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Molecular Phylogenetics and Evolution 28 (2003) 131–151
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Phylogenetic studies of Ophioglossaceae: evidence from *rbcL* and *trnL-F* plastid DNA sequences and morphology

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Received 5 August 2002; revised 19 December 2002

Abstract

Ophioglossaceae are a putatively ancient lineage of ferns in which the aerial portion of the plant is composed of a single leaf. The simplicity of foliar morphology has limited the number of characters available for constructing classifications and contributed to taxonomic difficulties at nearly every level of classification within the family. Analysis of plastid DNA *rbcL* sequences from 36 species representing the diversity of Ophioglossaceae supported the monophyly of the family. Intrafamilial relationships were examined using *rbcL* and *trnL-F* plastid DNA sequences and morphological data. Individual and combined analyses of the three data sets revealed two main clades within the family, here termed ophioglossoid and botrychioid. In the botrychioid clade, *Helminthostachys* was sister to a broadly defined *Botrychium*, within which *Botrychium* in the narrow sense of some authors and *Sceptridium* were sister. *Botrypus* was paraphyletic, with *Botrypus virginianus* sister to *Botrychium* plus *Sceptridium*, and with *Botrypus strictus* sister to all other botrychioid species except *Helminthostachys*. In the ophioglossoid clade, *Ophioglossum* in the narrow sense was sister to *Cheiroglossa* plus *Ophioderma*, but relationships within *Ophioglossum* were not well supported.

Keywords: *Ophioglossum*; *Botrychium*; Moonwort; Ophioglossaceae; Phylogeny; *rbcL*; *trnL-F*

1. Introduction

Ophioglossaceae, commonly called the adder's tongue ferns, are a putatively ancient lineage of pteridophytes (Bower, 1926; Campbell, 1905; Clausen, 1938; Eames, 1936; Gifford and Foster, 1989; Kato, 1987; Prantl, 1884; Presl, 1845; Tryon and Tryon, 1982; Wagner, 1990) with an evolutionary history that is enigmatic largely because fossils of the family date only from the earliest Tertiary (Rothwell and Stockey, 1989). Relative to other ferns, unusual features of the family include the presence of eusporangia, limited secondary growth in the rhizome, sheathing leaf bases, circular bordered pits, subterranean and non-chlorophyllous gametophytes, and the absence of circinate vernation, root hairs, and sclerenchyma (Bower, 1926; Kato, 1988; Tryon and Tryon, 1982; Wagner, 1990). The principal attribute distinguishing Ophioglossaceae from other pteridophytes is the division of the frond into separate sterile (trophophore) and fertile (sporophore) segments (Bower, 1926; Smith, 1955; Wagner, 1990). The sporophore/trophophore construction of the leaf provides a clear synapomorphy for members of the family (Bierhorst, 1971; Bower, 1926; Campbell, 1905; Wagner, 1990), and monophyly of Ophioglossaceae has not been questioned.

Within Ophioglossaceae, the entire aerial portion of the plant is a single, often inconspicuous leaf (Clausen, 1938; Eames, 1936). Most members of Ophioglossaceae do not exhibit the morphological complexity typical of "higher" (i.e., leptosporangiate) ferns (Clausen, 1938; Eames, 1936). The simplicity of foliar morphology has limited the number of characters available for constructing classifications and understanding relationships, thereby forcing investigators to rely on "trivial" details of frond size and dissection as diagnostic markers

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doi:10.1016/S1055-7903(03)00032-0

(Clausen, 1938). Problems associated with the interpretation of these subtle differences have created taxonomic difficulties at nearly every level of classification within the family (Clausen, 1938; Hauk, 1995; Tryon and Tryon, 1982).

