Sequence</th>
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<td>White et al. 1990</td>
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<td>Simon et al. 1994</td>
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<td>M13F</td>
<td>5'-GCCAGGGTTTTCCAGTCACGA-3'</td>
<td>Perkin Elmer 1994</td>
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<td>M13R</td>
<td>5'-GAGCGGATAAACATTTCACAGG-3'</td>
<td>Perkin Elmer 1994</td>
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</tbody>
</table>
DNA Isolation

Only tissue from adult moths was used for DNA isolation. The moths were prepared for DNA isolation by removing the wings followed by grinding in liquid nitrogen or sterilized acid-washed sand in a mortar and pestle. DNA was isolated by the protocol of Doyle and Doyle (1990) using a 2% formulation of the CTAB isolation buffer. This protocol was the same as for plant material but scaled down to one-tenth volume for all buffers and reagents. This allowed all stages of the isolation to be carried out in 1.5 ml centrifuge tubes and all centrifuge spins were in a microfuge.

Polymerase Chain Reaction (PCR) Amplification of Target Sequences, Cloning, and Automated Cycle Sequencing

The procedures for these three phases of the study were the same as for the plant material. See chapter two for these protocols.

Data Analysis and Phylogeny Construction

Aligned sequences were entered as MacClade version 3.05 data matrices for all subsequent database manipulations and cladistic analyses. Cladistic analyses were performed with PAUP version 3.1.1 using parsimony based algorithms for phylogeny construction. The PAUP analyses consisted of heuristic searches for the most parsimonious trees using both branch swapping with tree bisection/reconstruction and stepwise random addition methodologies. This insured that a full range of possible trees was derived and evaluated to yield maximal parsimony. Both heuristic search methodologies gave the same results in all cases so that only one tree is presented for each data set in the results section below. Heuristic searches were followed by branch and bound search.
routines to test the stability of tree topologies under repeated resampling and rearrangement. This gave a quantitative measure of the support in the data for each branch in the resultant tree.

Results

Sequence alignments were attempted for both the ITS 1 and COI-COII datasets but only the COI-COII dataset could be confidently aligned due to greater divergence between the outgroup species and *Exyra* in the ITS 1 dataset. The *Exyra* sequences themselves were fully alignable and it was possible to calculate pairwise genetic distances between species as described below.

The COI-COII dataset contained 8 taxa and 4484 characters. There were 41 phylogenetically informative characters. Each *Exyra* species was represented by two accessions. The outgroup for this data set contained one accession of *Tarachidia semiflava* and one of *Thioptera nigrofimbria*.

The phylogenetic tree obtained for this dataset can be found in Fig. 4.1. Heuristic random stepwise addition and branch/bound searches each yielded two most parsimonious trees of length 51. A fifty percent majority rule consensus tree was computed on the basis of these two most parsimonious trees.

Several indices are used to evaluate the goodness of fit between the data set and tree topology. They are the consistency index or C.I., the homoplasy index or H.I. and the retention index or R.I. The values were 0.88, 0.12 and 0.90 respectively. These indices range between zero and one. Large values (>0.80) of the C.I. and R.I. and small values (<0.2) of the H.I. are considered indicators of very good fit between the data and the tree output. In the topology reported here there is 100% support for all
Fig. 4.1. Fifty percent majority rule consensus tree for Exyra species based COI-COII DNA sequence data. Heuristic search branch support values shown above nodes and branch bound values shown below nodes.
branches in the branch/bound search results. The consensus tree topology shows *E. ridingsii* to be the basal group in the genus with *E. fax* and *E. semicrocea* as sister taxa though there is incomplete resolution because one of the *E. semicrocea* accessions is more closely related to both *E. fax* accessions than it is to the other *E. semicrocea* accession. The data set was reanalyzed using only the *E. semicrocea* accession that was distinguishable from *E. fax*. The topology from this second analysis is shown in Fig. 4.2 along with the companion phylogeny for *Sarracenia*. This was done to illustrate the relationship between host use in the moth and speciation in the plant host.

Pairwise intertaxa genetic distances were computed with the distance algorithm available in PAUP. The resulting distance matrices for the COI-COI dataset and the *Exyra* accessions in the ITS1 dataset are presented in Tables 4.3 and 4.4 respectively. Table 4.5 contains intertaxa genetic distances across COI-COI and ITS 1 for the *Exyra* accessions.

