

## The phylogenetic position of river-weeds (Podostemaceae): Insights from *rbcL* sequence data

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### Abstract

The systematic position of the river-weed family Podostemaceae remains enigmatic due to taxonomic difficulties imposed by the radically altered morphology of these alga-like angiosperms. Although previous workers have placed this group phylogenetically among a wide variety of monocotyledons and dicotyledons, most contemporary authors have proposed that river-weeds are closely related to members of the dicotyledonous order Rosales. A diversity of opinion also exists as to whether the Hydrostachyaceae are related to Podostemaceae. We have investigated the phylogeny of river-weeds by comparing DNA sequences of the chloroplast encoded *rbcL* gene for eight river-weed genera together with 84 other angiosperm and 11 non-flowering seed plant taxa. The high level of sequence divergence in *rbcL* that exists between river-weeds, Hydrostachyaceae and other angiosperms presents systematic problems that parallel those associated with the highly divergent morphology of these groups. Rooting *rbcL* sequences with distant non-flowering plant outgroups results in a topology where Podostemaceae comprise a basal angiosperm clade, but in which other renditions of angiosperm family relationships are depicted unreasonably. Restricting the comparison of river-weed sequences entirely with angiosperms places the group as a sister clade to the Hydrostachyaceae as some authors had anticipated, but this result is only weakly supported. The high level of both morphological and molecular divergence in the river-weed clade confounds efforts to correctly ascertain their phylogenetic relationships. A tentative hypothesis from *rbcL* data is that the Hydrostachyaceae and Podostemaceae are sister taxa whose closest relatives are the rosoid families Crassulaceae and Haloragaceae. © 1997 Elsevier Science B.V.

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## 1. Introduction

Phylogenetic relationships of aquatic angiosperms often are difficult to reconcile because adaptation to hydric habitats typically is accompanied by extensive structural reduction and modification that can obscure or even eliminate taxonomically useful characters. This problem is exacerbated in the 'river-weeds' (Podostemaceae) whose lotic river rapid habitats have selected for highly unusual and extreme structural modifications. Taxonomists have treated the order Podostemales Lindley as comprising either the single family Podostemaceae Rich. ex C. Agardh (e.g. Cronquist, 1981; Steenis, 1981; Cook, 1990) or two distinct families, Tristichaceae Willis and Podostemaceae (e.g. Willis, 1915, 1926; Cusset and Cusset, 1988a). The former (*sensu lato*) concept usually recognizes two subfamilies, Tristichoideae and Podostemoideae (Van Royen, 1951; Takhtajan, 1980; Thorne, 1992). Although most taxonomists have followed a *sensu lato* concept of Podostemaceae, recognition of Tristichaceae has merit (see discussion). The Podostemaceae (*s.l.*) have also been considered as close relatives of Hydrostachyaceae, because of various similarities and their unusual affinity for rheophytic habitats.

River-weed adaptation has resulted in an unorthodox morphological organization that authors have described as "alga-like", "fucoid", "lichen-like", "moss-like" or "webbed" (Graham and Wood, 1975; Cronquist, 1981; Van Steenis, 1981). Among the peculiarities of river-weeds are a 'thallus' and specialized adhesive 'haptera' that facilitate their attachment to rock substrates (Graham and Wood, 1975; Cronquist, 1981; Van Steenis, 1981; Jäger-Zürn, 1992; Rutishauser and Huber, 1995; Rutishauser, 1997). Some features of river-weeds are so extensively modified that they superficially resemble monocotyledon characteristics. The presence of silica bodies, trimerous gynoechia, and fused stamen filaments are reminiscent of conditions found in the highly specialized monocotyledon family Orchidaceae (Dahlgren, 1980). The Podostemaceae uniquely possess a 'pseudoembryo sac' that results from disintegration of nucellar cells below the embryo sac; there is also no triple fusion, and hence no endosperm development in the group (Went, 1910; Bhojwani and Bhatnagar, 1979). There is evidence that the plumule aborts rapidly in seedlings, in which case the thalloid growth form could represent a highly specialized, photosynthetic root system (Dormer, 1972; Fahn, 1982). However, Rutishauser (1997) described shoots as rosulate, thalloid or elongate in form and arising endogenously from roots. Even the interpretation of basic leaf structure in Podostemaceae is complex (Rutishauser, 1995, 1997).

The combination of unconventional morphology, unusual embryology, and reduced anatomy has led to a vast difference of opinion regarding the systematic relationships of river-weeds. As summarized in an early taxonomic history (Van Royen, 1951), Podostemaceae were first described (Humboldt et al., 1816) as a family related to monocotyledons (Alismataceae, Butomaceae, Juncaceae), a convention followed by many subsequent authors. Later opinions suggested affinities to other monocotyledons

including Najadaceae (Schultz, 1832), Lemnaceae and even Orchidaceae (Presl, 1830). Other unconventional associations were made with the families Ceratophyllaceae, Callitrichaceae, and even the algal Characeae (Schultz, 1832; Endlicher, 1837, 1839).

Lindley (1830), Schleiden (1839) and Gardner (1847) demonstrated the dicotyledon embryo of Podostemaceae. However, even with this interpretation, the affinities suggested for the family (including Piperaceae, Nepenthaceae, Polygonaceae, Scrophulariaceae, Lentibulariaceae, and Caryophyllaceae) remained vague (Van Royen, 1951; Graham and Wood, 1975). Eventually, embryological studies focused attention on Rosaceae, Saxifragaceae and Crassulaceae as possible relatives of Podostemaceae (Eichler, 1886; Warming, 1888; Willis, 1902; Went, 1910; Rombach, 1911). Arber (1920) recounted earlier opinions that Podostemaceae represented an ancient group related to Rosales and Sarraceniales and she suggested affinities to taxa such as *Nepenthes* and Saxifragaceae. Subsequent authors (Hutchinson, 1926; Engler, 1930; Mauritzon, 1933) continued to support the relationship of Podostemaceae to these families. Van Royen (1951) concluded that Saxifragaceae were essentially the sister group to a complex comprising Rosaceae, Crassulaceae and Podostemaceae, with the latter two families related most closely. Sculthorpe (1967, p. 21) noted that the phylogenetic position of Podostemaceae was “obscured by the very strange morphology of all its members”, but believed there was sufficient evidence to suggest a relationship to Saxifragaceae (superior ovary, free styles, numerous anatropous ovules) and Crassulaceae (various embryological features). Takhtajan (1980) regarded the Podostemaceae as “related to and derived from” the order Saxifragales, and followed several authors (e.g. Mauritzon, 1933; Kapil, 1970; Maheshwari, 1945) who concluded that the family was derived from Crassulaceae.

The rosalean alliance of Podostemaceae has not been accepted without reservation. Cronquist (1981) shared the opinion that Podostemaceae are related to either Saxifragaceae or Crassulaceae but still considered them to be “taxonomically isolated”. Van Steenis (1981) also assumed that Podostemaceae were related to Rosales, but emphasized that further details of their ancestry were “obscure”. Dahlgren (1980) placed Podostemaceae in superorder Podostemiflorae, commenting that “...they are so specialized that they cannot be associated with any other superorder without severe reservations”. Corner (1976) argued that the seed structure of Podostemaceae was so different from Crassulaceae or Saxifragaceae “as to discredit the idea” of their relationship, and believed that the group was more likely derived from piperealean ancestors. An extreme view has been taken by Cusset and Cusset (1988b,c), who proposed the Podostemopsida as a class of angiosperms equivalent in rank to monocotyledons and dicotyledons. Thus, even after more than a century and a half, the relationships of river-weeds remain unresolved at nearly every higher level of classification.