As typically circumscribed, three genera constitute Ophioglossaceae: *Ophioglossum* L., *Helminthostachys* Kaulf., and *Botrychium* Sw. (Bower, 1926; Clausen, 1938; Eames, 1936; Tryon and Tryon, 1982; Wagner, 1990). A fourth genus, *Mankyua* B.-Y. Sun, M.H. Kim, and C.H. Kim, has also been described (Sun et al., 2001). *Ophioglossum* (adder's tongue fern, hand fern) is nearly cosmopolitan, *Botrychium* (moonwort, grapefern, and rattlesnake fern) is principally temperate/boreal, and monotypic *Helminthostachys* is distributed more narrowly in lowland Indo-Malayan and Australasian regions (Wagner, 1990). *Mankyua* is reported only from Cheju Island off the coast of Korea (Sun et al., 2001). The classification of *Botrychium* used here will follow that of Kato (1987), who advocated division of a broadly defined genus *Botrychium* sensu lato (s.l.) into four separate genera: *Botrychium* sensu stricto (s.s.), *Sceptridium* Lyon, *Japanobotrychium* Masam., and *Botrypus* Michx. (=subgenus *Osmundopteris* (Milde) R.T. Clausen). Kato's system is employed not to validate particular generic concepts or ranking philosophies, but rather to subdivide *Botrychium* s.l. into manageable units for the purposes of analysis and discussion. Likewise, after Presl (1845), we treat *Ophioglossum* s.l. as four separate genera, *Ophioglossum* s.s., *Rhizoglossum* C. Presl, *Ophioderma* (Blume) Endl., and *Cheiroglossa* C. Presl.

Assessments of evolutionary relationships in Ophioglossaceae are usually based on trends present in relatively few morphological characters such as leaf venation, degree of leaf dissection, sporangia structure, vernation, and gametophyte construction (Bower, 1926; Clausen, 1938). However, the genera of Ophioglossaceae possess a confusing mixture of presumed primitive and derived character states for many of these features (Bower, 1926; Chrysler, 1945; Clausen, 1938; Kato, 1978, 1987). In *Ophioglossum* s.l., reticulate venation, entire leaves, and embedded sporangia are generally considered derived character states, but the cylindrical, branched gametophytes are thought to be primitive (Bower, 1926; Clausen, 1938; Kato, 1987). In *Botrychium* s.l., free dichotomous venation, dissected leaves, and stalked sporangia are regarded as ancestral character states, but the simple, unbranched, and dorsiventrally flattened gametophytes are putatively more derived (Bower, 1926; Clausen, 1938; Kato, 1987). *Helminthostachys* possesses both the free venation typical of *Botrychium* s.l. and the anastomosing venation present in *Ophioglossum* s.l. (Bhambie and Madan, 1982) while exhibiting unique features such as a vertical, dorsiventral rhizome, and sterile appendages within the sporophore (Bierhorst, 1971). Bower (1926), Chrysler (1941), Clausen (1938), and Kato (1978) regarded *Ophioglossum* s.s. as relatively derived in the family, and *Botrychium* s.l. (or subgroups of *Botrychium* s.l.) as diverging earlier from the common ancestor. Clausen (1938) considered *Helminthostachys* nearly intermediate between *Ophioglossum* s.l. and *Botrychium* s.l., whereas Bower (1926) suggested that *Helminthostachys* is relatively isolated from other members of the family.

Interpretation of character evolution in Ophioglossaceae has long been problematic, perhaps because evolutionary scenarios have been deduced based on trends present in other plant groups that either may not be closely related or are not subject to similar environmental conditions or evolutionary constraints. Competing hypotheses of phylogenetic reconstruction within *Botrychium* s.l. document the ambiguity involved in interpreting character evolution within Ophioglossaceae. The relatively large, dissected trophophores of *Botrypus* and *Japanobotrychium* are often considered primitive and the much-reduced trophophores of *Botrychium* s.s. derived, whereas erect leaves of *Botrychium* s.s. are regarded as primitive and the reflexed leaves of *Sceptridium*, *Botrypus*, and *Japanobotrychium* derived (Clausen, 1938). Bower (1926) concluded that *Botrychium* s.l. is the most "primitive" group, whereas Kato (1987) considered *Botrypus* sister to all other genera and *Botrychium* s.s. and *Ophioglossum* s.l. more recently derived. Clausen (1938) regarded *Sceptridium* as "primitive" and *Ophioglossum* s.l. more recently derived. Clearly, there has been no consensus.

