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# Phylogenetic relationships in Sarraceniaceae based on rbcL and ITS sequences

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**ABSTRACT.** Sarraceniaceae, a New World family of carnivorous plants, consist of *Sarracenia, Heliamphora,* and the monotypic *Darlingtonia.* Despite extensive interest in these plants, few explicit phylogenetic hypotheses are available for the family. We investigate phylogenetic relationships in the family using sequences of the internal transcribed spacer regions of nuclear ribosomal DNA (ITS) and of the chloroplast gene *rbcL.* One representative of each genus was sequenced for *rbcL,* whereas representatives of all species in the family, except for *H. heterodoxa* and *H. ionasii,* were sequenced for ITS. Both the ITS- and *rbcL*-based phylogenies demonstrate that *Sarracenia* and *Heliamphora* form a monophyletic group that is the sister group of *Darlingtonia. Sarracenia* and *Heliamphora* are each well supported monophyletic genera. In our restricted sampling of *Heliamphora, H. nutans* is the sister of *H. minor* and *H. tatei.* Our results provide little resolution of relationships among species of *Sarracenia,* but identify one clade that consists of all species except *S. alata.* Within this clade are two smaller clades comprising:—1) *S. purpurea* and *S. leucophylla,*—and—2) *S. flava, S. minor,* and *S. psittacina.*

Sarraceniaceae have received considerable attention from biologists because of carnivory throughout the family. All members of Sarraceniaceae are found in nutrient-poor habitats and like other carnivorous plants use animals as a supplemental source of nitrogen that is taken up in the form of ammonia (Christensen 1977; Bradshaw and Creelman 1984; Jaffe et al. 1992). Sarraceniaceae, Cephalotaceae, and Nepenthaceae use pitcher-like (ascidiate) leaves as passive traps for the collection of animals. As has been suggested previously (Thanikaimoni and Vasanthy 1972; DeBuhr 1975) and shown in recent phylogenetic analyses (Albert et al. 1992; Chase et al. 1993), the ascidiate leaves shared by these three families are evolutionary parallelisms.

Sarraceniaceae consist of three distinct genera, *Darlingtonia* Torr., *Heliamphora* Benth., and *Sarracenia* L. The North American genera *Darlingtonia* and *Sarracenia* are relatively well known compared to the South American *Heliamphora. Darlingtonia* is monospecific and located in western Oregon and northern California. *Darlingtonia californica* Torr. occurs in areas with standing water that are associated with serpentine outcrops or other rocks with a high metal content (Ziemer 1973; Juniper et al. 1989). Most species of *Sarracenia* occur in the southeastern United States; however, *S. purpurea* L., the most widespread species, is distributed from the Gulf Coast, along the Atlantic seaboard, and across the northern United States and boreal Canada west to British Columbia. Species of *Sarracenia* are found in bogs, savannas, and flatwoods (McDaniel 1971). The circumscription of species of *Sarracenia* has varied, sometimes considerably (Wherry 1933; Bell 1949; Case and Case 1976; McDaniel 1971). The recent revision by McDaniel (1971) clarified patterns of population variation and provided reasonable circumscriptions of eight species. Since McDaniel's (1971) revision, *S. alabamensis* Case and Case, a species restricted to northern Alabama, was described (Case and Case 1974). *Heliamphora* is a small genus of five (Steyermark 1979) or six (Maguire 1970, 1978) species restricted to Roraima sandstones of the Guayana Highland of Venezuela, Guyana, and Brazil in northeastern South America. Maguire (1978) noted that "no clear-cut distinctions within the genus are discernible" (p. 37). His species circumscriptions were based largely on interpopulational differences in pitcher morphology.

Few explicit hypotheses of intergeneric relationships have been presented for Sarraceniaceae. Macfarlane (1893), Maguire (1978), and Juniper et al. (1989) placed the North American genera *Darlingtonia* and *Sarracenia* as sister taxa either derived within or as the sister group of the South American *Heliamphora.* In contrast, an analysis of *rbcL* sequences (Albert et al. 1992) placed *Heliamphora* and *Sarracenia* as sister taxa, which in turn are the sister group of *Darlingtonia*. Presumably derived morphological features have often been cited to suggest a close relationship between *Darlingtonia* and *Sarracenia* (Macfarlane 1893; Markgraf 1955; Maguire 1978), although it has been suggested that either this clade or *Heliamphora* may have arisen first (DeBuhr 1977). No proposals regarding relationships among species of *Heliamphora* have been made and few have been suggested for *Sarracenia.*

We present a phylogenetic analysis of Sarraceniaceae using DNA sequence data. Relationships among the genera are reexamined using data from the chloroplast gene *rbcL* because of the unexpected position of *Darlingtonia* in the Albert et al. (1992) phylogeny of carnivorous plants, and because of the large amount of sequence divergence in the internal transcribed spacer region in *Darlingtonia.* In addition, relationships among species are examined using ITS sequence data.

## MATERIALS AND METHODS

***Outgroup Selection.*** Outgroup taxa were selected on the basis of the *rbcL* analysis of Chase et al. (1993; especially Figs. 12B and 13B). The outgroup for the *rbcL* analysis consisted of *Cornus* L., *Alangium* Lam., *Hydrangea* L., and *Philadelphus* L. The *rbcL* sequences for all taxa, except *Darlingtonia,* were obtained from GenBank. *Actinidia* Lindley alone was used as the outgroup in the ITS-based phylogeny reconstruction because it and *Roridula* Burm f. ex L. are closest relatives of Sarraceniaceae (Chase et al. 1993). Material of *Roridula* was, unfortunately, not available.

***Ingroup Sampling.*** We used the systematic treatments of Maguire (1978) and Steyermark (1984) as the basis for species circumscriptions for *Heliamphora,* and McDaniel (1971) for *Sarracenia,* except that we also recognize the more recently described *S. alabamensis* (Case and Case 1974). Sequences of the *rbcL* gene for *H. nutans* Benth. and *S. flava* L. were obtained from GenBank and *D. californica* was sequenced from fresh leaf material (Table 1). Sequences of ITS-1 and ITS-2 were obtained for all species of *Sarracenia, Darlingtonia californica,* and three species of the more inaccessible *Heliamphora.* Material of *Darlingtonia* was collected in the field; all other samples were obtained from botanic gardens or commercial nurseries (Table 1). In the *rbcL* analysis the extended ingroup consisted of all taxa used by Chase et al. (1993) in Fig. 13B (i.e. exemplar genera of the orders Diapensales, Ebenales, Ericales, Geraniales, Nepenthales, Primulales, Rosales, Solanales, Theales, and Violales) plus *Darlingtonia.*

***DNA Isolation, Amplification, and Sequencing.*** Total DNA was isolated from 0.7 to 1.0 gram of fresh leaf material or 0.2 to 0.4 gram of material dried in silica gel using a modification of the CTAB method of Doyle and Doyle (1987), where 1.0% β-mercaptoethanol (instead of 0.2%) was used in the extraction buffer. DNA was reprecipitated with ice-cold 95% EtOH, washed in 95% EtOH, and resuspended in TE.