**Discussion**

The genetic overlap between *S. fax* and *S. semicrocea* is intriguing. Looking at the genetic distance matrices in Tables 4.3 and 4.4 is instructive in this regard. The two *E. fax* specimens were collected at the same site on the Apalachicola National Forest and there is no detectable divergence between them in the COI-COI region. The same situation is found with reference to ITS 1. One *E. semicrocea* came from the Kisatchie National Forest in western Louisiana and the other specimen was collected on Lake Ramsey Wildlife Management Area in eastern Louisiana. The distance between them is 0.143 for COI-COI and their respective divergences relative to *E. fax* are 0.024 and 0.171. For ITS 1,
Fig. 4.2. Alignment between moth and host plant molecular phylogenies showing host plant use patterns for Exyra spp.
Table 4.3. Matrix of pairwise relative genetic distances in Exyra spp. calculated from COI-COII sequence data.

<table>
<thead>
<tr>
<th></th>
<th>E. sem 1</th>
<th>E. sem 2</th>
<th>E. rid 1</th>
<th>E. rid 2</th>
<th>E. fax 1</th>
<th>E. fax 2</th>
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<tbody>
<tr>
<td>E. sem 1</td>
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<td>0.146</td>
<td>0.390</td>
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<td>0.488</td>
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<td>0.463</td>
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<td>0.439</td>
<td>0.000</td>
</tr>
<tr>
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<td></td>
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<td>0.439</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
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<tr>
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<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>E. fax 2</td>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

E. sem=Exyra semicroea, E. rid=E. ridingsii, E. fax=E. fax
<table>
<thead>
<tr>
<th></th>
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<th>E. sem 2</th>
<th>E. rid 1</th>
<th>E. rid 2</th>
<th>E. fax 1</th>
<th>E. fax 2</th>
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<tbody>
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<td>0.565</td>
<td>0.609</td>
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<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>E. fax 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

E. sem = Exyra semicrocea, E. rid = E. ridingsii, E. fax = E. fax
Table 4.5. Matrix of pairwise genetic distance for Exyra spp. calculated from COI-COII and ITS 1 rDNA sequence data.

<table>
<thead>
<tr>
<th></th>
<th>E. sem 1</th>
<th>E. sem2</th>
<th>E. rid 1</th>
<th>E. rid 2</th>
<th>E. fax 1</th>
<th>E. fax 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. sem 1</td>
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<td>0.643</td>
<td>0.690</td>
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<td>0.561</td>
</tr>
<tr>
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<td>0.767</td>
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<td>0.429</td>
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<tr>
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<td>0.045</td>
<td>0.857</td>
<td>0.857</td>
<td></td>
</tr>
<tr>
<td>E. rid 2</td>
<td></td>
<td></td>
<td>0.000</td>
<td>0.810</td>
<td>0.810</td>
<td></td>
</tr>
<tr>
<td>E. fax 1</td>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>E. fax 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

E. sem = Exyra semiocraea, E. rid = Exyra ridingsii, E. fax = Exyra fax
there is no divergence seen between these *E. semicrocea* individuals and both are about 80% divergent from the two *E. fax* specimens.

The mitochondrial and ribosomal genetic distance matrices show patterns of divergence with different phylogenetic implications. Because of the overlap in mitochondrial genotype between *E. fax* and one *E. semicrocea* individual, the distance between these two species is confounded in the mitochondrial distance matrix. They are shown to be very close to one another and about equidistant from *E. ridingsii* which is reflected in the phylogenetic reconstruction (Fig. 4.1) by the basal position of the latter. In the ITS 1 distance matrix, *E. semicrocea* and *E. ridingsii* are the more closely related of the three species and are about equidistant from *E. fax*. This pattern is incompatible with the phylogenetic reconstruction based on the mitochondrial sequence data. Distances calculated on the basis of the combined data set tend to reinforce the phylogenetic reconstruction (Fig. 4.1) with *E. fax* and *E. semicrocea* more closely allied (average distance=0.495), *E. semicrocea* and *E. ridingsii* intermediate in divergence (average distance=0.717), and *E. fax* and *E. ridingsii* the most distant pairing (average distance=0.834).