Kato (1987) was the first to apply cladistic methods to questions of character evolution and phylogeny in Ophioglossaceae. His analysis included 13 morphological characters from the six groups within Ophioglossaceae that he apparently presumed were monophyletic: *Ophioglossum* s.l., *Helminthostachys*, *Botrypus*, *Japanobotrychium*, *Sceptridium*, and *Botrychium* s.s. Kato's results explicitly challenged the monophyly of *Botrychium* s.l. by placing *Sceptridium* and *Helminthostachys* in a clade sister to *Ophioglossum* s.l. and *Botrychium* s.s. Accordingly, Kato recognized *Botrypus*, *Japanobotrychium*, *Sceptridium*, and *Botrychium* s.s. as distinct genera.

Within lineages of great antiquity (e.g., ferns), high levels of homoplasy, hybridization, and extinction of intermediate groups have complicated attempts to interpret morphological character evolution (Pryer et al., 1995). DNA sequencing is currently providing important insights into fern phylogeny (Pryer et al., 2001a). A combined analysis of four plastid and one nuclear DNA regions has established well-supported relationships among ancient ferns and fern allies, with Psilotaceae sister to Ophioglossaceae (Pryer et al., 2001a). Analysis of sequence variation in the protein-coding gene *rbcL*

has produced explicit hypotheses of phylogenetic relationships among leptosporangiate fern families (Hasebe et al., 1993, 1994, 1995; Pryer et al., 2001a; Pryer et al., 1995; Wolf et al., 1994; Wolf et al., 1999). Several studies have utilized molecular techniques to facilitate phylogenetic reconstruction within leptosporangiate fern families (Crane et al., 1995; Dubuisson, 1997; Gastony and Rollo, 1995, 1998; Gastony and Ungerer, 1997; Haufler and Ranker, 1995; Murikami et al., 1999; Pryer et al., 2001b; Sano et al., 2000; Wolf, 1995; Wolf et al., 1994). However, no molecular studies have addressed generic-level relationships within the eusporangiate ferns.

To address questions of phylogenetic relationships and character evolution within Ophioglossaceae, we used protein-coding plastid DNA sequences (*rbcL*), non-coding plastid DNA sequences (*trn*L_{UAA}-F_{GAA} intergenic spacer region; Taberlet et al., 1991), and morphological data. Our objectives for this study were to: (1) evaluate the monophyly of Ophioglossaceae, (2) identify monophyletic species groups within Ophioglossaceae, (3) establish sister–group relationships among these monophyletic groups, (4) compare results based on coding and non-coding plastid DNA sequences, and (5) compare molecular patterns to those derived from morphological data to provide a sound basis for interpreting character evolution within the family.

2. Materials and methods

2.1. DNA extraction, PCR amplification, and sequencing

Leaves from 36 species representing five of the six presumably monophyletic groups cited in Kato (1987) (i.e., *Ophioglossum* s.l., *Helminthostachys*, *Botrypus*, *Japanobotrychium*, *Sceptridium*, and *Botrychium* s.s.) were silica-gel dried (Chase and Hills, 1990). Material from *Japanobotrychium lanuginosum*, *Rhizoglossum* (= *Ophioglossum bergianum*), and the recently described *Mankyua chejuense* was not available for analysis. Table 1 lists all taxa included in this study, voucher information, collection sites, and GenBank accession numbers for both *rbcL* and *trnL-F* sequences. One species of *Sceptridium* from India was not determined to species and is referred to as "*Sceptridium* sp." DNA extraction, amplification, purification of amplified products, and sequencing of *rbcL* follow procedures outlined in Hauk (1995). Eight internal sequencing primers for *rbcL* were designed for manual sequencing of Ophioglossaceae. The *trnL-F* spacer region was amplified and sequenced using the "e" and "f" primers of Taberlet et al. (1991). Procedures for amplification and sequencing of the *trnL-F* spacer were identical to those used for *rbcL*, except that no internal primers were necessary.