The ITS region was amplified via the polymerase chain reaction (PCR) using Replitherm, DNA polymerase (Epicentre Technologies, Madison, WI). The PCR reaction mixture consisted of 5 µl of 20X reaction buffer, 6 µl of 25mM magnesium chloride solution, 16 µl of a 1.25 mM dNTP solution in equimolar ratio, 25 pmol of each primer, 10-50 ng of template DNA, and 0.5 units of Replitherm all in a total volume of 100 µl. The PCR samples were heated to 94° C for two minutes prior to the addition of Replitherm to denature unwanted proteases and nucleases. The double-stranded PCR products were produced via 30 cycles of denaturation (94° C for 1.5 min), primer annealing (55° C for 2 min), and extension (72° C for 3 min). A 15-min final extension cycle at 72° C followed the 30th cycle.

The two ITS regions were amplified separately. The 1407F (D. Nickrent, Univ. of Southern Illinois, pers. comm.) and ITS2 primers (White et al. 1990) were used in equal proportions to produce double-stranded product that included ITS-1, whereas ITS3 (White et al. 1990) and 307R (D. Nickrent, Univ. of Southern Illinois, pers. comm.) were used to amplify ITS-2. Double-stranded PCR products were then used as templates to produce single-stranded DNA following the same general PCR protocol as above, except only the ITS2 primer was used to produce single-stranded DNA of the ITS-1 region and ITS3 was used to produce single-stranded DNA of the ITS-2 region. The resultant single-stranded DNAs were precipitated with 20% PEG/2.5 M NaCl, washed in 70% EtOH, and washed a second time in 95% EtOH before being resuspended in 7 µl of TE buffer (Morgan and Soltis 1993) prior to sequencing.

TABLE 1. Collections of *Actinidia, Darlingtonia, Heliamphora,* and *Sarracenia* used in the ITS sequencing study. Presented are species, origin (location of voucher), and accession numbers. Voucher specimens are deposited at ALTA, B, K, and WS. GenBank accession numbers for the ITS sequences for all taxa, as well as the *rbcL* sequence accession number for *Darlingtonia*, are given.

| Species | Origin (voucher location) | Accession number | GenBank Accession numbers (ITS-1/ITS-2 respectively) | GenBank Accession number (*rbcL*) |
|---|---|---|---|---|
| *Actinidia deliciosa* (A. Chev.) Laing and Ferguson | Royal Botanic Gardens, Kew (K) | 394-85.04544 | L42184/L42197 | — |
| *Darlingtonia californica* Torr. | U.S.A.: Oregon: Josephine Co. (ALTA) | Bayer, et al. *OR-840* | L42185/L42199 | L42211 |
| *Heliamphora minor* Gleason | Berlin Botanic Garden (B) | 116-36-74-80 | L42186/L42200 | — |
| *Heliamphora nutans* Benth. | Berlin Botanic Garden (B) | 056-95-74-80 | L42187/L42201 | — |
| *Heliamphora tatei* Gleason | Royal Botanic Gardens, Kew (K) | 388-91.01838 | L42188/L42202 | — |
| *Sarracenia alabamensis* Case & Case | Royal Botanic Gardens, Kew (K) | 154-89.01071 | L42187/L42203 | — |
| *Sarracenia alata* (Wood) Wood | Orgel's Orchids, Miami, FL (WS) | Hufford *707* | L42278/L42279 | — |
| *Sarracenia flava* L. | Orgel's Orchids, Miami, FL (WS) | Hufford *708* | L42190/L42204 | — |
| *Sarracenia leucophylla* Raf. | Orgel's Orchids, Miami, FL (WS) | Hufford *705* | L42191/L42205 | — |
| *Sarracenia minor* Walter | Orgel's Orchids, Miami, FL (WS) | no voucher | L42192/L42206 | — |
| *Sarracenia oreophila* (Kearney) Wherry | Royal Botanic Gardens, Kew (K) | 539-87.0381C Case | L42193/L42207 | — |
| *Sarracenia psittacina* Michx. | Orgel's Orchids, Miami, FL (WS) | Hufford *709* | L42194/L42208 | — |
| *Sarracenia purpurea* L. | North Carolina, cultivated (WS) | Xiang *252* | L42195/L42209 | — |
| *Sarracenia rubra* Walter | Royal Botanic Gardens, Kew (K) | 276-85.03251 | L42196/L42210 | — |

The single-stranded DNA's were sequenced using the dideoxy chain termination method (Sanger et al. 1977) with the use of the Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, OH) and $^{35}$S-dATP. Single-stranded reactions were first carried out without the addition of manganese. In some instances, a second sequencing reaction was performed using manganese to increase the yield of short fragments (U.S. Biochemical). The ITS1 and ITS4 primers (White et al. 1990) were used, respectively, to sequence the ITS-1 and the ITS-2 regions. The sequence fragments were separated in 6.0% polyacrylamide gels (0.4 mm thickness; 1X TBE buffer). The gels were fixed in 10% acetic acid for 20 minutes, washed in distilled water, and allowed to air dry. They were then used to expose Kodak X-Omat AR film for 24-36 hr.

*Darlingtonia* was resequenced for *rbcL* for the reasons noted above. The amplification and sequencing of *rbcL* for *Darlingtonia* followed methods we have used previously (e.g., Morgan and Soltis 1993).

***Sequence Analysis and Phylogenetic Reconstruction.*** The *rbcL* sequences were aligned visually, whereas the ITS sequences were initially aligned using Clustal V (Higgins et al. 1992; Thompson et al. 1994; vers. 1.4), and the alignments were subsequently refined manually (Swofford and Olsen 1990). Several divergence weights were explored for the ITS sequence alignment (Delay Divergence Option of Clustal V) including 20%, 40%, 60% (the default), and 80%. Additionally, several combinations of the gap opening penalty (GOP) and gap extension penalty (GEP) were investigated. GOPs of 10 (the default) and 100 were explored in all permutations with GEPs of 5 (the default) and 10. The alignment of the ITS sequences necessitated inference of several insertions and deletions.