The phylogenetic position of the unusual family Hydrostachyaceae is also of interest, because these highly modified plants of river rapid habitats have often been allied with Podostemaceae. Tulasne (1849), Weddell (1873) and Bentham and Hooker (1880) recognized the Hydrostachyaceae as a subordinate taxon of Podostemaceae corresponding to either subfamilial or tribal rank. Hutchinson (1926) united the families Hydrostachyaceae and Podostemaceae in the order Podostemales. Other authors (e.g. Engler, 1930; Takhtajan, 1980; Cronquist, 1981; Dahlgren, 1989) have doubted their relation-

Table 1

Sources of *rbcL* sequences used in this study. GenBank accession numbers are indicated in parentheses

| I. Non-flowering seed plants          | II. Angiosperms (cont.)           | II. Angiosperms (cont.)           | II. Angiosperms (cont.)          |
|---------------------------------------|-----------------------------------|-----------------------------------|----------------------------------|
| Cupressaceae                          | Callitricaceae                    | Hydrostachyaceae                  | Rosaceae                         |
| 1. <i>Chamaecyparis</i> (L12570)      | 22. <i>Callitriche</i> (L11681)   | 43. <i>Hydrostachys</i> (U17879)  | 66. <i>Adenostoma</i> (UO6790)   |
| Cycadaceae                            | Caryophyllaceae                   | Lemnaceae                         | 67. <i>Crataegus</i> (UO6799)    |
| 2. <i>Cycas</i> (L12674)              | 23. <i>Dianthus</i> (M77699)      | Lentibulariaceae                  | 68. <i>Exochorda</i> (UO6801)    |
| 3. <i>Zamia</i> (D10736)              | Celastraceae                      | 45. <i>Utricularia</i> (L13190)   | 69. <i>Fallugia</i> (UO6802)     |
| Ephedraceae                           | 24. <i>Euonymus</i> (L13184)      | Loasaceae                         | 70. <i>Filipendula</i> (UO6803)  |
| 4. <i>Ephedra sinica</i> (D10732)     | Ceratophyllaceae                  | 46. <i>Loasa</i> (U17876)         | 71. <i>Fragaria</i> (UO6805)     |
| 5. <i>Ephedra tweediana</i> (L12677)  | 25. <i>Ceratophyllum</i> (M77030) | 47. <i>Schismocarpus</i> (U17878) | 72. <i>Geum</i> (L01921)         |
| Ginkgoaceae                           | Chenopodiaceae                    | Magnoliaceae                      | 73. <i>Holodiscus</i> (UO6807)   |
| 6. <i>Ginkgo</i> (D10733)             | 26. <i>Spinacia</i> (J01443)      | 48. <i>Magnolia</i> (X54345)      | 74. <i>Kagneckia</i> (UO6808)    |
| Gnetaceae                             | Chloranthaceae                    | Myricaceae                        | 75. <i>Lyonothamnus</i> (UO6811) |
| 7. <i>Gnetum gemon</i> (L12680)       | 27. <i>Chloranthus</i> (L12640)   | 49. <i>Myrica</i> (L01934)        | 76. <i>Neillia</i> (UO6813)      |
| 8. <i>Gnetum parvifolium</i> (D10734) | Cornaceae                         | Nepenthaceae                      | 77. <i>Neviusia</i> (UO6815)     |
| Pinaceae                              | 28. <i>Cornus</i> (L11215)        | 50. <i>Nepenthes</i> (L01936)     | 78. <i>Potentilla</i> (UO6818)   |
| 9. <i>Pinus</i> (X58134)              | 29. <i>Aucuba</i> (L11210)        | Nyssaceae                         | 79. <i>Prinsepia</i> (UO6819)    |
| 10. <i>Cedrus</i> (X63662)            | Crassulaceae                      | 51. <i>Camptotheca</i> (L11211)   | 80. <i>Prunus</i> (L01947)       |
| Welwitschiaceae                       | 30. <i>Crassula</i> (L01899)      | 52. <i>Nyssa</i> (L01937)         | 81. <i>Purshia</i> (UO6821)      |
| 11. <i>Welwitschia</i> (D10735)       | 31. <i>Kalanchoe</i> (L11189)     |                                   | 82. <i>Rhodotypos</i> (UO6823)   |

- II. Angiosperms
- Acoraceae  
12. *Acorus* (M91625)
- Alangiaceae  
13. *Alangium* (L11209)
- Apiaceae  
14. *Sanicula* (L11170)
- Aponogetonaceae  
15. *Aponogeton* (U68091)
- Araceae  
16. *Pistia* (M96963)
- Aristolochiaceae  
17. *Asarum* (L14290)
- Asteraceae  
18. *Barnadesia* (L13859)
- Begoniaceae  
19. *Begonia* (L01888)
- Cabombaceae  
20. *Brasenia* (M77028)
- Cannabaceae  
21. *Humulus* (U02729)
32. *Sedum* (L01956)
- Cucurbitaceae  
33. *Cucurbita* (L21938)
- Eucommiaceae  
34. *Eucommia* (L01917)
- Fagaceae  
35. *Nothofagus* (L13342)
- Garryaceae  
36. *Garrya* (L01919)
- Geraniaceae  
37. *Pelargonium* (L01941)
- Gunneraceae  
38. *Gunnera* (L11186)
- Haloragaceae  
39. *Myriophyllum* (L11195)
- Hippuridaceae  
40. *Hippuris* (L36443)
- Hydrangeaceae  
41. *Hydrangea* (L11187)
42. *Philadelphus* (L11198)
- Piperaceae  
53. *Peperomia* (L12661)
- PODOSTEMACEAE  
54. *Apinagia* (U68083)
55. *Cladopus* (U68084)
56. *Marathrum* (U68085)
57. *Mourera* (U68086)
58. *Oserya* (U68087)
59. *Podostemum* (U68088)
60. *Tristicha* (U68089)
61. *Vanroyenella* (U68090)
- Polygalaceae  
62. *Polygala* (L01945)
- Polygonaceae  
63. *Rheum* (M77702)
- Ranunculaceae  
64. *Ranunculus* (L08766)
- Rhamnaceae  
65. *Rhamnus* (L13189)
83. *Rubus* (U06825)
84. *Sorbaria* (U06826)
85. *Sorbus* (U06827)
86. *Spiraea* (L11206)
- Saururaceae  
87. *Saururus* (L14294)
- Saxifragaceae  
88. *Astilboides* (U06207)
89. *Bergenia* (U06208)
90. *Boykinia* (L11175)
91. *Darmera* (L11180)
92. *Heuchera* (L01925)
93. *Mukdenia* (U06212)
94. *Parnassia* (L01939)
95. *Saxifraga mertensiana* (U06216)
96. *Saxifraga punctata* (U06218)
97. *Sullivantia* (U06219)
98. *Tanakaea* (U06220)
99. *Telesonix* (U06221)
100. *Toimiaea* (U06223)
- Scrophulariaceae  
101. *Antirrhinum* (L11688)
102. *Digitalis* (L01902)
- Solanaceae  
103. *Solanum* (M76402)

ship, and have suggested placement of Hydrostachyaceae among various families in the subclass Asteridae, a result consistent with a preliminary analysis of *rbcL* data (Hempel et al., 1995).

Molecular data have been applied only sparingly to the systematics of Podostemaceae. Several micromolecular (phytochemical) studies have been conducted (e.g., Burkhardt et al., 1992; Romo Contreras et al., 1993), but these have essentially been inconclusive for clarifying the relationship of Podostemaceae to other angiosperms, including Hydrostachyaceae (Scogin, 1992). Macromolecular (i.e. DNA) data are virtually non-existent for Podostemaceae, but have yielded valuable insights into phylogenetic relationships at essentially all taxonomic levels in other angiosperms (Soltis et al., 1992). There are now more than 2000 sequences (representing most extant seed plant families) for the chloroplast encoded gene *rbcL* in GenBank (a DNA sequence database accessible via the Internet). Indeed, *rbcL* data have been an important resource for investigating higher level phylogenetic relationships in angiosperms (Chase et al., 1993). Comprehensive studies of the Saxifragaceae and Rosaceae (Soltis et al., 1993; Morgan et al., 1994) as well as a sequence from Hydrostachyaceae (Hempel et al., 1995) have provided *rbcL* sequences for a thorough sampling of taxa deemed critical in the question of relationships of Podostemaceae.