There is no apparent explanation for this anomaly in which a specimen of *E. semicrocea* has an *E. semicrocea* genotype for its nuclear genome and an *E. fax* genotype for its mitochondrial genome. Contamination can be ruled out because the *E. fax* specimens were acquired two years after the isolation of DNA from the *E. semicrocea* specimens. Hybridization is not a likely factor either as the *E. semicrocea* specimens were collected at sites that are 400-500 miles from the range of *E. fax*. These moths are very sedentary and do not migrate making a
chance out of range occurrence for _E. fax_ unlikely as well. Misidentification of specimens is not a viable explanation because the two species are readily distinguishable having different body and wing coloration.

The effect on the tree topology of this anomalous _E. semicrocea_ mitochondrial genotype is that _E. semicrocea_ and _E. fax_ are not a full sister clade but are instead a grade. This makes for a somewhat fuzzy species boundary but the tree is still informative with regard to overall trends in host specialization. When the trait of host specialization is mapped on the tree in Fig. 4.1, the specialization on _S. flava_ by _E. ridingsii_ is indicated to be the ancestral state for the group. There appears to be incipient host switching to _S. purpurea_ in the evolution of _E. fax_. The fact that the outgroup species _T. semiflava_ (Tietz 1972) also further suggests that host specialization is the ancestral state in this group. With host specialization being ancestral in the group, a more recent origin is suggested for the generalist tendencies seen in _E. semicrocea_.

Comparing the moth phylogeny with the companion phylogeny for _Sarracenia_ indicates some interesting matches (Fig. 4.2). The position for the host _S. flava_ in the plant phylogeny aligns with the positioning of _E. ridingsii_ in the moth phylogeny as does the more recent derivations of _S. purpurea_ and _E. fax_ in their respective phylogenies. This is an independent indication of a possible link between speciation in the moth by host plant switching and speciation in the host plant itself.

Another set of parallel trends in moth and host that may be of relevance is that of size. _S. flava_ is the largest pitcher host plant species
reaching a height of one meter. *S. leucophylla* is also a robust species though shorter in stature than *S. flava*. The progression is to smaller statured species less than 50 cm in height in the evolution of *S. purpurea*, *S. rubra* and *S. alata*. A comparable reduction in size is seen for the moths with *E. ridingsii* about double in size relative to *E. fax* and *E. semicrocea*. With the entire life cycle of the moth taking place inside the pitcher a change in the size of the available food resource may have been accompanied by a similar size reduction in the moth.

**Literature Cited**


CHAPTER 5
A BIOGEOGRAPHIC SPECIATION MODEL
FOR PITCHER PLANTS AND THEIR MOTHS

Introduction

Chapters two, three and four described phylogenetic analyses of biological data in the form of DNA sequences and morphological characters. These analyses yielded pictorial inferences about the sequence of origination of species in pitcher plants and in their moths in the form of phylogenetic trees. By giving the phylogenetic trees the added geographic dimensions of space and time a biogeographic speciation model is created. This is accomplished by mapping the phylogeny onto the spatial map of the landscapes occupied by the taxa. In this way we can see how the chronology of speciation inferred in the phylogenetic tree is played out in the area cladogram inferred in the spatial arrangement of the extant taxa. If there is a good fit between the these two types of data there should be congruence in the overlay of one type of tree on the other.

For the origination of genera there are two phylogenetic trees and one area cladogram. The two phylogenetic trees are the one derived from DNA sequence data (Fig. 2.5) and the one derived from morphological data (Fig. 3.1). These two trees differ in the placement of Darlingtonia, but Darlingtonia is more closely linked morphologically to Sarracenia than it is to Heliamphora as the ten synapomorphies uniting Darlingtonia and Sarracenia attest (chapter three).

The morphology based phylogeny is strongly supported by the genetic distance data of Table 2.6. Between Darlingtonia and Sarracenia, the genetic distance is half (average distance=0.227) that between
Sarracenia and Heliamphora (average distance=0.381) or between Darlingtonia and Heliamphora (average distance=0.411). These results yield a topology of (((Darlingtonia, Sarracenia) Heliamphora) Roridula) which is the same as Fig. 3.1.

The distance data also show Darlingtonia to be less diverged from Roridula at distance=0.68 than are Sarracenia and Heliamphora at distances=0.78-0.83. This may be causing Darlingtonia to appear basal in the molecular phylogenetic reconstruction. A possible explanation for this discrepancy is that base substitutions in Darlingtonia have reverted to like character states found in Roridula at a higher frequency than in Heliamphora or Sarracenia, giving these shared character states the appearance of synapomorphies which they are not.