The proportion of nucleotide differences among

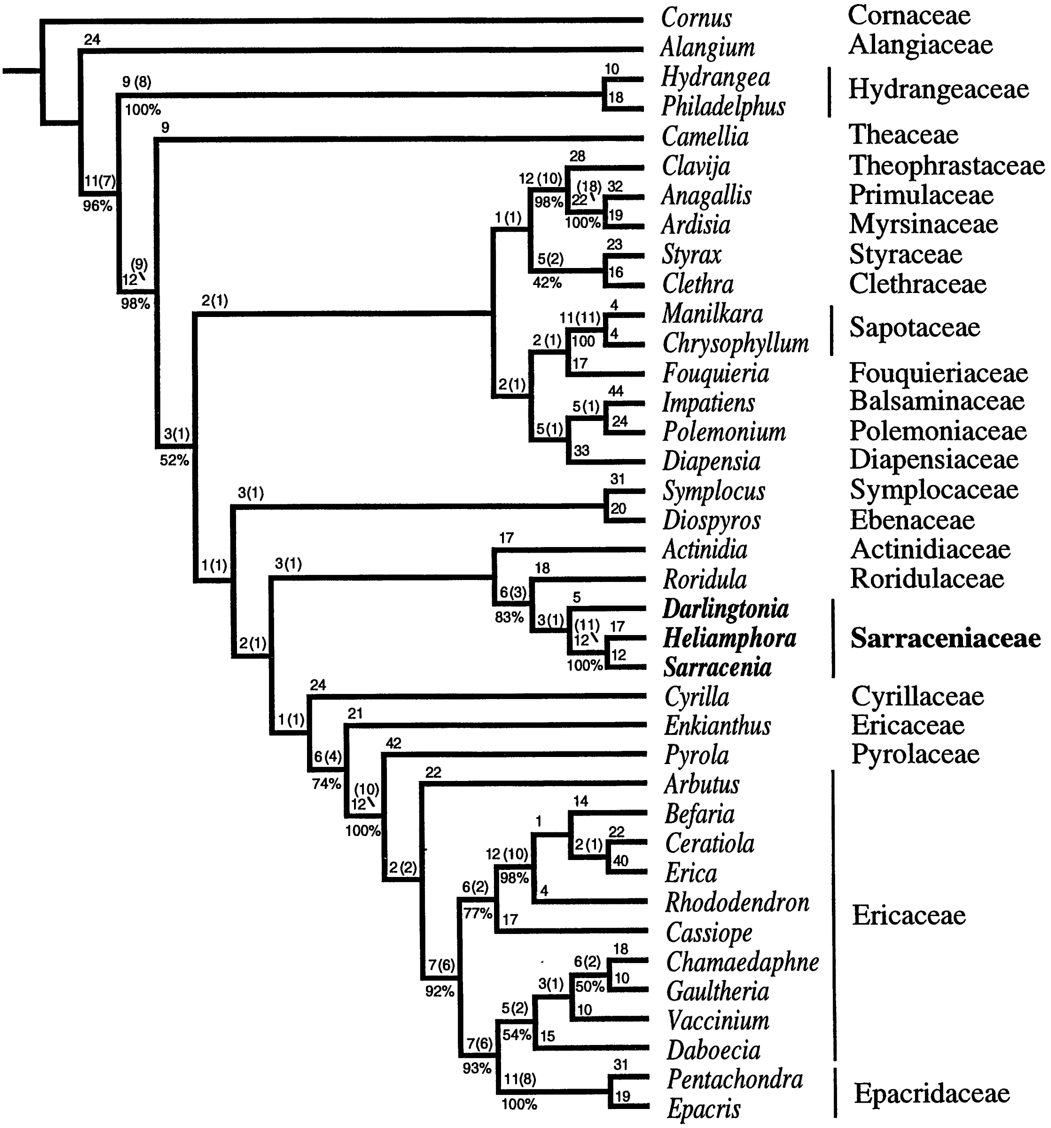

FIG. 1. One of six equally most parsimonious trees resulting from phylogenetic analysis of *rbcL* sequence data. The most equally parsimonious trees have a length of 899 (consistency index = 0.43). The tree gives the unambiguous base pair changes above the branches, decay index values in parentheses above the branch, and bootstrap values as percentages below each branch.

the taxa was calculated using "Show Distance Matrix" option of PAUP. A total of 142 phylogenetically informative base pairs of ITS-1 and ITS-2 was used in the analysis of the 14 taxa, whereas 37 phylogenetically informative sites from the *rbcL* sequences were used in the generic level analysis. ITS sequences for all taxa (Appendix 1) and the *rbcL* sequence for *Darlingtonia* have been submitted to GenBank (see Table 1 for accession numbers). Invariant sites and strictly autapomorphic base changes were treated as missing in the phylogenetic reconstructions ("ignore uninformative characters" option). Insertion/deletion events (indels) were ignored in the analysis, but were later mapped on the cladograms to assess their phylogenetic utility (Fig. 2).

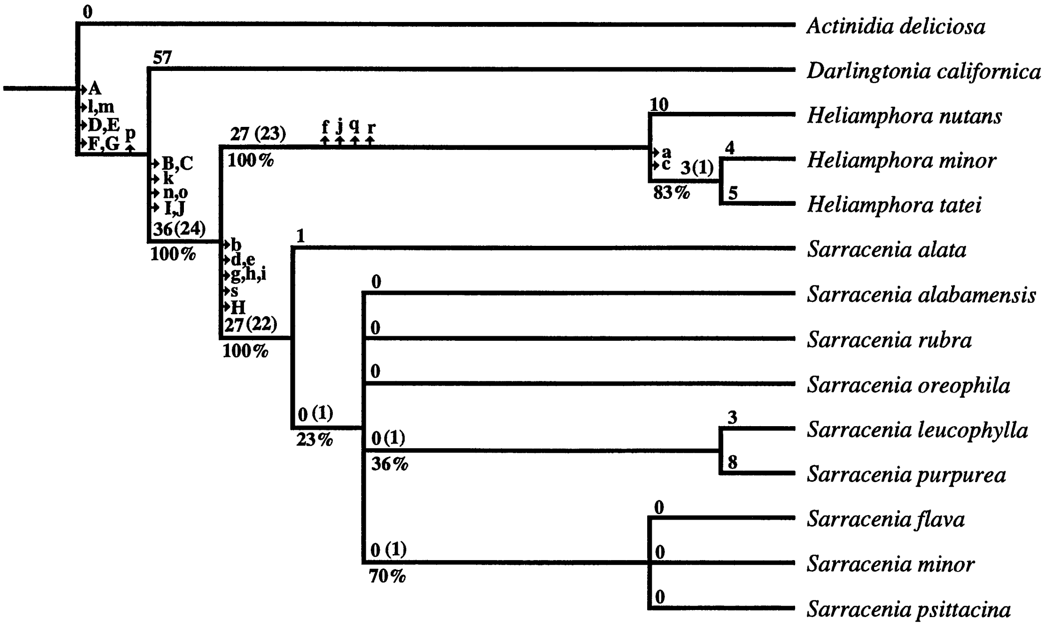

FIG. 2. Strict consensus tree of species of Sarraceniaceae and *Actinidia* (Actinidiaceae) based on ITS sequences. Thirteen equally most parsimonious trees of length 251 (consistency index = 0.789) were used in the construction of the tree. Unambiguous base pair changes are indicated above the branches. Decay index values are given in parentheses above the branch, whereas bootstrap values are given as percentages below each branch. Phylogenetically informative indels are indicated above or to the right of branches where they occur where upper case letters indicate base pair insertions and lower case letters show deletions relative to the sequence of *Actinidia*.

Sequence data were analyzed using PAUP version 3.1.1 (Swofford 1991). Phylogenetic reconstruction was performed on unweighted characters by a branch-and-bound search with simple addition of taxa. Strict consensus trees (Margush and McMorris 1981) were constructed for the set of equally most parsimonious cladograms in both *rbcL* and ITS analyses. MacClade (version 3.0; Maddison and Maddison 1992) was used to examine the distribution of molecular and morphological attributes on the cladogram topologies.

Bootstrap (Felsenstein 1985) and decay (Bremer 1988; Donoghue et al. 1992) analyses were used to estimate the robustness of clades. The bootstrap analysis employed 1000 replicates of branch-and-bound searching for ITS and 100 replicates of heuristic (SIMPLE addition sequence) searching for *rbcL*. Heuristic searches employing 100 replicates of random taxon addition were conducted on the *rbcL* data to search for islands of other most parsimonious trees (Maddison 1991). Decay analyses were performed using the converse constraint method of Baum et al. (1994).