2.2. Molecular data and phylogenetic analyses

All *trnL-F* sequences were aligned using the computer program CLUSTAL W (Thompson et al., 1994) followed by minor manual adjustments. Deletions were coded as missing data; all unambiguous indels were added to the sequence data matrix by coding them as binary (present/absent) characters. Alignment of *rbcL* sequences was accomplished easily by eye because no indels were present. For the *rbcL* and combined analyses, outgroups for Ophioglossaceae (Psilotaceae and Marattiaceae) were selected based on analyses of extant fern relationships (Hauk and Chase, 1991; Manhart, 1995; Pryer et al., 2001a) and are listed in Table 1. *Equisetum* was not used in our analyses because, although *Equisetum* was sister to Marattiaceae in the multigene analysis of Pryer et al. (2001a), bootstrap support for that relationship was relatively weak (62%), and *Equisetum* was extremely sequence divergent. In single gene analyses, the placement of *Equisetum* close to Ophioglossaceae, Psilotaceae, or Marattiaceae was not well supported (Manhart, 1994; Manhart, 1995). A larger analysis (not shown), including 30 taxa representing the diversity of leptosporangiate ferns, was conducted and showed relationships consistent with those produced by use of only Psilotaceae and Marattiaceae.

Sequences were analyzed using PAUP* 4.0b6 (Swofford, 2001). A maximum parsimony (heuristic) analysis of 1000 random taxon entries was conducted on the *rbcL* data using TBR (tree-bisection–reconnection) swapping and MulTrees on (saving multiple, equally parsimonious trees), no tree limit with all characters weighted equally and unordered (Fitch parsimony; Fitch, 1971). Bootstrap analyses (Felsenstein, 1985) of 1000 iterations with 10 random additions per iteration were conducted to evaluate internal support (Figs. 1, 2, and 4). Because outgroup sequences could not be aligned, the *trnL-F* tree (Fig. 2) was arranged using midpoint rooting (as implemented in PAUP* 4.02b6). This was done to determine whether the root within Ophioglossaceae would be attached to the same node as in the analysis of *rbcL* including outgroups, which was the case, so we show the *trnL-F* trees oriented in the same manner (with the same hierarchical structure) as those produced by the analysis of *rbcL*. The basic assumption of mid-point rooting is that the node at which divergence is greatest is the root node (i.e., it is the deepest node in the tree). The morphological data (Fig. 3) were analyzed in the same manner as the *trnL-F* sequences (see below).

A combined Fitch analysis of all three datasets was also conducted (Fig. 4) using the same procedures as in the *rbcL* analysis. No combinability tests were performed because these have been shown to be unreliable (Reeves et al., 2001; Yoder et al., 2001). Node by node inspection has been advocated as the best way to infer

Table 1
List of species, voucher numbers, collection sites, and GenBank accession numbers for 36 species of Ophioglossaceae sequenced for *rbcL* and/or *trnL-F*