Radiation at the Generic Level

With independent corroboration from the distance data, the morphological phylogeny is used as the hypothesis for the sequence of origination of taxa at the generic level. Superimposing the phylogeny on a geographic map as shown in Fig. 5.1, results in a scenario in which a Roridula-like ancestor colonized the Venezuelan Guayana. There, a pitchered carnivorous form evolved which became the Heliamphora lineage. A second colonization involving a Heliamphora-like ancestor, occurred in the southeastern U.S. probably in peninsular Florida giving rise to the Sarracenia-Darlingtonia lineage. The final stage was the colonization of the Pacific Northwest by a Sarrenica-Darlingtonia-like ancestor which led to the derivation of Darlingtonia.
Fig. 5.1 Sequence of origination of genera in Sarraceniaceae. ROR=Roridula, HEL=Heliamphora, SAR=Sarracenia, DAR=Darlingtonia, anc=ancestor
Radiation Within *Heliamphora*

According to the molecular phylogeny, *H. heterodoxa* is the basal member of the genus. The distribution for this species is centered in the Gran Sabana, a vast lowland marsh savanna and tepui complex in the heart of the Venezuelan Guayana. If the base of the phylogeny is aligned over this region, the spatial arrangement of taxa is largely concordant with the phylogeny (Fig. 5.2). From the initial colonization of the Gran Sabana by a *Roridula* type ancestor (internode labeled r in Fig. 5.2), a pitchered type emerged, founding the *Heliamphora* lineage. The lineage then split, giving rise to *H. heterodoxa* on Kavanyen Tepui and to the ancestor of the rest of the *Heliamphora* lineage (internode denoted as h in Fig. 5.2). A second round of lineage splitting involved colonization in the Roraima Tepui chain to the east by a *H. nutans*-like ancestor and to the west by a *H. minor*-like ancestor. Further splitting by colonization of Illu and Roraima Tepuis gave rise to *H. nutans* and *H. ionasii*. Westward expansion of the range led to colonization of Chimanta Tepui and the derivation of *H. minor*. The *H. tatei*-like ancestor that arose out of this split underwent another round of speciation with subsequent colonization of Duida-Huachamacari Tepuis and Cerro de la Neblina, resulting in *H. tatei* and *H. neblinae*.

Radiation Within *Sarracenia*

The Gulf and Atlantic Coastal Plains present a different arena for speciation than the tepuis of the Venezuelan Guayana. The landscape is one of great contiguity in both landform and plant formation (Walker and Coleman 1987, Noss 1988). Historically, the longleaf pine savanna in which pitcher plants are found extended from Texas to Virginia, its vast expanse broken only by the floodplains of great rivers like the Mississippi,
Fig. 5.2. Sequence of origination of taxa in Heliamphora in relation to location on tepuis.
the Mobile and Apalachicola. It is a subtle landscape on a regional scale but encompasses marked differences in substrate and hydrology within short distances at the local scale (Norquist 1984). Symparity is evident in the overlapping ranges of *Sarracenia* species but radiation within the group appears to be accompanied by strong selection for habitat specialization. McDaniel (1971) observed that when *Sarracenia* species occur in the same area they occupy different microsites. The current range of each *Sarracenia* species is shown in Fig. 5.3.

A plausible scenario for speciation in *Sarracenia* entails an initial introduction from South America into peninsular Florida. This is where the distribution of the basal (*S. psittacina, S. minor, S. oreophila*) clade is centered. If the molecular phylogeny is aligned at this starting point some interesting spatial patterns emerge. Speciation in the group appears to have proceeded as a two-pronged affair with radiation east and west along the expanding northern perimeter of the genus to give taxa of primarily either Gulf Coastal Plain or Atlantic Coastal Plain affinities. This is illustrated in Fig. 5.4 in the alternation between Gulf Coastal Plain and Atlantic Coastal Plain species in the phylogeny.