## RESULTS

**rbcL.** The *rbcL* sequence of *Darlingtonia* was 1428 bp in length and confirmed the earlier sequence of Albert et al. (1992). The proportion of nucleotide differences between pairs of genera of Sarraceniaceae ranged from 2.4% to 2.7%. Between genera of Sarraceniaceae and the outgroup genera the values ranged from 4.4% to 7.2%. Two hundred fifty-four sites (17.8% of the gene sequence) from *rbcL* have the potential to provide phylogenetic information.

***ITS Region.*** The length of the ITS-1 region in Sarraceniaceae ranged from 240 to 249 bp. The proportion of nucleotide differences ranged from 51.3–61.7% between species of Sarraceniaceae and *Actinidia*; from 25.5–54.4% between species of different genera of Sarraceniaceae; and from 0.5–3.8% between pairs of species of *Sarracenia*. ITS-2 is shorter than ITS-1 in Sarraceniaceae, ranging from 218 to 232 bp. For ITS-2, divergence values ranged from 39.3–46.5% between species of Sarraceniaceae and *Actinidia*; from 15.6–44.3% between pairs of ingroup genera; and from 0.4–7.4% between pairs of species within *Sarracenia.* In the entire ITS region, the divergence ranged from 47.0–49.3% between Sarraceniaceae and *Actinidia;* from 20.3–46.9% between pairs of ingroup genera; and from 0.5–5.7% between pairs of species of *Sarracenia.* In the combined ITS-1 and ITS-2 regions, 142 sites (29.4% of the sequence length) potentially provide phylogenetic information. The remaining sites (70.6%) are either invariant or are strictly autapomorphic.

Numerous indels, ranging in length from one to 10 bp, were needed to align both ITS-1 and ITS-2 sequences, particularly at the generic level. Many of the indels support the phylogenetic relationships based on nucleotide substitutions alone.

***Phylogenetic Reconstructions.*** Both the ITS- and *rbcL*-based phylogenies (Figs. 1, 2) show similar phylogenetic relationships among the genera of Sarraceniaceae. The phylogenetic analysis of the *rbcL* sequence data yielded six equally parsimonious trees of 899 steps (consistency index = 0.43; retention index = 0.57; Fig. 1). All trees indicate that Sarraceniaceae are monophyletic and place *Darlingtonia* as the sister to the *Heliamphora-Sarracenia* clade. The sister group relationship of *Heliamphora* and *Sarracenia* is well supported in both gene trees, with a bootstrap value of 100% and a decay index of 11. The Sarraceniaceae-*Roridula* clade occurred in 83% of the bootstrap replicates but decayed in trees 3 steps longer than the most parsimonious.

All ITS alignments produced by Clustal V yielded trees with similar topologies. The generic relationships in trees derived from the various alignments were identical. The differences among the trees were all within *Sarracenia,* where *S. rubra* Walt. and *S. oreophila* (Kearney) Wherry were sister taxa in a few, but not all, trees. The alignment using standard defaults (Divergence = 40%; GOP = 10; GEP = 5) was chosen for presentation here because it was representative of the majority of the alignments. Thirteen equally parsimonious trees of 251 steps (consistency index = 0.789; consensus cladogram in Fig. 2) were found in the analysis of the combined ITS-1 and ITS-2 sequences. There is strong support for the monophyly of both *Sarracenia* (27 base pair synapomorphies and 8 indels; bootstrap value of 100% and a decay index of 22) and *Heliamphora* (27 synapomorphies and 4 indels; bootstrap value of 100% and a decay index of 23). The ITS results demonstrate strong support for the monophyly of *Heliamphora* plus *Sarracenia* (Fig. 2). This clade is supported by 36 synapomorphies and seven indels. The clade occurred in all bootstrap replicates and occurred in all less parsimonious cladogram topologies up to 24 steps longer than the most parsimonious trees. *Heliamphora* and *Sarracenia* are consistently placed as the sister group of *Darlingtonia.* In *Heliamphora, H. tatei* Gleason and *H. minor* Gleason are sister taxa (bootstrap value of 83% and decay index of 1). In contrast, there is little resolution of relationships among species of *Sarracenia. Sarracenia alata* (Wood) Wood was placed as the sister group of all other species in the genus with weak support. The equally parsimonious trees differ in the placement of groups within the large subclade of *Sarracenia* that is the sister group of *S. alata.* In this large subclade are two monophyletic groups:—1) *S. purpurea* and *S. leucophylla* Raf.,—and—2) *S. flava, S. minor* Walt., and *S. psittacina* Michx. The *S. purpurea-S. leucophylla* clade has a low bootstrap value (36%) and decays in trees one step longer than the most parsimonious trees. The monophyly of *S. minor, S. flava,* and *S. psittacina* has only moderate support (bootstrap value of 70% and decay index of 1).

## DISCUSSION

***Familial Circumscription.*** All of the six most parsimonious trees that resulted from analysis of the *rbcL* data placed *Roridula* as the sister to a clade containing the three genera of Sarraceniaceae, *Darlingtonia, Heliamphora* and *Sarracenia* (Fig. 1). The ascidiate leaves, rhizomatous shoot architecture, nodding flowers, and polystaminate androecia of *Darlingtonia, Heliamphora,* and *Sarracenia* provide morphological synapomorphies that support monophyly of the Sarraceniaceae as traditionally circumscribed (Uphof 1936). We suggest that the placement of *Roridula* relative to Sarraceniaceae and other Ericales, a relationship that was also demonstrated using morphological data by Conran and Dowd (1993), be examined more extensively with sequence data from other genes.

***Generic Relationships.*** Both chloroplast (*rbcL*) and nuclear (ITS) data sets indicate the same relationships among the genera of Sarraceniaceae. Trees from both gene sequences place *Darlingtonia* as the sister of a strongly supported *Heliamphora-Sarracenia* clade (Figs. 1, 2). *Sarracenia* and *Heliamphora* are each well-defined, monophyletic groups supported by a large number of synapomorphies in the ITS trees (Fig. 2). The placement of *Darlingtonia* as the sister group of *Sarracenia* plus *Heliamphora* is consistent with the relationships proposed by Albert et al. (1992), but contrasts with the common perception that *Sarracenia* and *Darlingtonia* are more closely related to each other than either is to *Heliamphora*. *Heliamphora* has often been perceived as either the basal member of the family or the sister group to a lineage consisting of *Sarracenia* and *Darlingtonia* (Macfarlane 1893; Maguire 1978; Juniper et al. 1989).