A study of *rbcL* sequence data that includes Podostemaceae presents an invaluable opportunity to investigate and perhaps clarify the relationships of this bizarre angiosperm family. Similar studies have helped to clarify a variety of systematic questions in several aquatic plant groups where reduction and structural divergence have complicated efforts to ascertain relationships (e.g. Les et al., 1991, 1993; Les and Haynes, 1995).

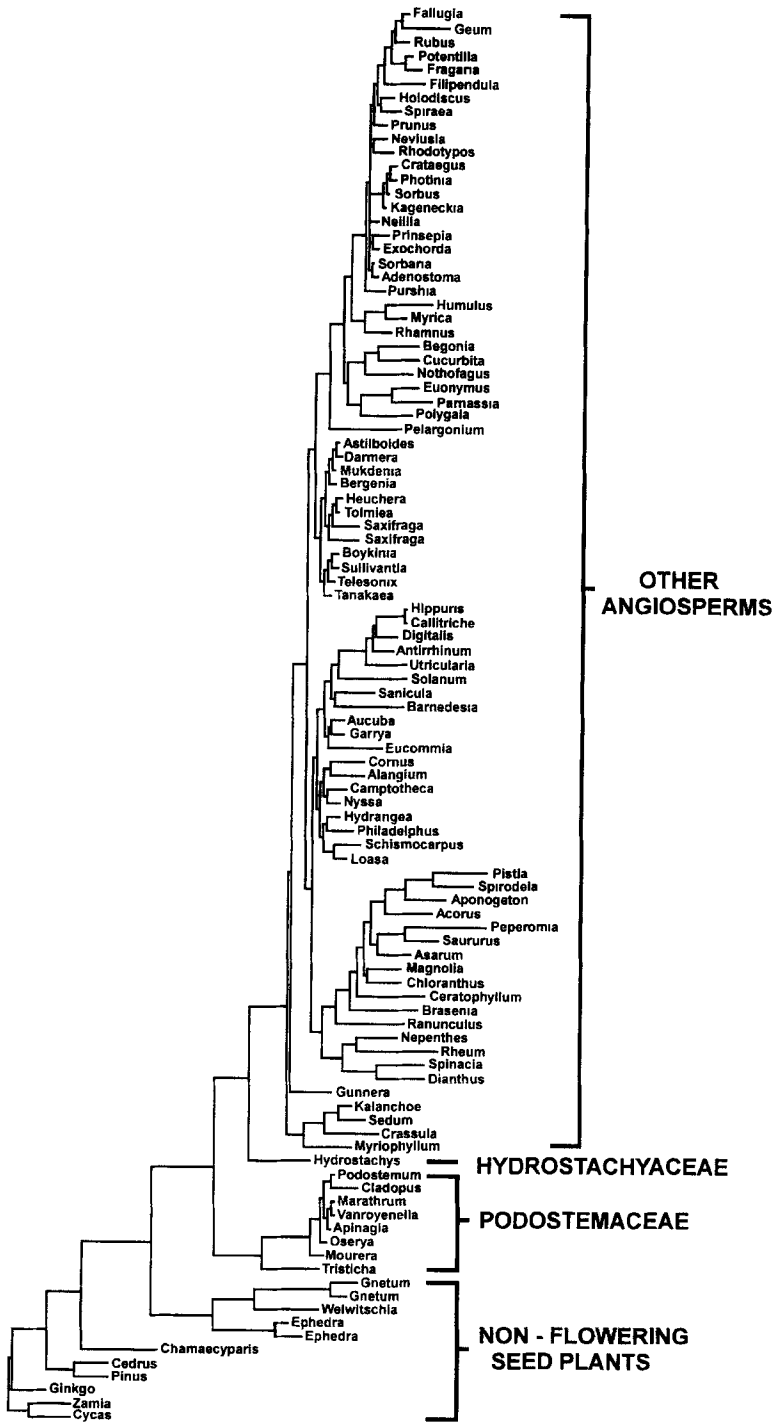
In this study, we report on a phylogenetic analysis of *rbcL* sequences from eight species representing eight different genera of Podostemaceae *s.l.* These sequences have been analysed along with those from most major groups of angiosperms with an emphasis on taxa that have been suggested previously as possible relatives of river-weeds. As far as we are aware, this study is the first use of molecular (DNA) data, and the first explicit use of cladistic approaches to evaluate the phylogenetic position of river-weeds among angiosperms.

## 2. Methods

We retrieved from GenBank 82 *rbcL* sequences of taxa from families putatively allied to the Podostemaceae in former taxonomic treatments as well as angiosperm

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Fig. 1. Cladogram of angiosperms and non-flowering seed plants constructed from *rbcL* sequence data indicated a basal position of the Podostemaceae among angiosperms. All branch lengths are proportional. Topology shown is one of 160 equally parsimonious trees (3840 steps) recovered in this analysis. The strict consensus tree differed by only minor details (see Results). The placement of Podostemaceae is probably a rooting artifact caused by long branch attraction to the divergent non-flowering seed plant outgroups. Hydrostachyaceae and Podostemaceae are adjacent groups.



families from a wide sampling of monocotyledons and dicotyledons (Table 1). We also retrieved 11 non-flowering seed plant sequences to include as outgroups. Treatments of the Saxifragaceae and Rosaceae (Soltis et al., 1993; Morgan et al., 1994) were used to guide the initial selection of 'exemplars' from these large families, to represent the major clades identified in those studies. In addition, we sequenced *rbcL* for *Apinagia yguazuensis* Chodat et Vischer (1348 nucleotides), *Cladopus austrosatsumensis* (Koidz.) Ohwi (1349 nucleotides), *Marathrum rubrum* Novelo & Philbrick (1354 nucleotides), *Mourera asperau* (Bong.) Tul. (1169 nucleotides), *Oserya coulteriana* Tul. (1348 nucleotides), *Podostemum ceratophyllum* Michx. (1348 nucleotides), *Tristicha trifaria* (Bory ex Willd.) Sprengel (1402 nucleotides), and *Vanroyenella plumosa* Novelo and Philbrick (1348 nucleotides). To improve representation of some angiosperm taxa, we also sequenced *rbcL* from *Aponogeton elongatus* F. Muell. ex Benth. (Aponogetonaceae; 1183 nucleotides) and *Spirodela intermedia* W. Koch (Lemnaceae; 1348 nucleotides). Methods for DNA isolation, amplification and sequencing followed those reported by Les et al. (1993). A complete list of taxa and accession numbers is provided in Table 1.