**Parallel Speciation Patterns In Southern Pines**

In their entry through the Florida gateway, *Sarracenia* seems to be tracking a similar colonization pattern seen for the southern pines. *Pinus elliottii* var. *densa* is found predominantly in southern Florida and the Florida Keys. *Pinus elliottii* var. *elliottii* is found in northern Florida and the Florida panhandle. The closest relative of *P. elliottii* is *P. caribaea* which is found in western Cuba, the Isle of pines and along the coast in the Yucatan Peninsula (Rushforth 1987). *P. elliottii* is also known to readily
<table>
<thead>
<tr>
<th>GULF COASTAL PLAIN</th>
<th>ATLANTIC COASTAL PLAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. alata</td>
<td>S. rubra</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>S. leucophylla</td>
<td>S. purpurea</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>S. oreophila</td>
<td>S. flavum</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>S. psittacina</td>
<td>S. minor</td>
</tr>
</tbody>
</table>

Fig. 5.3. Southeastern U.S. ranges for Sarracenia showing coastal plain affinities.
Fig. 5.4. Sequence of origination in Sarracenia.
GCP=Gulf Coastal Plain, ACP=Atlantic Coastal Plain,
ALA=S. alata, RUB=S. rubra, PUR=S. purpurea,
LEU=S. leucophylla, FLA=S. flava, ORE=S. oreophila,
PSI=S. psittacina, hel=Heliamphora-like ancestor
colonize barrier islands as is seen by the presence of var. densa in the Florida Keys. The Florida invasion by southern pines has most likely come about by island hopping from the Yucatan Peninsula during the last low sea stand. For the southern pines there is palynological evidence that they first appeared in the southern tip of Florida 18,000 years ago and moved simultaneously northeast and northwest into the Atlantic and Gulf Coastal Plains, reaching their present distribution in the last 3,000 years (Wright 1989). This circumstantial evidence suggests southern pines and *Sarracenia* may share a similar pathway and time frame for their respective radiations.

**Speciation Model for Pitcher Plant Moths**

The speciation model for pitcher plants moths is found in chapter four. In this model, the host phylogeny is used in place of geographic range maps as the spatial component. Host switching is then the analog of habitat colonization.

Genetic distances between individual moth species are as great as the distances between entire genera in the plants though the chronological timeframes are likely to be approximately equivalent. This is an order of magnitude difference in the relative rate of evolution in moths vs. plants.

The two groups differ dramatically in a number of life history traits that have been implicated in influencing rates of evolution such as generation time, breeding system, population size and level of fragmentation (Li 1996). Pitcher plants are long-lived perennials with generation times that may be measured in years. They reproduce primarily by vegetative means, form dense swards, and are inbreeding.
when they do reproduce sexually. The moths on the other hand, are short-lived and have multiple generations per year, are outcrossed, and populations are clumped within pitcher plants stands. Any of these factors may be contributing to the large difference observed in rate of evolution but there is no way of knowing what is the underlying cause without additional study.

Time Span for Evolution in Sarraceniaceae

Because of their acidic substrates and topographic positions as closed basins and depressions, the heathland landscapes occupied by Sarraceniaceae and Roridulaceae are ideal sites for pollen preservation and palynological reconstructions of past vegetation history. The ages of these landscapes have been dated by radiocarbon analysis as well. The youngest landscapes are the tepui marsh savannas at 3 000-6 000 years (Steyermark 1995). The Coastal Plain savannas of the Southeastern U.S. and the wet fynbos of South Africa are of comparable age at 18 000 (Wright 1989) and >14 000 years (Meadows and Sugden 1991) respectively. Ages of 6 000-8 000 years are suggested for sites in the Pacific Northwest (Wright 1989). When coupled with the low rates of 1-5% divergence (Table 2.6) between species within genera, the picture is one of recent radiations in youthful landscapes.

Literature Cited


VITA

The eldest of four children, Margaret Ellen Oard was born December 18, 1950, in St. Joseph, Missouri. Her father was a civil engineer and her mother was a musician and a college instructor of music.

Two aspects of her character were apparent from earliest childhood. These traits were an innate love of the outdoors and an incessant inquisitiveness. These eventually found a natural outlet in the study of biology.

The interest in biology was pursued as an undergraduate and in subsequent graduate training. She received a Bachelor of Arts Degree in biology and religion from Macalester College in St. Paul, Minnesota, in 1972. This was followed by a Master of Science Degree in botany from Iowa State University, Ames, Iowa, in 1977 and a Master of Science Degree in genetics from the University of California, Davis, California, in 1991.

She enter the doctoral program at Louisiana State University, Baton Rouge, Louisiana, in September, 1992. The Doctor of Philosophy Degree from the Department of Entomology will be conferred in December, 1997.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Margaret Ellen Oard

Major Field: Entomology

Title of Dissertation: The Evolution of Landscapes and Lineages in Pitcher Plants and Their Moths

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Thomas J. Riley

[Signatures]

[Signatures]

Date of Examination:

June 27, 1997