The pattern of generic relationships indicated by both chloroplast and nuclear sequences requires that we address previous assumptions about historical biogeography in the family. Sarraceniaceae only occur in the New World. It has been assumed that the earliest members of the family had a South American distribution and that a northward dispersal was associated with the origin of derived members of the group (Maguire 1970, 1978; Juniper et al. 1989). Maguire (1970), for example, suggested that *Sarracenia* might have come to Florida from South America via the Antillean Arc. This south to north hypothesis rests on the assumption that *Heliamphora* is more primitive than *Darlingtonia* and *Sarracenia*. For example, leaves of *Heliamphora* are less complex than those of *Darlingtonia* and *Sarracenia*, and this has been used to support an hypothesis of primitiveness for the former genus (Macfarlane 1893; Markgraf 1955). A south to north evolution in the family may also be consistent with the hypothesis that *Roridula*, a southern African taxon placed as the sister group of Sarraceniaceae by Albert et al. (1992), Chase et al. (1993), and this paper, shares a common ancestor in the southern hemisphere with Sarraceniaceae. Maguire (1970) suggested that Guayana Highland groups have affinities with various floras, including that of southern Africa. In addition to the possible linkage of the South African *Roridula* with *Heliamphora*, Maguire (1970) suggested that Guayana Highland Rutaceae and *Ledothamnus* Meissner of Ericaceae may have their closest relatives in southern Africa.

The placement of *Darlingtonia* as the sister genus of *Heliamphora* and *Sarracenia* requires that we consider alternatives to the south to north dispersal of Sarraceniaceae. Although this phylogenetic pattern is logically consistent with the hypothesis that the common ancestor of Sarraceniaceae and *Roridula* had a southern hemisphere distribution (Fig. 3), it is equally possible that the family originated in North America. Croizat (1960) previously suggested that Sarraceniaceae may have originated in western North America. Some support for this hypothesis lies in the presence of Ericaceae, currently considered to be a reasonable sister group to the *Roridula*-Sarraceniaceae clade (Fig. 1), in North America. Actinidiaceae also have a historical record in North America, although they are now limited in distribution (Gilg and Werdermann 1925) to tropical and subtropical Asia and tropical South America (only *Saurauia* Willd.). Fossil seeds of *Actinidia* have been described from the Eocene of Oregon (Manchester 1994) and are most similar to those of *Heliamphora* (Hufford and Bayer, pers. obs.). Fossil flowers similar to those of *Saurauia* have been described from Late Cretaceous of Georgia (Keller et al. 1996).

The origin of the current distribution of Sarraceniaceae is equivocal. There is no fossil evidence for the hypothesis that the current distribution results from fragmentation of an ancestral group with a range covering all or nearly all that of the extant members. Sarraceniaceae are restricted to moist (generally standing water), nutrient-poor areas, and extant *Darlingtonia* and some *Heliamphora* appear to have edaphic limitations that also argue against extremely widespread distributions. Hence, any previous widespread distribution that is more continuous than that displayed by the extant genera must have been patchy. Contrary to some previous authors who have considered Sarraceniaceae to have limited dispersability (e.g. Maguire 1978), we hypothesize that their small seeds (about 1.5 mm in length) may be readily dispersed by birds. *Darlingtonia* seeds in particular are notable for possessing a tail and trichomes (Hufford and Bayer, pers. obs.), features making them well suited for relatively long distance dispersal (Carlquist 1965).

No morphological characters unambiguously support the generic relationships, particularly the sister-taxon relationship of *Heliamphora* and *Sarracenia*, found using *rbcL* and ITS sequence data. In contrast, a few potentially apomorphic character states from morphological data support an hypothesis of monophyly for *Darlingtonia* and *Sarracenia* and their placement as the sister group of *Heliamphora* (Hufford unpubl.), including one wing on the ventral side of the ascidiate leaf, a tightly rolled lip at the opening of the ascidiate leaf, and an inflorescence of a solitary flower.

Some have suggested previously that certain character states suggest that *Heliamphora* is primitive relative to *Darlingtonia* and *Sarracenia*. Character states, including ascidiate leaves with two ventral wings (Macfarlane 1889), long vessel elements and tracheids (DeBuhr 1977), a high number of bars on perforation plates of vessel elements

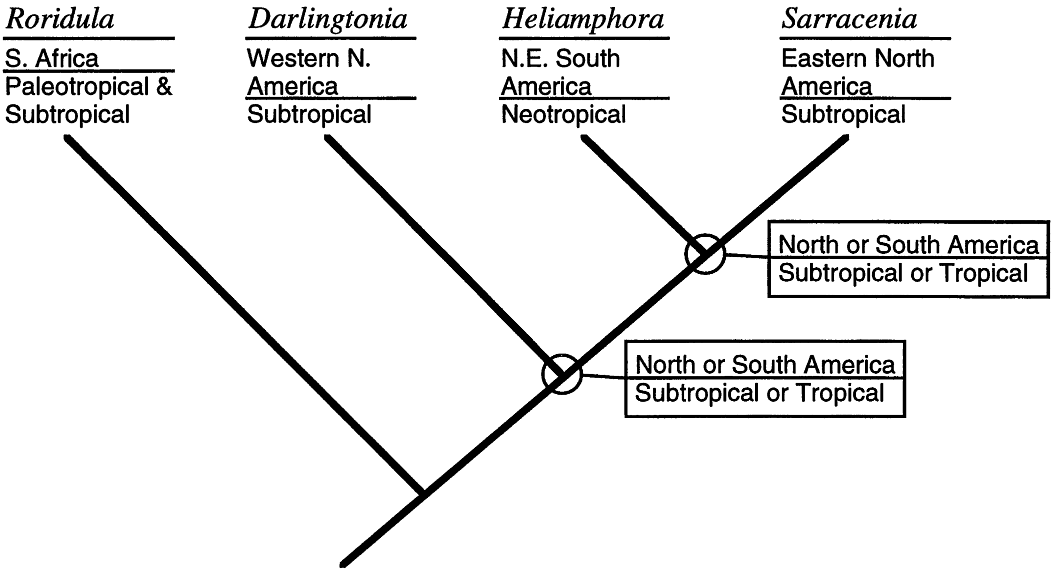

FIG. 3. Cladogram showing generic relationships of Sarraceniaceae and *Roridula* based on one of the equally most parsimonious trees from the *rbcL* analysis. General geographic and latitudinal distributions are indicated for each of the genera. The hypothesis shows that each of the interior nodes of the Sarraceniaceae cladogram must be considered to have either a tropical or subtropical and either a North American or South American origin. These results may indicate that if Sarraceniaceae originated in the North American subtropics, then *Heliamphora* may have originated via long distance dispersal or migration. In contrast, if Sarraceniaceae originated in the Neotropics, then two long distance dispersal or migration events would be necessary to account for the origin of the two North American genera.

(DeBuhr 1977), and a low number of apertures on pollen grains (Thanikaimoni and Vasanthy 1972), have been used to identify the primitiveness of *Heliamphora*. It is unrealistic, however, to attempt to use any one syndrome of characters to suggest the primitiveness of a particular taxon. To ascertain the phylogenetic informativeness of character states, we need to compare their distribution among Sarraceniaceae to that among outgroups. Three outgroups of Sarraceniaceae, Actinidiaceae, Ericaceae, and *Roridula* (Fig. 1), lack ascidiate leaves. Hence, the two wings on *Heliamphora* leaves compared to the single wing on *Darlingtonia* and *Sarracenia* leaves cannot be polarized. Wood anatomy is also ambiguous because variation in both Ericaceae and *Roridula* is similar to that found among *Heliamphora, Darlingtonia,* and *Sarracenia.* Most members of outgroups of Sarraceniaceae have triaperturate pollen (some Ericaceae have more than three apertures), and this character state may only indicate that *Heliamphora* and *Darlingtonia* share the plesiomorphy of having fewer apertures than *Sarracenia.*

All three genera of Sarraceniaceae have floral features that are derived or specialized relative to the outgroups, although Renner (1989) suggested that *Darlingtonia* has the least specialized floral morphology in the family. For example, *Darlingtonia* has petaloid sepals, *Sarracenia* has an umbricullate style, and *Heliamphora* has a unicyclic perianth, reflexed stamens, and a restricted pattern of anther dehiscence. The floral specializations of both *Sarracenia* and *Heliamphora* may be tied to particular pollination strategies. The umbricullate style of *Sarracenia* may have a role in secondary pollen presentation (Mandossian 1965; Schnell 1983) and the androecial features of *Heliamphora* with buzz pollination (Renner 1989). The floral morphological "specializations" of *Heliamphora* for porate anther dehiscence and buzz pollination, however, may be plesiomorphies because they are shared with *Roridula* (Marloth 1903; Vogel 1978) and also occur in the related Actinidiaceae (Vijayaraghavan 1965) and Ericaceae (Matthews and Knox 1926).