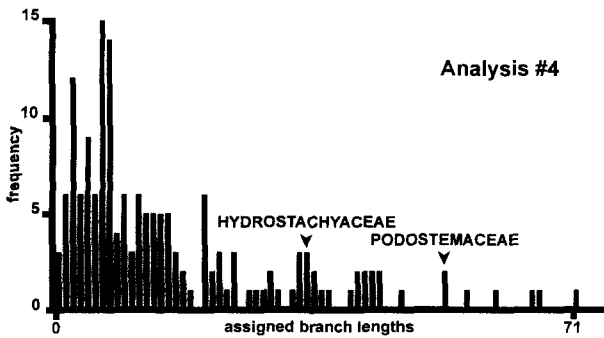
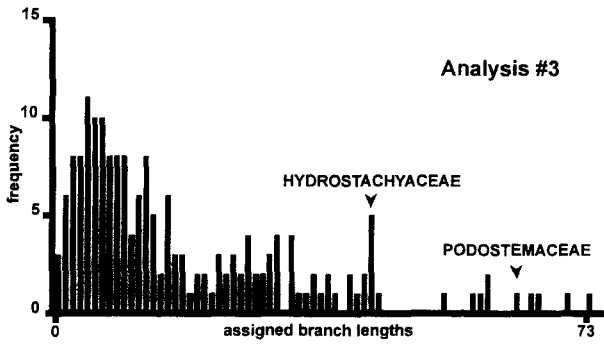
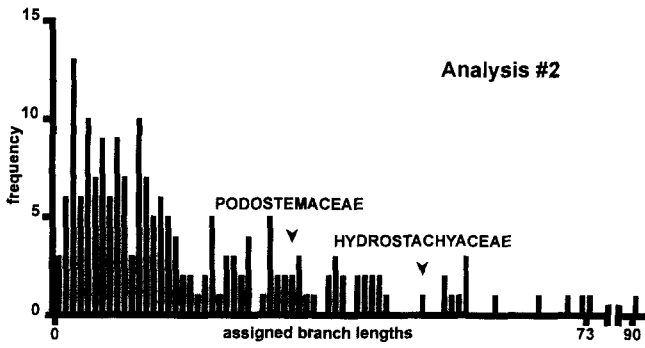
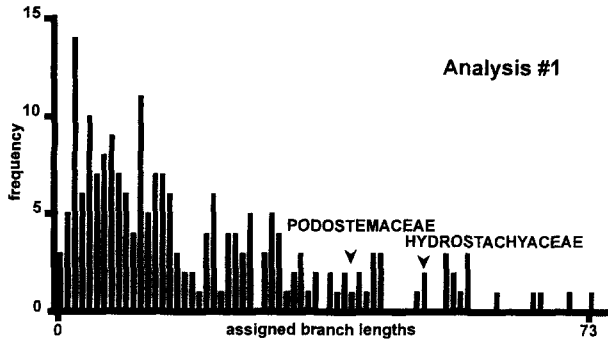
The final data set represented 1403 nucleotides for the 103 taxa compared. Incomplete sections of sequences were coded as missing data. Phylogenetic analysis of the sequence data was conducted using PAUP version 3.1.1 (Swofford, 1993) to perform an unweighted maximum parsimony analysis. We employed SIMPLE addition sequence, COLLAPSE, MULPARS, STEEPEST DESCENT and TBR branch-swapping options in all analyses. Strict consensus was used to assess results where multiple equally minimal length trees were recovered. We selected one representative tree from each analysis to depict the relative branch lengths of taxa.

Four analyses were conducted. The first analysis designated two cycad taxa (*Zamia*, *Cycas*) as outgroups to direct the rooting of the recovered topology. Because the results of this analysis indicated potential problems with attraction of long ingroup branches by the outgroup (see Discussion), we also performed a series of analyses that excluded taxa of progressively distant relationship to angiosperms. The second analysis retained the same angiosperm sequences, but included only three sequences from the division Gnetophyta, which resolved as the closest sister group to angiosperms in the first analysis. The third analysis excluded all non-flowering plants and used *Ceratophyllum* to root the tree of angiosperm sequences as suggested by results from a prior 'global' analysis of seed plant *rbcL* sequences (Chase et al., 1993). The fourth analysis excluded *Ceratophyllum*, all magnoliid, and all monocotyledon angiosperm sequences. The resulting network was rooted using *Ranunculus*, which fell among the basal group of 'eudicot' taxa in the global *rbcL* analysis (Chase et al., 1993).

In the fourth analysis, random taxon addition (35 replicates; MULPARS ON; STEEPEST DESCENT OFF) was used to search for the presence of multiple islands of

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Fig. 2. Histograms of branch lengths for four analyses of *rbcL* sequence data. The branches leading to the Podostemaceae and Hydrostachyaceae are extremely long and susceptible to attraction from other long branches in all four analyses. The relative branch lengths of Podostemaceae and Hydrostachyaceae shifted as distant outgroups were excluded from the analyses (analysis sequence 1–4).



minimal length trees (Maddison, 1991). We computed bootstrap support (Felsenstein, 1985) for the indicated monophyly of clades using 200 replicates (SIMPLE addition sequence; MULPARS OFF; STEEPEST DESCENT OFF). An uncorrected estimate of sequence divergence among Podostemaceae species was obtained by comparing a 1348 nucleotide region surveyed for all species (except *Mourera* where the results are based on 1169 sites). The distribution of branch lengths was examined for a representative tree in each analysis to provide an overview of relative branch lengths for Podostemaceae and other groups.