Species	Voucher/Herbarium	Site	GenBank *rbcL*	GenBank *trnL-F*
Botrychium ascendens W.H. Wagner	Hauk 529/NCU	Hurricane Creek, Oregon, USA	L40982*	AY138422
Bo. campestre W.H. Wagner and Farrar	Farrar s.n./ISC	Iowa, USA	L40961*	AY138426
Bo. crenulatum W.H. Wagner	Hauk 616/NCU	Hurricane Creek, Oregon, USA	L40959*	AY138431
Bo. lanceolatum (S.G. Gmel.) Ångstr.	Hauk 571/NCU	Taquamenon Falls, Michigan, USA	L40963*	AY138432
Bo. lineare W.H. Wagner	Hauk 581/NCU	Pike's Peak, Colorado, USA	L40964*	AY138425
Bo. lunaria (L.) Sw.	Hauk 564/NCU	Marathon, Ontario, Canada	L40965*	AY138430
Bo. montanum W.H. Wagner	Hauk 607/NCU	Lake Co., Montana, USA	L40916*	AY138429
Bo. paradoxum W.H. Wagner	Hauk 610/NCU	Waterton, Alberta, Canada	L40972*	AY138424
Bo. pedunculosum W.H. Wagner	Hauk 615/NCU	Lostine River, Oregon, USA	L40973*	AY138434
Bo. pinnatum H. St. John	Hauk 604/NCU	Stagger Inn, Washington, USA	L40974*	AY138433
Bo. pumicola Coville	Hauk 618/NCU	Newberry Caldera, Oregon, USA	L40976*	AY138428
Bo. simplex E. Hitchc.	Hauk 619/NCU	Mt. Ashland, Oregon, USA	L40978*	AY138427
Bo. spathulatum W.H. Wagner	Wagner 88036/MICH	Angler, Ontario, Canada	L40980*	AY138423
Botrypus strictus (Underw.) Holub	Sahashi s.n./OS	Japan	AY138408	AY138444
Bp. virginianus (L.) Michx.	Hauk 575/OS	Grand Sable Dunes, Michigan, USA	AY138407	AY138443
Cheiroglossa palmata (L.) C. Presl	R. Peet s.n./NCU	Toosohotchee State Reserve, Florida, USA	AY138421	AY138455
Helminthostachys zeylanica (L.) Hook.	Sahashi s.n./TOHO Hauk/OS	Japan	AY138409	AY138445
Ophioderma pendula (L.) Presl	RGB Kew 1976-06412/K	Kew Gardens, London, UK	AY138420	–
Ophioglossum costatum R. Br.	Burrows 5766/K	Zambia	AY138418	AY138453
Op. crotalophoroides Walter	Leonard 9380/NCU	Mississippi, USA	AY138417	AY138452
Op. gomezianum Welw. ex A. Braun	Burrows 5767/K	Zambia	AY138419	AY138454
Op. gramineum Willd.	RGB Kew 1981-6838/K	London, UK	AY138412	AY138448
Op. nudicaule L.	Wurdack s.n./NCU	Georgia, USA	AY138416	–
Op. petiolatum Hook.	RGB Kew 1988-03791/K	London, UK	AY138411	AY138447
Op. pusillum Raf.	Nekola 8069/COE	Coggon Pond, Iowa, USA	AY138413	AY138449
Op. reticulatum L.	Moran 5644/MO	Honduras	AY138410	AY138446
Op. richardsiae J.E. Burrows	Burrows 5756/K	Zambia	AY138415	AY138451
Op. vulgatum L.	Burrows 5752/K	Zambia	AY138414	AY138450
Sceptridium atrovirens Sahashi	Sahashi s.n./TOHO	Japan	AY138402	AY138438
S. dissectum (Spreng.) Lyon	Hauk 621/NCU	Chapel Hill, North Carolina, USA	AY138401	AY138436
S. japonicum (Prantl) Lyon	Sahashi s.n./TOHO	Japan	AY138403	AY138439
S. lunarioides (Michx.) Holub	Watkins 29/ISC	Pleasant Hill Cemetery, Dale Co., Alabama, USA	AY138406	AY138442
S. multifidum (S.G. Gmel.) Nishida ex Tagawa	Hauk 577/NCU	Grand Sable Dunes, Michigan, USA	AY138400	AY138435
S. oneidense (Gilbert) Holub	Wagner 8600/MICH	Michigan, USA	AY138898	AY138437
S. ternatum (Thunb.) Lyon	Sahashi s.n./TOHO	Japan	AY138404	AY138440
Sceptridium. sp.	Madhusoodanan s.n./ CUH	India	AY138405	AY138441
Angiopteris evecta (G. Forst.) Hoffm.	Nagata 12/20/88/	Philippines	AY138397	–
Danaea nodosa (L.) Sm.	Sharpe JS94105/COA	Puerto Rico, USA	AY138398	–
Marattia sp.	Hauk 697/NCU	Philippines	AY138399	–
Psilotum nudum (L.) P. Beauv.			L1 1059	–
Tmesipteris sp.			U30836	–

Outgroup taxa for the *rbcL* analysis are included. Asterisk indicates GenBank numbers published in Hauk (1995). No *trnL-F* sequence was recovered from *Ophioglossum nudicaule, Ophioderma pendulum*, or the outgroups.