**Heliamphora.** Relatively few specimens of *Heliamphora* have been available for systematic study. Maguire's (1978) review of *Heliamphora* recognized six species, although he noted that there were no clear-cut distinctions among species and he emphasized "pitcher" characteristics in his circumscriptions. Steyermark (1984) accepted only five species, merging *H. nebulinae* Maguire into *H. tatei*. Because of limited material, we sampled only three of the species recognized by Steyermark (1984). Our sampling does include *H. tatei*, the only dendroid member of the genus, and covers the key geographic distinction between eastern Guayana and Amazonian Guayana recognized by Maguire (1978). The ITS sequence analysis (Fig. 2) indicates that *H. nutans* is the sister group of *H. minor* and *H. tatei*. *Heliamphora nutans* and *H. minor* both occur on the tepuí complexes of the Gran Sabana region in eastern Guayana, whereas *H. tatei* is found on the adjacent Duida and Huachamarcari mesas over 500 km to the west (Maguire 1978). The more geographically restricted Amazonian *H. tatei* may be more recently derived than *H. nutans*, *H. minor*, and their relatives, all of which are more widespread in eastern Guayana. Our results also suggest that the dendroid form of *H. tatei* is derived within the genus, contrary to Maguire's (1978) assumption that this architecture indicates the primitiveness of *Heliamphora* among Sarraceniaceae.

**Sarracenia.** Our results provide little resolution of relationships among species of *Sarracenia* (Fig. 2). Two factors may be especially important in this lack of resolution. First, sequences of ITS-1 and ITS-2 may not evolve at a rate that is sufficient to be phylogenetically informative for *Sarracenia*. For example, our data failed to resolve an expected relationship between *S. rubra* and *S. alabamensis* (Case and Case 1974, 1976; Romeo et al. 1977; Schnell 1977). Second, interspecific hybridization, particularly introgression, may have led to incorporation of both parental ITS sequences in an individual followed by concerted evolution in subsequent generations toward one parent without the accompanying morphological evidence that such hybridization has occurred. The identification of our samples based on morphological attributes using MacDaniel's (1971) circumscriptions does not preclude the possibility of introgressed genomes in the materials used to extract nuclear DNA. This could have produced spurious results and even a lack of resolution in our cladograms, as discussed in a recent review of the effect of hybridization on phylogenetic analysis (McDade 1995). For example, the placement of *S. flava* on the branch including *S. minor* and *S. psittacina* is unexpected and may be a consequence of interspecific hybridization. Bell (1949) and McDaniel (1971) have reported natural hybrids between *S. flava* and *S. minor*. The ITS data did not reveal an expected relationship between *S. flava* and *S. oreophila*. These two species have similar leaf and floral forms and have been hypothesized to share a derived musty or feline floral scent (Wherry 1933; Schnell 1980). To resolve the relationships among species of *Sarracenia*, we suggest that a DNA sequence that evolves more rapidly than ITS be used for phylogeny reconstruction and that sampling utilize multiple natural populations of each species in areas where they occur in isolation from other species of *Sarracenia*.

The placement of *Sarracenia leucophylla* and *S. purpurea* as sister species in our results is consistent with their position in a phenetic analysis of the genus by Schnell and Krider (1976) that clustered these two species. These two species and *S. alata* have scalloped margins on the hoods of their ascidiate leaves in contrast to the other species in which the hoods are entire. *Sarracenia leucophylla* and *S. purpurea* share uniquely the presence of presumed anthocyanin pigments demonstrated in a chromatographic study (Schnell 1978).

In conclusion, phylogenetic analysis of sequences of both the chloroplast gene *rbcL* and nuclear ITS-1 and ITS-2 resulted in most parsimonious cladograms in which *Heliamphora* and *Sarracenia* formed a monophyletic group that was the sister group of *Darlingtonia*. Although *Roridula* and Sarraceniaceae share carnivory, the leaf modifications that underlie insect capture differ in the two groups (Juniper et al. 1989). A sister group relationship of *Roridula* and Sarraceniaceae has been demonstrated not only by the broad analysis of Chase et al. (1993) using *rbcL* gene sequences but also by Conran and Dowd (1993) using morphological data (they placed *Roridula* in the broad Rosidae data matrix of Hufford (1992). A next significant step, using the results of such studies, will be the analysis of morphological character state transitions associated with the origin of carnivory and the divergent leaf forms that facilitate it in the *Roridula*-Sarraceniaceae clade.

The results of the ITS sequence analysis provide limited resolution of the relationships among species of *Sarracenia*. Some patterns of relationship that were found are counter to expectations based on data and previous hypotheses in the literature. To resolve relationships in *Sarracenia*, we advocate use of DNA sequences that evolve more rapidly than ITS. Additionally, because hybridization is known between species of *Sarracenia*, we suggest that sampling utilize multiple natural populations of each species. The populations of each species should be sampled in areas where particular species occur in isolation from others in the genus.

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APPENDIX 1. The ITS-1 and ITS-2 data matrix. The sites are numbered sequentially from the beginning of the ITS-1 region and ending at the end of the ITS-2 region. Dots indicate that the nucleotide is the same as that given for *Actinidia*, dashes are deleted base pairs, and question marks are missing or ambiguous data.