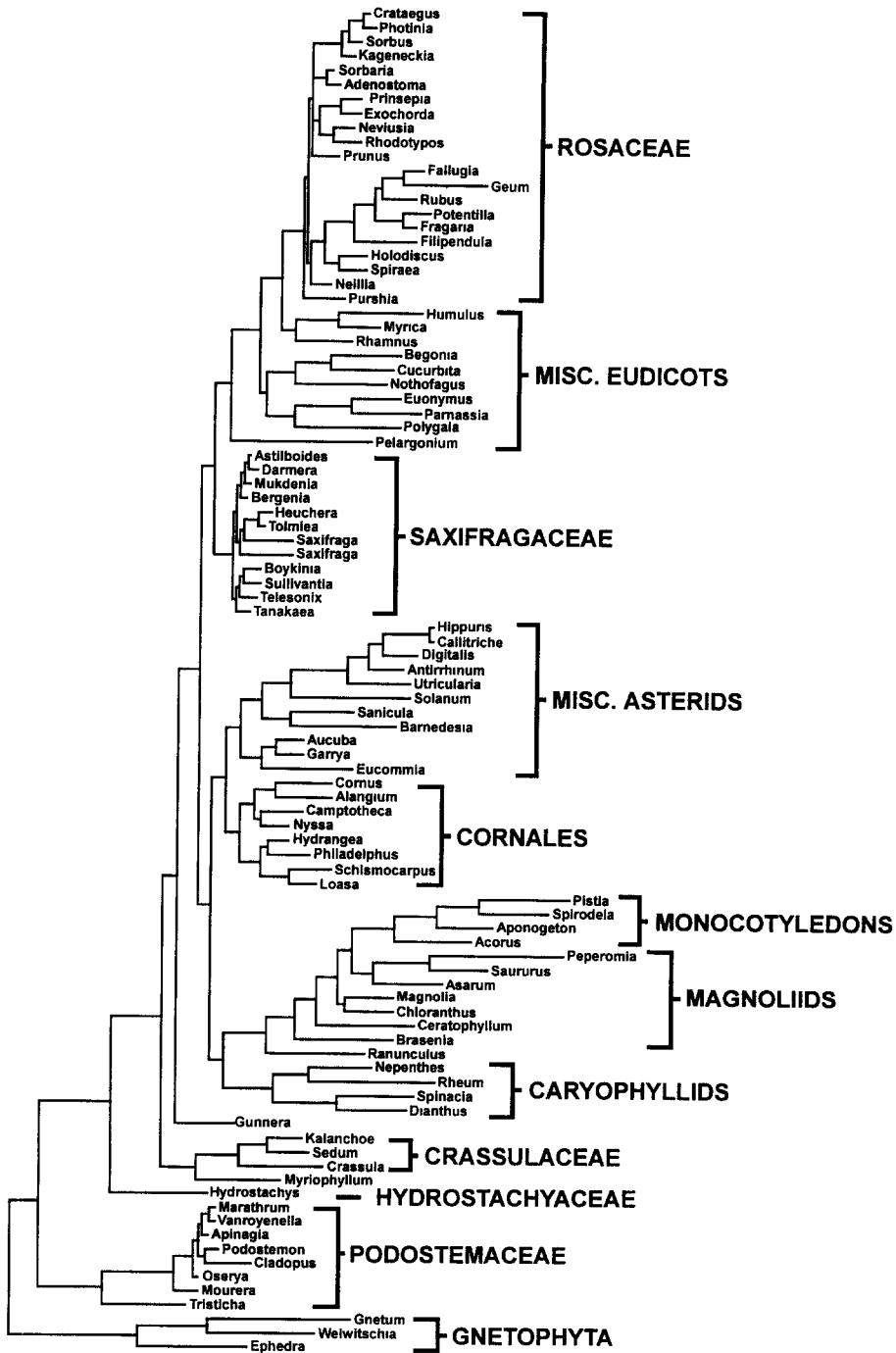
### 3. Results

From our analysis of the complete data set using cycad sequences for outgroup rooting, we recovered a total of 160 trees (3840 steps; consistency index, CI = 0.28, 0.25 excluding uninformative characters; retention index, RI = 0.62; Fig. 1). This topology showed the Podostemaceae as basal to other angiosperms, but resolved the ingroup topology in a highly irregular fashion. Hydrostachyaceae were the closest group to Podostemaceae, followed by Haloragaceae, Crassulaceae and Gunneraceae which were basal to other angiosperms. Successively, the Saxifragaceae and Rosaceae appeared next with members of the Cornales, subclass Asteridae, subclass Caryophyllidae, subclass Magnoliidae and monocotyledons derived ultimately (Fig. 1). The topology depicted in this analysis, where monocotyledons and magnoliid dicotyledons were among the groups that appeared to be most highly derived, resembled an inverted version of relationships that are generally assumed for angiosperms (e.g. Cronquist, 1981), or that have been obtained in similar analyses excluding Podostemaceae taxa (e.g. Chase et al., 1993).

The branch leading to the Podostemaceae (40 steps) was the 16th longest of the 203 branches resolved in the cladogram; the branch to Hydrostachyaceae (50 steps) was tenth longest (Fig. 2). In perspective, the branch separating monocotyledons from dicotyledons was only 21 steps, that separating the ingroup (angiosperms) from the outgroup (non-flowering plants) was 53 steps, and that separating *Tristicha* from other Podostemaceae was 43 steps. The strict consensus tree was virtually identical to the one presented, and differed essentially by the collapse of some nodes within the Saxifragaceae (*Astilbooides*, *Bergenia*, *Darmera*, *Mukdenia*) and Podostemaceae (*Apinagia*, *Marathrum*, *Vanroyenella*).

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Fig. 3. Cladogram constructed from *rbcL* data for various groups of angiosperms using three outgroup genera from the division Gnetophyta to root the network. All branch lengths are proportional. The topology shown is one of 80 equally parsimonious trees (3458 steps) recovered in this analysis. Again, the strict consensus tree differed by only minor details (see Results). The position of Podostemaceae remained basal to other angiosperms in this analysis, but the topology of other angiosperms also remained 'inverted' relative to relationships typically perceived by contemporary taxonomists. Phylogenetically, the Hydrostachyaceae, Haloragaceae (*Myriophyllum*) and Crassulaceae were the closest angiosperm families to the Podostemaceae in this analysis.



Limiting the outgroup to three Gnetophyte sequences (second analysis) resulted in 80 trees (3458 steps; CI = 0.30, 0.26 excluding uninformative characters; RI = 0.60). This analysis (Fig. 3) retained the position of Podostemaceae as basal to other angiosperms, again with Hydrostachyaceae, Haloragaceae and Crassulaceae as the successively closest angiosperm families (Fig. 3). Details of this topology differed little from those of the first analysis (Fig. 1). The branch leading to Podostemaceae (32 steps) was the 23rd longest among the 188 branches in this analysis (Fig. 2); the remainder of the ingroup was supported by a branch of 38 steps. Other branch lengths for relative comparison were: monocotyledons (21 steps), outgroup (90 steps), Hydrostachyaceae (50 steps) and *Tristicha*/other Podostemaceae (42 steps). The strict consensus tree differed only by the collapse of some nodes in the Rosaceae (*Prinsepia*/*Exochorda*, *Neuiusia*/*Rhodotypos*), Saxifragaceae (*Astilboides*, *Bergenia*, *Darmera*, *Mukdenia*) and Podostemaceae (*Apinagia*, *Marathrum*, *Vanroyenella*).

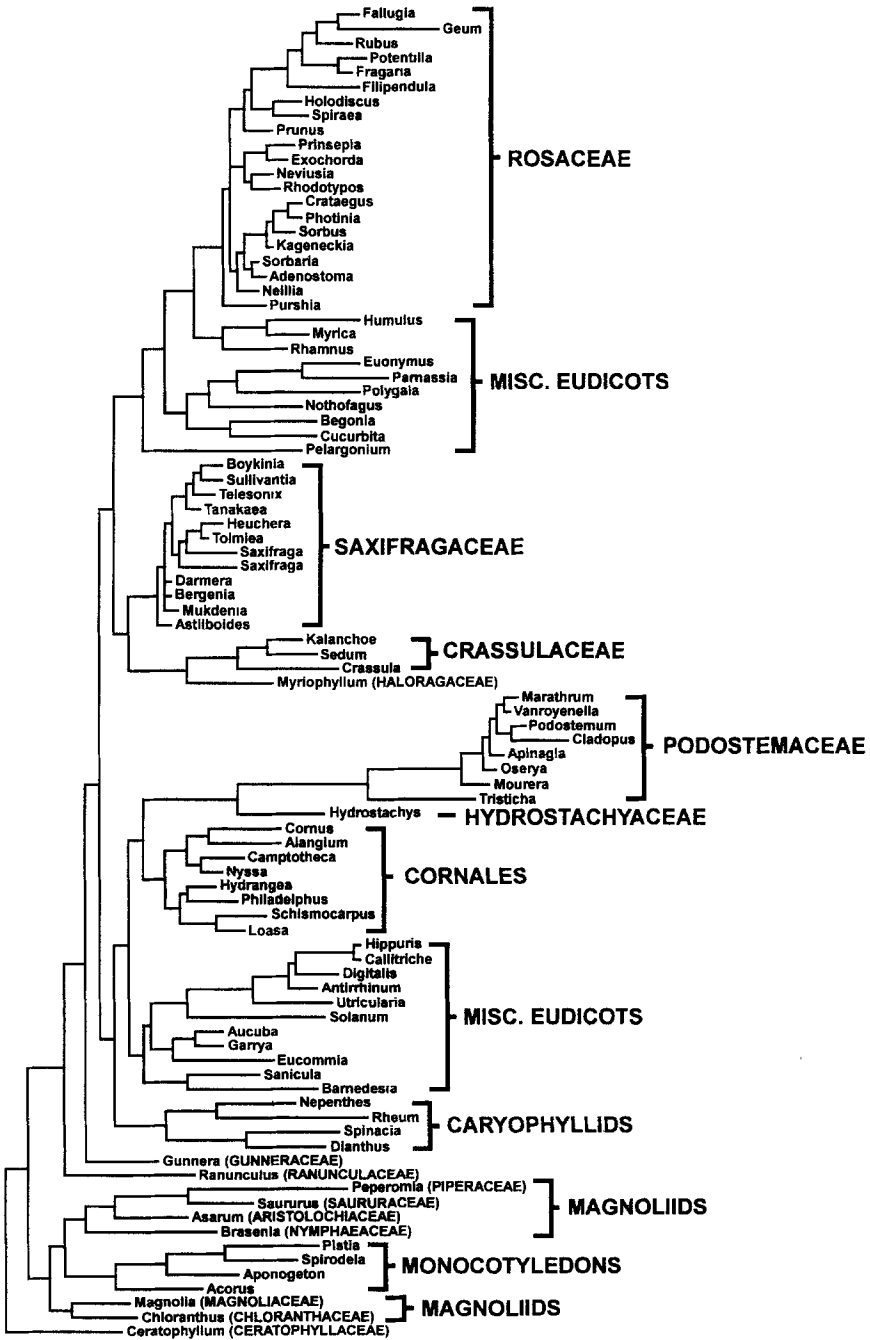
The third analysis, which excluded all non-flowering plant sequences and used *Ceratophyllum* to root the remaining sequences, generated 8300 trees before reaching the tree buffer overflow imposed by computer memory limitations. Thus, this search was possibly ineffective at estimating the minimal length topology. The trees recovered were 3148 steps in length (CI = 0.31, 0.26 excluding uninformative characters; RI = 0.61). Strict consensus of these trees (not shown) produced a topology compatible with the results of similar analyses (e.g. Chase et al., 1993), where monocotyledons and magnoliid dicotyledons occurred in a position basal to a 'eudicot' clade. The position of Podostemaceae was poorly resolved, but nested within a clade that included various members of subclasses Asteridae and Caryophyllidae. Although far less resolved than the example presented (Fig. 4), the strict consensus tree also depicted the Hydrostachyaceae as the sister group to Podostemaceae. Ranunculaceae and Gunneraceae were basal to the 'eudicot' clade that contained the members of Podostemaceae.