which phenomena could be responsible for incongruence among independent data sets (Reeves et al., 2001; Wiens, 1998), and we use this procedure here. For both the *trnL-F* and morphological data sets, midpoint rooting was consistent with placing the root where the *rbcL* analysis indicated (i.e., between *Cheiroglossa* + *Ophioderma* + *Ophioglossum* s.s. and the remainder of the family). Thus, in the combined analyses, the root was determined by outgroup comparison via the *rbcL* data set. A combined analysis of *rbcL* and *trnL-F* data was conducted to provide a template for examining morphological character/character state changes within the family (Fig. 5) but this could as well have been conducted on the combined analysis of all data because the tree topology was virtually identical (i.e., only the position of *O. crotalophoroides* of *Ophioglossum* s.s. differed in the two trees). Analyses, choice of outgroups, and root placement for the *rbcL* + *trnL-F* analysis were

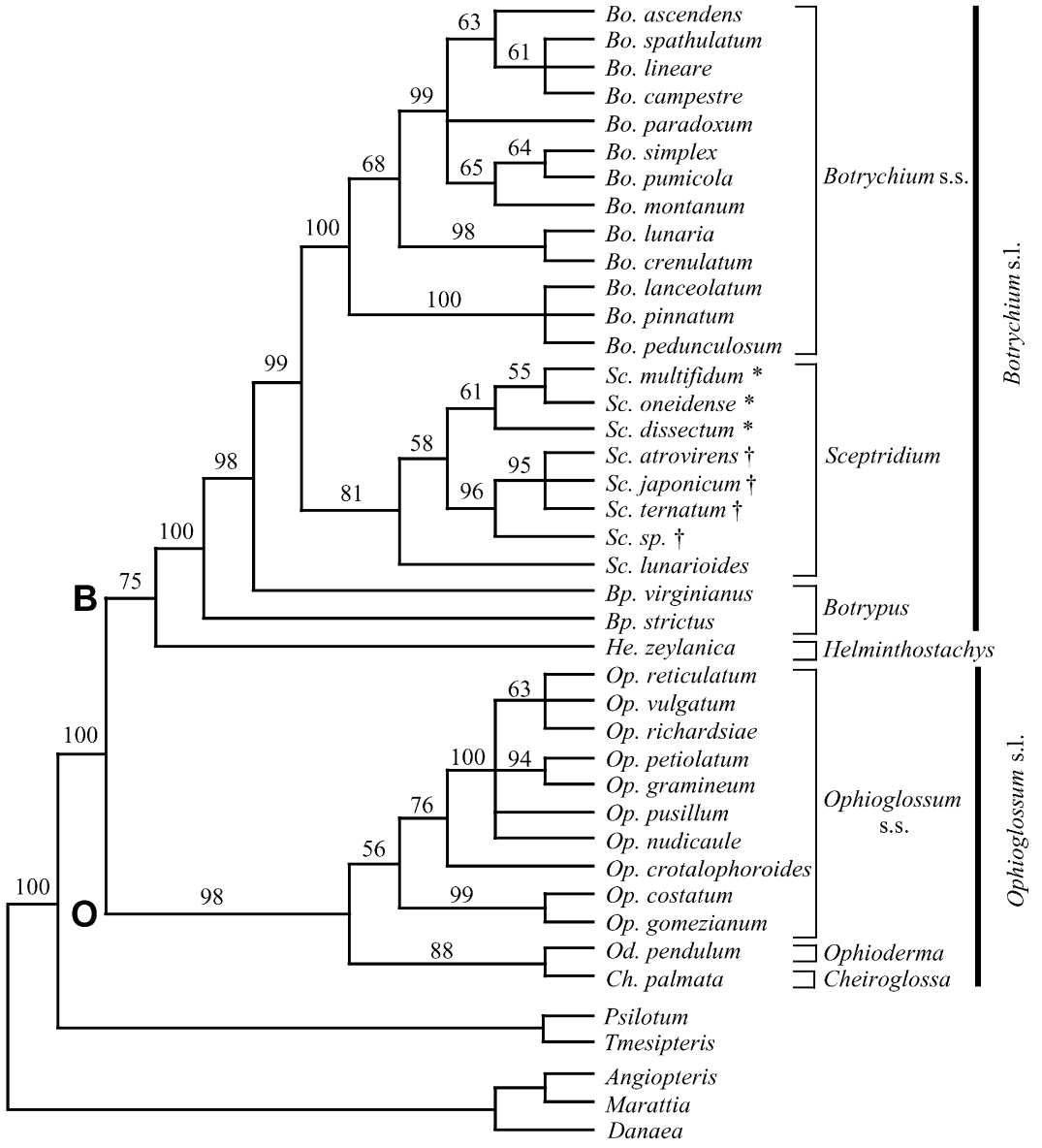

Fig. 1. Strict consensus of four equally most parsimonious trees (length = 889) generated from analysis of *rbcL* data. Numbers above branches are bootstrap percentages. Large, bold print "B" and "O" indicate the base of the botrychioid and ophioglossoid lineages, respectively. An asterisk (*) indicates North American collections of *Sceptridium* species and dagger (†) indicates Asian collections. Psilotaceae and Marattiaceae were designated as outgroups. *Bo.*, *Botrychium* s.s.; *Sc.*, *Sceptridium*; *Bp.*, *Botrypus*; *He.*, *Helminthostachys*; *Op.*, *Ophioglossum* s.s.; *Od.*, *Ophioderma*; and *Ch.*, *Cheiroglossa*.

the same as for the combined *rbcL*, *trnL-F*, and morphological analysis.

2.3. Morphological data and analyses

Morphological characters used in this investigation (Tables 2 and 3) were assembled primarily from previously published sources. All characters were evaluated for independence prior to data matrix construction. Midpoint rooting was employed instead of outgroup rooting because the extreme reduction in morphological complexity of Psilotaceae precludes insight into ancestral states of Ophioglossaceae for all characters of the trophophore (e.g., architecture, primordia pubescence, venation, stomata, and leaf sheath covering), sporophore (e.g., branching), and perhaps characters associated with the sporangium (e.g., initiation, attachment, and dehiscence). The unique structure and enigmatic origin of the ophioglossaceous "leaf" make direct comparisons to outgroups tenuous for many characters (Chrysler, 1945; Clausen, 1938; however, see Nishida, 1957).

(1) Wintergreen phenology—absent (0) or present (1). Species of *Sceptridium* possess leaves that emerge in