| | \|ITS-1><br>1 50 | 51 100 |
|---|---|---|
| *Actinidia deliciosa* | ?????CCCTG CAAAGCAGAACGACCCGTGAACTTGTC--TAATATTCTT? | ?GGAAGAAAGT--TTGG--TTTT-----TATAACCCTACTCTTTCTTCCC |
| *Darlingtonia californica* | CGAAA ..-...C........T....?.C.CGGC-.---A.-.?.CCT.AC | ACA.?.TGT..TT..TAATA.AAATCTA.T--?.TT.GGCTA??GC???A |
| *Heliamphora minor* | ?????....A .G..............C....A...TT--....CCA..G | G.....T.G.GGG....--CC..------.TGG-.T..TGCT??GGCC..A |
| *Heliamphora nutans* | ?????....A ..........T...........A...TTT.....CCA..G | G.....T.G.GGG....--.CG.------.TGGC.T..CGCTA..GCC..A |
| *Heliamphora tatei* | ?????....A ..........T...........A...TT--....CCA..G | G.....T.G.GGG....--CC..------.TG--.T..TGCTA.GGCC..A |
| *Sarracenia alabamensis* | ????? ......?....................A...TTA.--..CCA..G | G..G..T.GC.TG..C.CC.C..CCC-A.T-GC.T.ATAC.GAGGG.GTG |
| *Sarracenia alata* | ?????..... .C....................A...TTA.--..CCA..G | G..G..T.GC.TG....CC.C..CCC-A.T-GC.T.ATAC.GAGGG.GTG |
| *Sarracenia flava* | ?????...... .....................A...TTA.--..CCA..A | G..G..T.GC.TG..?.CC.C..CCC-A.T-GC.T.ATAC.GAGGG.G.G |
| *Sarracenia leucophylla* | ?????. .....C....................A...TTA.--..CCA..G | G..G..T.GC.TG..?.CC.C..CCC-A.T-GC.T.ATAC.GAGGG.GTG |
| *Sarracenia minor* | ?????...... C....................A...TTA.--..CCA..G | G..G..T.GC.TG..C.CC.C..CCC-A..-GT.T.ATAC.GAGGG.G.A |
| *Sarracenia oreophila* | ?????? ???..?...A................A...TTA.--..CCA..G | G..G..T.GC.TG..?.CC.C..CCC-A.T-GC.T.ATAC.GAGGG.GTG |
| *Sarracenia psittacina* | ?????. ..........................A...TTA.--..CCA..G | G..G..T.GC.TG....CC.C..CCC-A.T-GC.T.ATAC.GAGGG.G.G |
| *Sarracenia purpurea* | ?????....... ....................A...TTA.--..CCA..G | G..G..T.GC.TG..C.CC.C..CCC-A.T-GC.T.ATGC.GAGGG.GTG |
| *Sarracenia rubra* | ?????....... ....................A...TTA.--..CCA..G | G..G..T.GC.TG..C.CC.C..CCC-A.T-GC.T.ATAC.GAGGG.GTG |

APPENDIX 1. Continued.

| | 101 150 | 151 200 |
|---|---|---|
| *Actinidia deliciosa* | TTT?CCGGGTGTGCTCGTGTTGCCCTCTGGTGACACTCTCATTCCCCGGT | CAAACAACGAA?CCCGGCGCAAAACGCGTCAAGGAACTTGAACAATGC?A |
| *Darlingtonia californica* | .GCTAG..AA?G?.???GAA?C...GTGA..TTA..AAATGAA...A?.C | GCG.ACTGCGTAA--C.GAA??--TT--G-C.ACC.AGAACTGC.-CAG- |
| *Heliamphora minor* | .GCCGG.....GCT.T.GAA.C..T.TG.ACCCGTAGTCATCCTT.G.TC | G.T.A.TGA.--...C.G-?CG.G?TGCGTC.A.G.AC.AG.AC.A.AG. |
| *Heliamphora nutans* | .GCCGG.....CCT.T.GAA.C..T.TG.ACCCGTAGTCATCCTT.G.T. | G.T.A.TGA.--...C.GCGTGCGTTGCGTC.A.G.AC.AG.AC.A.AG. |
| *Heliamphora tatei* | .GCCGG.....CCT.T.GAA.C..T.TG.ACCCGTAGTCATCCTT.G.TC | G.T.A.TGA.--...C.GC?CG.GTTGCGTC.A.G.AC.AG.AC.A.AG. |
| *Sarracenia alabamensis* | ..C---..A.CCCT.T.GACAT.GTA..CA.CCTTTGG.TGA-------- | --T.A.T.A..A...-.GCGCG.TTTGTG.C.A.G.AC..G.AC.A.AG. |
| *Sarracenia alata* | ..C---..A.CCCT.T.GACAT.GTA..CA.CCTTTGG.TGA-------- | --T.A.T.A..A...-.GCGCG.TTTGTG.C.A.G.AC..G.AC.A.AG. |
| *Sarracenia flava* | ..C---..A.CCCT.T.GACAT.GTA..CA.CCTTTGG.TGA-------- | --T.A.T.A..A...-.GCACG.TTTGTG.C.A.G.AC..G.AC.A.AG. |
| *Sarracenia leucophylla* | ..C---..A.CCCT.T.GACAT.GTA..CA.CCTTTGG.TGA-------- | --T.A.T.A..A...-.GCGCG.TTTGTG.C.A.G.AC..G.AC.A.AG. |
| *Sarracenia minor* | ..C---..A.CCCT.T.GACAT.GTA..CA.CCTTTG?.TGA-------- | --T.A.T.A..A...-.GCACG.TTTGTG.C.A.G.AC..G.AC.A.AG. |
| *Sarracenia oreophila* | | --T.A.T.A..A...-.GCACG.TTTGTG.C.A.G.AC..G.AC.A.AG. |
| *Sarracenia psittacina* | ..C---..A.CCCT.T.G.CAT.GTA..CA.CCTTTGG.TGA-------- | --T.A.T.A..A...-.G?GC?.TTTGTG.C.A.G.AC..G.AC.A.AG. |
| *Sarracenia purpurea* | ..C---..A.CCCT.T.GACAT.GTA..CA.CCTTTGG.TGA-------- | --T.A.T.A..A...-.GC?C?.TTTGTG.C.A.G.AC..G.AC.A.AG. |
| *Sarracenia rubra* | ..C---..A.CCCT.T.GACAT.GTA..CA.CCTTTGG.TGA-------- | --T.A.T.A..A...-.GCACG.TTTGTG.C.A.G.AC..G.AC.A.AG. |

APPENDIX 1. Continued.