The branch supporting the Podostemaceae (63 steps) remained relatively long (fifth longest of the 179 branches in this analysis; Fig. 2) and indicated the considerable divergence of this family from the other angiosperms whose sequences were included in the analysis. Branch lengths for relative comparison were: outgroup (65 steps), *Tristicha*/other Podostemaceae (43 steps), monocotyledons (22 steps) and 'eudicots' (17 steps). The branch supporting the Hydrostachyaceae/Podostemaceae clade was 43 steps.

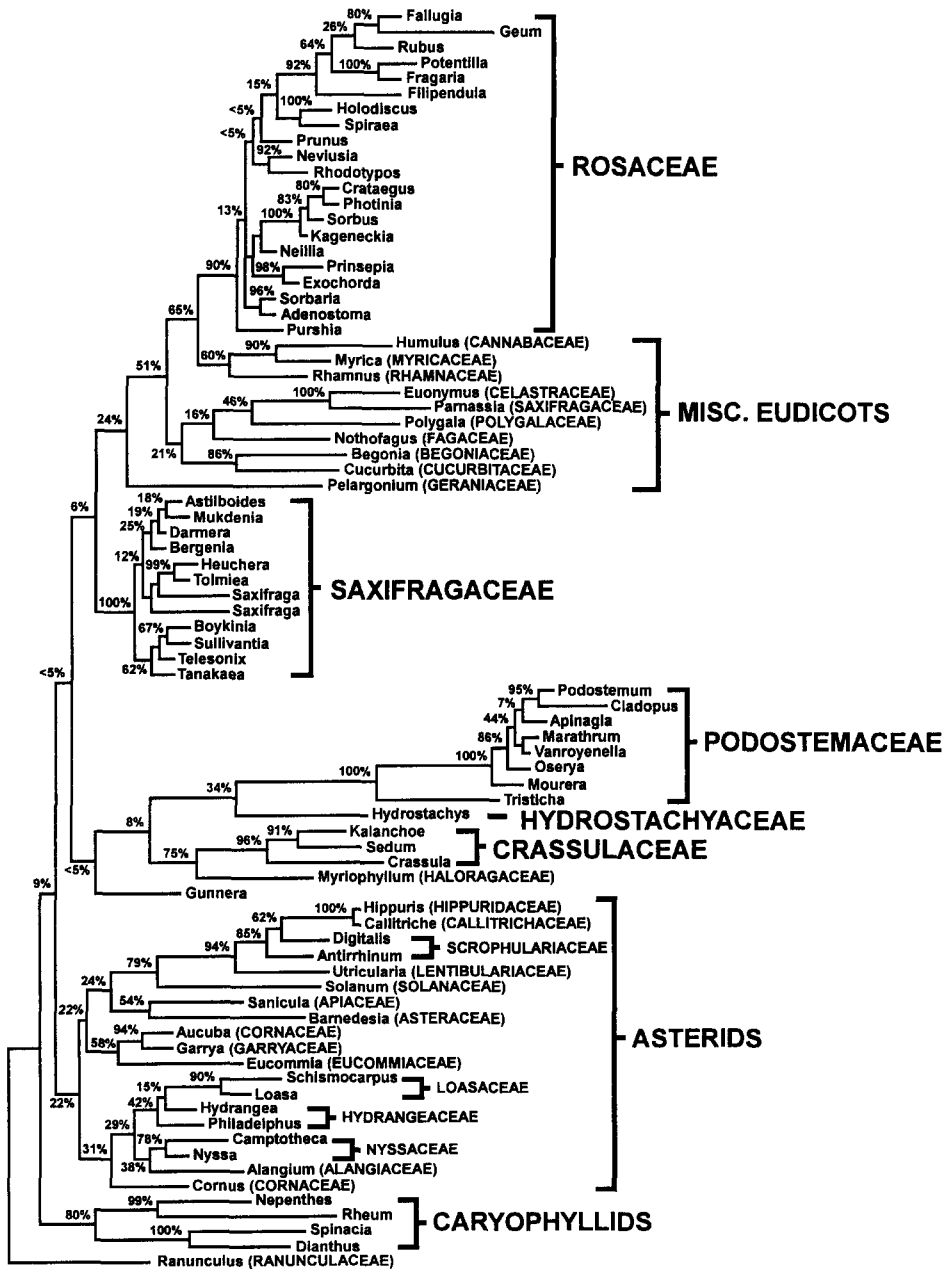
The fourth analysis (restricted to 'eudicot' sequences and rooted by *Ranunculus*) produced 160 trees (2535 steps) which showed the Hydrostachyaceae/Podostemaceae as a sister clade to the subclass Caryophyllidae (results not shown). However, 35 random taxon additions recovered an island of shorter trees (2534 steps) that differed considerably in topology. Here, too, the analysis was impaired by a tree-buffer overflow.

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Fig. 4. Cladogram constructed from *rbcL* sequences of various angiosperms using *Ceratophyllum* to root the network. All branch lengths are proportional. This topology is one of 8,300 equally parsimonious trees (3,148 steps) recovered. The strict consensus tree was similar overall to the result shown, but contained a greater proportion of unresolved nodes. Here, Podostemaceae fell within a clade comprising Hydrostachyaceae and families of the order Cornales, although this result may represent a suboptimal solution (see Discussion). Other angiosperm groups occupied positions that are reasonably compatible with contemporary phylogenetic hypotheses.



The topology of the 640 trees recovered in this island (2534 steps; CI = 0.35, 0.29 excluding uninformative characters; RI = 0.64) joined Hydrostachyaceae and Podostemaceae as sister taxa and placed the two families in a clade with Crassulaceae and Haloragaceae. These families nested in a clade that comprised Saxifragaceae, Rosaceae,



and a variety of eudicot families (Fig. 5). The strict consensus showed much the same topology with less resolution as noted for certain members of Podostemaceae, Rosaceae and Saxifragaceae in previous analyses; resolution of several other clades was slightly reduced in the consensus tree, but the major portions of the topology did not differ fundamentally from that presented in Fig. 5.

The Podostemaceae branch (53 steps) was the sixth longest of the 165 branches (Fig. 2); *Tristicha* was separated from other Podostemaceae by a branch length of 43 steps. The Hydrostachyaceae and Podostemaceae were joined by a branch of 34 steps. The branch supporting a clade with Hydrostachyaceae, Podostemaceae, Haloragaceae and Crassulaceae was 20 steps. Bootstrap support for several deep internal nodes was low (< 5–9%), including the association of Podostemaceae and Hydrostachyaceae with Crassulaceae and Haloragaceae (8%). Support for the monophyly of Podostemaceae was high (100%), but support for an association with Hydrostachyaceae was much lower (34%). Monophyly of the Crassulaceae, Rosaceae, and Saxifragaceae (excluding *Parnassia*) was also strongly supported, with bootstrap values of 96, 90 and 100% respectively (Fig. 5).

The *rbcL* sequence of *Tristicha* was 6.8–8.6% divergent from sequences of other Podostemaceae genera (Table 2). The *rbcL* variation among the remaining five genera of Podostemaceae surveyed ranged from 0.7 to 3.2%, with the highest level observed between the sequences of *Cladopus* and *Mourera* (Table 2).

#### 4. Discussion

The *rbcL* data indicate a substantial degree of sequence divergence between members of the Podostemaceae that we examined. Sequence divergence between *Tristicha* and other Podostemaceae exceeded twice the level of divergence observed among the five other genera of Podostemaceae surveyed from different continents (Table 2). Although not definitive, this result lends support to taxonomic treatments that have maintained Tristichaceae and Podostemaceae as separate families. *Tristicha* was basal to other genera of Podostemaceae in all analyses, and the monophyly of *Tristicha* and other Podostemaceae was supported by 100% bootstrap values.

In all analyses, a high degree of sequence divergence was evident between both the

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Fig. 5. A cladogram of 'eudicot' taxa constructed from *rbcL* sequences using *Ranunculus* to root the network. All branch lengths are proportional. The topology shown is one of 640 equally parsimonious trees recovered (2534 steps) using 35 random input additions of taxa. The strict consensus tree was not as well resolved, but depicted the major features of the topology illustrated by this example. The level of internal support (bootstrap value) is indicated at each node (a few nodes lack values because of space limitations). The Podostemaceae reside in a clade along with the Hydrostachyaceae as its immediate sister group. The Crassulaceae and Haloragaceae occur as an adjacent clade. Although this clade lacked strong internal support (8% bootstrap value), the association of these families coincides with the opinions of several contemporary taxonomists. Other renditions of relationships are similar to those resolved in global analyses of *rbcL* data and are fairly representative of traditional taxonomic perspectives. The Podostemaceae were strongly supported as a monophyletic group in this analysis. Internal support for the association of Hydrostachyaceae and Podostemaceae (34% bootstrap value) was markedly reduced.

Table 2  
 Estimated sequence divergence (uncorrected distances) in *rbcL* compared among eight genera of Podostemaceae. Country of origin is indicated in parentheses

|                     | <i>Apinagia</i><br>(Argentina) | <i>Cladopus</i><br>(Japan) | <i>Marathrum</i><br>(Mexico) | <i>Mourera</i><br>(Argentina) | <i>Oserya</i><br>(Mexico) | <i>Podostemum</i><br>(US) | <i>Tristicha</i><br>(Mexico) | <i>Vanroyenella</i><br>(Mexico) |
|---------------------|--------------------------------|----------------------------|------------------------------|-------------------------------|---------------------------|---------------------------|------------------------------|---------------------------------|
| <i>Apinagia</i>     | -                              |                            |                              |                               |                           |                           |                              |                                 |
| <i>Cladopus</i>     | 0.027                          | -                          |                              |                               |                           |                           |                              |                                 |
| <i>Marathrum</i>    | 0.007                          | 0.025                      | -                            |                               |                           |                           |                              |                                 |
| <i>Mourera</i>      | 0.020                          | 0.032                      | 0.021                        | -                             |                           |                           |                              |                                 |
| <i>Oserya</i>       | 0.010                          | 0.028                      | 0.010                        | 0.019                         | -                         |                           |                              |                                 |
| <i>Podostemum</i>   | 0.013                          | 0.020                      | 0.014                        | 0.019                         | 0.014                     | -                         |                              |                                 |
| <i>Tristicha</i>    | 0.068                          | 0.086                      | 0.072                        | 0.071                         | 0.073                     | 0.077                     | -                            |                                 |
| <i>Vanroyenella</i> | 0.007                          | 0.024                      | 0.004                        | 0.017                         | 0.008                     | 0.010                     | 0.070                        | -                               |

Podostemaceae and Hydrostachyaceae and other angiosperms. The branch separating the Podostemaceae clade from other angiosperm taxa (Figs. 1 and 3–5) ranged from 32–63 steps in length and always occurred in the top 12% of reported branch lengths. In all analyses (Figs. 1 and 3–5), the branch leading to the Podostemaceae was substantially longer than that which separated monocotyledons and dicotyledons. This high level of sequence divergence is compatible with taxonomic opinions that have suggested the segregation of Podostemaceae as an isolated order, Podostemales (e.g. Dahlgren, 1980; Takhtajan, 1980). The branch leading to Hydrostachyaceae (50 steps) was only three steps longer than the outgroup branch in the analysis that included all taxa.

Analysis of *rbcL* data indicates that the extreme morphological divergence of the Podostemaceae and Hydrostachyaceae correlates with a comparable level of divergence at the DNA sequence level. This type of evolutionary history (i.e. highly divergent characters) is as problematic for parsimony analyses of molecular data as it is with morphological data. The divergence of both types of characters makes it difficult to trace the relationship of these groups with any high degree of certainty, due to a problem known as long branch 'attraction' (Felsenstein, 1978; Hendy and Penny, 1989; Olmstead and Palmer, 1994). Simply stated, long branches created by character states (molecular or morphological) of extremely divergent taxa may converge to an incorrect tree topology as the true phylogenetic signal in the data becomes progressively diluted. Because the branch between the 'ingroup' and 'outgroup' is generally long, other long branches within the ingroup are susceptible to rooting errors, i.e. they may move the root of the ingroup by attraction of long branches in the ingroup and outgroup. In practice, it has proven extremely difficult to decide in such cases whether the placement of a divergent taxon is correct or erroneous.

Adding additional taxa that potentially could break up the long branch is one solution, but no other extant families with a potentially close relationship to either Hydrostachyaceae or Podostemaceae have been identified.

The *Ceratophyllum* sequence is also quite divergent and is also suspect as a possible long branch (Qiu et al., 1993). However, the association of taxa using the *Ceratophyllum* rooting in our second analysis was similar to that of the larger analysis of angiosperm *rbcL* sequences by Chase et al. (1993). It is interesting that the long branches of Podostemaceae and Hydrostachyaceae did not converge with *Ceratophyllum* in any of the analyses.

We believe that our initial analyses of Podostemaceae illustrated precisely the phenomenon of long branch attraction when non-flowering plant sequences were used for outgroup rooting. Those analyses yielded a topology depicting Podostemaceae as a basal angiosperm lineage (Figs. 1 and 3). Superficially, this result is not entirely implausible, given the recent suggestion to recognize the Podostemopsida as a distinct angiosperm class (Cusset and Cusset, 1988b,c). However, in addition to extensive morphological studies by Jäger-Zürn (1995) which do not support this interpretation, we are also reluctant to accept the basal position of Podostemaceae in the light of results from subsequent analyses. Manipulations of our data set showed that exclusion of even a single outgroup sequence (e.g. from Gnetophyta) in one case moved the position of the Podostemaceae clade to resolve as a sister group to the Gnetophyta (results not shown). This is not particularly surprising (nor acceptable taxonomically), given the extremely

long branches that separate taxa in the Gnetophyta, the clade that resolves as the closest extant sister group to angiosperms. *Gnetum* and *Welwitschia* were separated by a branch of 65 steps; the clade of *Ephedra* species by a branch of 50 steps, and the *Gnetum* and *Welwitschia* clade by a branch of 35 steps in the first analysis (Fig. 1). This aggregation of long branches would be a likely place for other long branches (e.g. Podostemaceae, Hydrostachyaceae) to converge.