| | <ITS-1 \| ITS-2><br>201 250 | 251 300 |
|---|---|---|
| *Actinidia deliciosa* | ACATCCATGC????GTTTTTGG?T?CTTGTGGTGCTTGCTCT ATTGTGT- | -TG---CCCACCCAAC-TCAAGTCTTACCAAGGATT?GGTGTGGGTGGG? |
| *Darlingtonia californica* | ....ATCG..CGCGC..C.ACCGGTTC.?????????????? .......- | -GT---...G......C.TCTCC..ATAA....G.AG.TGTGC......A |
| *Heliamphora minor* | G..CA.CGAT?CCTTG.C--------.ACA....T.G.GGG. ...T...C | ACCCTA..TTATTGC.TCACCACAG.GTAGGT..GCGATG.GT..-..CG |
| *Heliamphora nutans* | G.GCA.CGATGCCTTGCC----TGTT.ACA....T.?????? ...T...T | GCCCTA..TTATTGC.TCACCACAG.GTAGGT..GCGATG.GT..-..CG |
| *Heliamphora tatei* | G..CA.CGATGCCTTGCC----TGT..ACA....T.?????? ...T...C | ACCCTA..TTATTGC.TCACCACAG.GTAGGT..GCAATG.GT-.-..CG |
| *Sarracenia alabamensis* | G--CA.-GATCATGTCCAAGCATGTT.ACAT..TT.G.GGTG .......C | GCTATT....ATTGC.CCAC?TAAGAGT.G.T..GCAATG.GTAA-..CG |
| *Sarracenia alata* | G--CA.-GATCATGTCCAAGCATGTT.ACAT..TT.G.GGG. .......C | GCTATT....ATTGC.CCACTTAAGAGT.GGT..GCAATG.GTAA-..CG |
| *Sarracenia flava* | G--CA.-GATCATGTCCAAGCATGTT.ACAT...T.G.G??? .......C | GCTATT....ATTGC.CCACCTAAGAGT.GGT..GCAATG.GAAA-..CG |
| *Sarracenia leucophylla* | G--CA.-GATCATGTCCAAGCATGTT.ACAT.CTT.G.GG?? .......C | CGTATT....ATTGC.CCACCTAAGAGT.GGT..GCAATG.GCAA-..CG |
| *Sarracenia minor* | G--CA.-GATCATGTCCAAGCATGTT.ACAT..TT.G.GGTG .......C | GCTATT....ATTGC.CCACCTAAGAGT.GGT..GCAATG.GAAA-..CG |
| *Sarracenia oreophila* | G--CA.-GATCATGTCCAAGCATGTT.ACAT...T.G.G??? .......C | GCTATT....ATTGC.CCACTTAAGAGT.G-T..GCAATG.GCAA-..CG |
| *Sarracenia psittacina* | G--CA.-GATCATGTCCAAGCATGTT.ACAT..TT.G.G??? .......C | GCTATT....ATTGC.CCACCTAAGAGT.GGT..GCAATG.GAAA-..CG |
| *Sarracenia purpurea* | G--CA.-GATCATGTCCAAGCATGTT-ACAT...T.G.GGGG .......C | CGTATT....ATT.C.CCACCTAA.AGT.GGT..G.AATGTGTAA-..CG |
| *Sarracenia rubra* | G--CA.-GATCATGTCCAAGCATGTT.ACAT..TT.G.GGTG .......C | GCTATT....ATTGC.CCACTTAAGAGT.G-T..GCAATG.GCAA-..CG |

APPENDIX 1. Continued.

| | 301 350 | 351 400 |
|---|---|---|
| *Actinidia deliciosa* | GATATTGGCCCCCCGTGCACATTAGTGAACGGTCGGCC?AAAAATAAAGT | CCTTAGCAATGA-CGTCACAACAAGTGGTGGTTGACAAACACGTTGACA- |
| *Darlingtonia californica* | ...G.......T.....TG..C.C..T......T....T...T.AG.-.. | T..CGA.G.CAGA....G.G..............GT...G--CG.TG..T |
| *Heliamphora minor* | ......A....T......G..C.T..C......T....T......G.-.. | T...GA.G.CACA...T.TG.T..................--....C-.T |
| *Heliamphora nutans* | ......A....T......G..C.T..C......T....T......G.-.. | TG..GA.G.CACG...T..G.T..................--....C-.T |
| *Heliamphora tatei* | ......A...AT......G..C.T..C......T....T......G.-.. | T...GA.G.CACA...T.TG.T...C......C.......--....C-.T |
| *Sarracenia alabamensis* | T..........T......G..C.C..C......T....?......G.-.. | T...GA.G.CACA......G.T...........A......--....C-.T |
| *Sarracenia alata* | T..........T......G..C.C..C......T....T......G.-.. | T...GA.G.CACA......G.T...........A......--....C-.T |
| *Sarracenia flava* | T..........T......GT.C.C..C......T..???......G.-.. | T...GA.G.CACA......G.T...........A......--....C-.T |
| *Sarracenia leucophylla* | ...........T......G..C.C..C......T....T......G.-.. | T...GA.G.CACA......G.T...........A......--....C-.T |
| *Sarracenia minor* | T..........T......GA.C.C..C......T....T......G.-.. | T...GA.G.CACA......G.T...........A.....T--.....-.T |
| *Sarracenia oreophila* | T..........T......G..C.C..C......T....T......G.-.. | T...GA.G.CACA......G.T...........A......--....C-.T |
| *Sarracenia psittacina* | T..........T......GT.C.C..C......T....C......G.-.. | T...GA.G.CACA......G.T...........A......--....C-.T |
| *Sarracenia purpurea* | T..........T......G..C.C..C......T....T......G.-.. | T...GA.G.CACA......G.T...........A......--....C-.T |
| *Sarracenia rubra* | T..........T......G..C.C..C......T....T......G.-.. | T...GA.G.CACA......G.T...........A......--....C-.T |

APPENDIX 1. Continued.

| | <ITS-2\| | |
|---|---|---|
| | 401 450 | 451 483 |
| *Actinidia deliciosa* | -GAT--CC-TGTT-GTGCTTGCCCCCATTGCTAATGGTTTA--CTTTTGA | CCCTAATGTGCTGTTATCA-CGG--CTTCGATC |
| *Darlingtonia californica* | C...GT..G...GC..CAC..T.GGT.CG.A.T-G.C.CCT--T.GA.CC | TATAGCACCAT......GG--T.T-..-....A |
| *Heliamphora minor* | CCTGTCATGC..GCCCATG.CG..A..AG--.T-A.C.CAT--A.-..TG | A..C----------..AAG-.AATGTACT...T |
| *Heliamphora nutans* | CTTGTCATGC..GCCCATG.CG..A.GAG--GT-A.C.CAT--A.-..TG | ...C----------..AAG-.AATGTACT...T |
| *Heliamphora tatei* | CCTGTCATGC..GCCCATG.CG..A..AG--.C-A.C.CAT--A.-..TG | A..C----------..AAG-.AATGTACT...T |
| *Sarracenia alabamensis* | CCTGTCGTGC..GCCCATG.TG..A.GAG--.T-G.C.CAT--A.GA.T. | A....A...T...-GA.TA.AATGGAC....T |
| *Sarracenia alata* | CCTGTCGTGC..GCCCATG.TG..A.GAG--.T-G.C.CAT--A.GA.T. | A....A...T...-GA.TA.AATG.AC....T |
| *Sarracenia flava* | CCTATCGTGC..GCCCATG.TG..A.GAG--.T-G.C.CAT--A.GA.T. | A...G.A...T...-GA.TA.AATG.AC....T |
| *Sarracenia leucophylla* | CCTGTCGTGC..GCCCATG.TG..A.GAG--.T-G.C.CAT--A.GA.T. | A....A...T...-GA.TA.AATG.AC....T |
| *Sarracenia minor* | CCTATCGTGC..GCCCATG.TG..A.GAG--.T-G.C.?AT--A.GG.T. | A...G.A...T...-GA.TA.AATA.ACT...T |
| *Sarracenia oreophila* | CCTGTCGTGC..GCCCATG.TG..A.GAG--.T-G.C.CAT--A.GA.T. | A....A...T...-GA.TA.AATG.AC....T |
| *Sarracenia psittacina* | CCTATCGTGC..GCCCATG.TG..A.GAG--.T-G.C.CATAAA.GA.T. | A...G.A...T...-GA.TA.AATG.AC....T |
| *Sarracenia purpurea* | CCTATCGTGC..GCC.ATG.TG..ATGAG--.T-G.C.CAT--A.GA.T. | A..C..A...T...-GA.TA.AATG.AC....T |
| *Sarracenia rubra* | CCTGTCGTGC..GCCCATG.TG..A.GAG--.T-G.C.CAT--A.GA.T. | A....A...T...-GA.TA.AATG.AC....T |