The labile phylogenetic position of Podostemaceae was wholly evident in results from the analyses described above. With divergent non-flowering plant sequences included in the analysis, Podostemaceae resolved as a basal angiosperm clade; however, interrelationships within the angiosperm ingroup were resolved in a highly irregular fashion that conflicted with most taxonomic opinions. Limiting the outgroup to three Gnetophyte sequences retained the Podostemaceae in a position basal to other angiosperms, but also retained a topology of ingroup relationships that was 'inverted' phylogenetically. Groups that have been hypothesized to represent primitive taxa in countless systematic studies appeared in derived positions and vice versa. This outcome indicated that the long branches of the non-flowering plant sequences resulted in a misplaced root of angiosperm sequences at the long branches delimiting the Hydrostachyaceae and Podostemaceae rather than in the vicinity of magnoliid taxa where the angiosperm root is generally assumed. Nevertheless, the Crassulaceae and Haloragaceae resolved as the closest groups to Podostemaceae and Hydrostachyaceae in these inverted topologies (Figs. 1 and 3).

Limiting the analysis exclusively to angiosperms resulted in the nesting of Hydrostachyaceae and Podostemaceae within an alliance of asterid and caryophyllid groups (e.g. Cornales, Asteraceae, Callitrichaceae), a position that was fairly remote from Haloragaceae and Crassulaceae. We believe that this result may reflect our failure to search for other, possibly shorter, islands of trees during this analysis. A similar association of taxa was recovered in the initial heuristic search of analysis 4, but a search for shorter trees did recover a topology one step shorter which re-established the association of Hydrostachyaceae and Podostemaceae with the Haloragaceae and Crassulaceae (Fig. 5). These observations further indicate the extremely labile position of Hydrostachyaceae and Podostemaceae in *rbcL* analyses. Trees that differed by only a single step in length placed these families among associations of angiosperms that represent extremely different phylogenetic affiliations (i.e. subclass Rosidae versus Caryophyllidae).

Yet, even when we restricted the analysis to 'eudicot' families (which resolved a clade consisting of the Haloragaceae, Crassulaceae, Hydrostachyaceae and Podostemaceae), low bootstrap values rendered the monophyly of this clade debatable. There was only 8% bootstrap support for this association (which few systematists would regard as compelling), and only 34% bootstrap support for the association of Hydrostachyaceae and Podostemaceae. We emphasize that Hempel et al. (1995) obtained a similar level of bootstrap support (30–34%) for the association of *Hydrostachys* and *Decumaria* (Hydrangeaceae) in analyses that did not include members of Podostemaceae. Although we view the relationship of Hydrostachyaceae and Hydrangeaceae as highly improbable, we cannot provide much better support for the association of Hydrostachyaceae and Podostemaceae.

In any event, it is evident that removing the long outgroup branches resulted in the placement of Hydrostachyaceae and Podostemaceae as a sister group to the Crassulaceae and other roseaceous families. Without other phylogenetic analyses of these groups to serve as testable hypotheses, we can only restate the opinions of many taxonomists who have suggested the placement of the group somewhere in the vicinity of the Rosales.

Most opinions have favored an alliance of the Podostemaceae to either Saxifragaceae or Crassulaceae (Cronquist, 1981). In this respect, our results support earlier hypotheses. However, the low level of bootstrap support for the phylogenetic association of Crassulaceae and Podostemaceae (8%) does not inspire much confidence in the degree of internal support provided by the *rbcL* data. We do, however, admit that the phylogenetic association of Podostemaceae, Crassulaceae, and Haloragaceae represents a more reasonable hypothesis than the position of the order as a basal angiosperm lineage. Despite the many morphological peculiarities of river-weeds, they are unquestionably dicotyledonous, and possess several similarities to both Crassulaceae and Haloragaceae. All three groups have tetrasporangiate, dithecal anthers, binucleate tricolpate pollen, and distinct styles. Both Crassulaceae and Podostemaceae have many anatropous, bitegmic ovules; septicidal capsules occur in both groups. Aquatic species are found in all three families, but this may be superfluous if Willis (1915) was correct in concluding that the family evolved from terrestrial plants. Interestingly, many of these features are also shared with Hydrostachyaceae, which differ from Crassulaceae and Podostemaceae essentially by their unitegmic ovules and parietal placentation. Mauritzon (1933) argued strongly that embryological features indicated the reduction of Hydrostachyaceae and Podostemaceae from the Crassulaceae. The results of the *rbcL* analysis are in complete agreement with this scenario. However, Scogin (1992) compared the phytochemical profiles of Hydrostachyaceae and Podostemaceae, but found no evidence of a close relationship between the families. Hempel et al. (1995) emphasized the unitegmic ovules of Hydrostachyaceae to suggest its phylogenetic position somewhere among the subclass Asteridae. Verdcourt (1986, p. 1) suggested that evidence contrary to the relationship of Hydrostachyaceae and Podostemaceae had become "...too extensive to be argued against".

However, many taxonomists who have doubted the relationship of Podostemaceae and Hydrostachyaceae have generally concluded that the latter family should be placed phylogenetically near the Scrophulariaceae, Callitrichaceae, or Hippuridaceae. Clearly the *rbcL* data provide no evidence of such an association. Thus we are left to consider the tentative hypothesis offered by the current *rbcL* analysis, and continue to search for better evidence for an alternative placement of these families. There is no doubt that Hydrostachyaceae and Podostemaceae differ in a number of features (Rauh and Jäger-Zürm, 1967); however, their level of molecular divergence, as indicated by the *rbcL* analysis, does not make this an unexpected outcome. Thus, studies that have demonstrated various differences between the groups do not necessarily preclude the possibility of their relationship.

Although a definitive conclusion on the phylogenetic position of either the Podostemaceae or Hydrostachyaceae cannot be achieved, the analysis of *rbcL* data represents an important initial step in determining the correct relationships of these families. Because no other comprehensive cladistic analysis of angiosperms has yet been

conducted that includes Podostemaceae, previous hypotheses of relationships simply have reflected the opinion of how various authors have perceived the best 'match' of features in this group with those of other flowering plant families. The application of phylogenetic methods in this study presents a hypothesis that can serve as a reasonable forum for further testing and scrutiny. The observation that our results are compatible with the opinions of many contemporary taxonomists enhances the robustness of the present hypothesis of relationships for river-weeds. Studies at higher taxonomic levels are seldom likely to yield definitive evidence of relationships. The high level of homoplasy in *rbcL* data is evidenced by the low consistency indices (< 35%) in all analyses. However, the overall congruence of the molecular data with the topologies presented was respectable as indicated by retention indices of 60–64%. Thus, the degree of confidence rendered by these results may not be particularly high, but is arguably higher than for conclusions reached simply by nonempirical means.

## 5. Conclusions

The *rbcL* sequences of Podostemaceae genera are substantially divergent from those of other angiosperms, including the Hydrostachyaceae and other families believed to represent the closest extant sister group of river-weeds. The long resulting branch, that supports Podostemaceae in parsimony analyses of *rbcL* data, creates problems when attempting to root the sequences of the complete data set using distant non-flowering plant outgroups. A basal position of Podostemaceae in the angiosperms is resolved when non-flowering plant outgroup sequences are used. Although this result superficially supports claims that Podostemaceae deserve taxonomic rank equivalent to monocotyledons and dicotyledons (Cusset and Cusset, 1988b,c), we view this association as an incredulous artifact of long branch attraction.

By restricting sequence comparisons to 'eudicots' (where the highest probability of relationship was expected), a clade was resolved that included the Crassulaceae and Haloragaceae as the sister groups to Hydrostachyaceae and Podostemaceae. The monophyly of this clade was not strongly supported by bootstrap analysis, but was consistent with the taxonomic opinion of several contemporary systematists. The phylogenetic alliance of Podostemaceae with the Hydrostachyaceae, Crassulaceae and Haloragaceae, is presented as a specific hypothesis for further evaluation.

The morphological peculiarities of Podostemaceae are mirrored by extensive molecular divergence, which supports the family as a monophyletic group deserving of taxonomic recognition. A high degree of molecular divergence between *Tristicha* and other genera of Podostemaceae supports the recognition of Tristichaceae as a separate family.

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