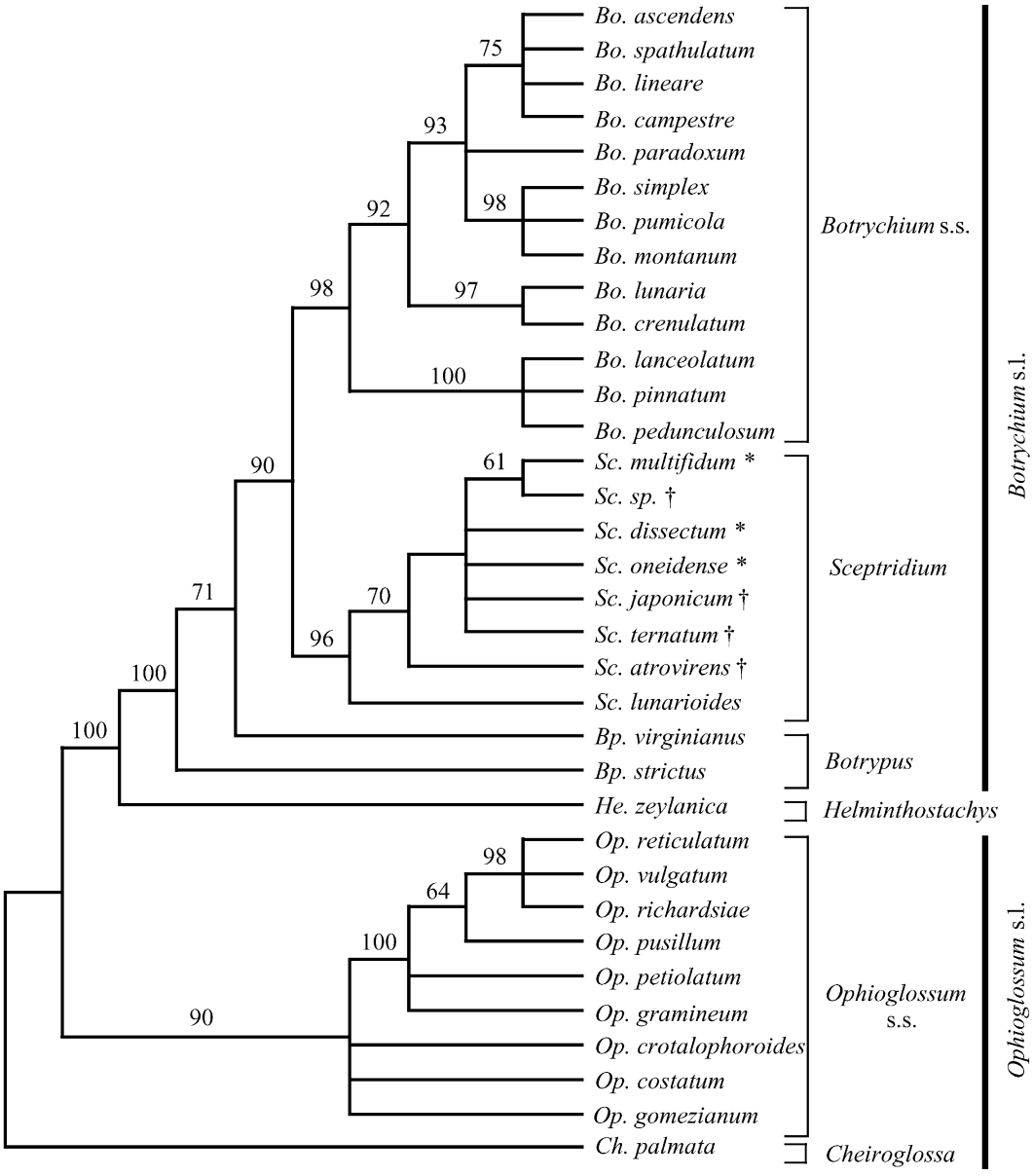

Fig. 2. Strict consensus of 277 equally most parsimonious trees (length = 605) from analysis of *trnL-F* data. Midpoint rooting was used for the analysis. Numbers above the branches are bootstrap percentages. An asterisk (*) indicates North American collections of *Sceptridium* species and dagger (†) indicates Asian collections. *Bo.*, *Botrychium* s.s.; *Sc.*, *Sceptridium*; *Bp.*, *Botrypus*; *He.*, *Helminthostachys*; *Op.*, *Ophioglossum* s.s.; *Od.*, *Ophioderma*; and *Ch.*, *Cheiroglossa*.

midspring to summer and persist throughout winter into the following spring or summer (Wagner, 1990). All other temperate species groups in Ophioglossaceae emerge during early spring or summer and subsequently senesce, remaining dormant until the next spring.

(2) Trophophore architecture—ternate (0), lobed/pinnate (1), simple/entire (2), or bifurcating/entire (3). *Ophioglossum* s.s. exhibits simple, entire trophophores, whereas *Cheiroglossa* and *Ophioderma* have simple, bifurcating leaves (Clausen, 1938). *Helminthostachys*, *Botrypus*, and *Sceptridium* have ternate trophophore construction, whereas all species of *Botrychium* s.s. except *Botrychium lanceolatum* have lobed/pinnate construction (Clausen, 1938).

(3) Leaf primordia pubescence—present (0) or absent (1). Wagner (1990) reported *Sceptridium*, *Botrypus*, and *Japanobotrychium* have pilose developing leaf primordia. *Botrychium* s.s. does not possess hairy leaf primordia, and we have found no record of leaf primordia pubescence in *Ophioglossum* s.s., *Ophioderma*, *Cheiroglossa*, or *Helminthostachys*.

(4) Leaf sheath covering—incomplete (0) or complete (1). In Ophioglossaceae the primary meristem and leaf

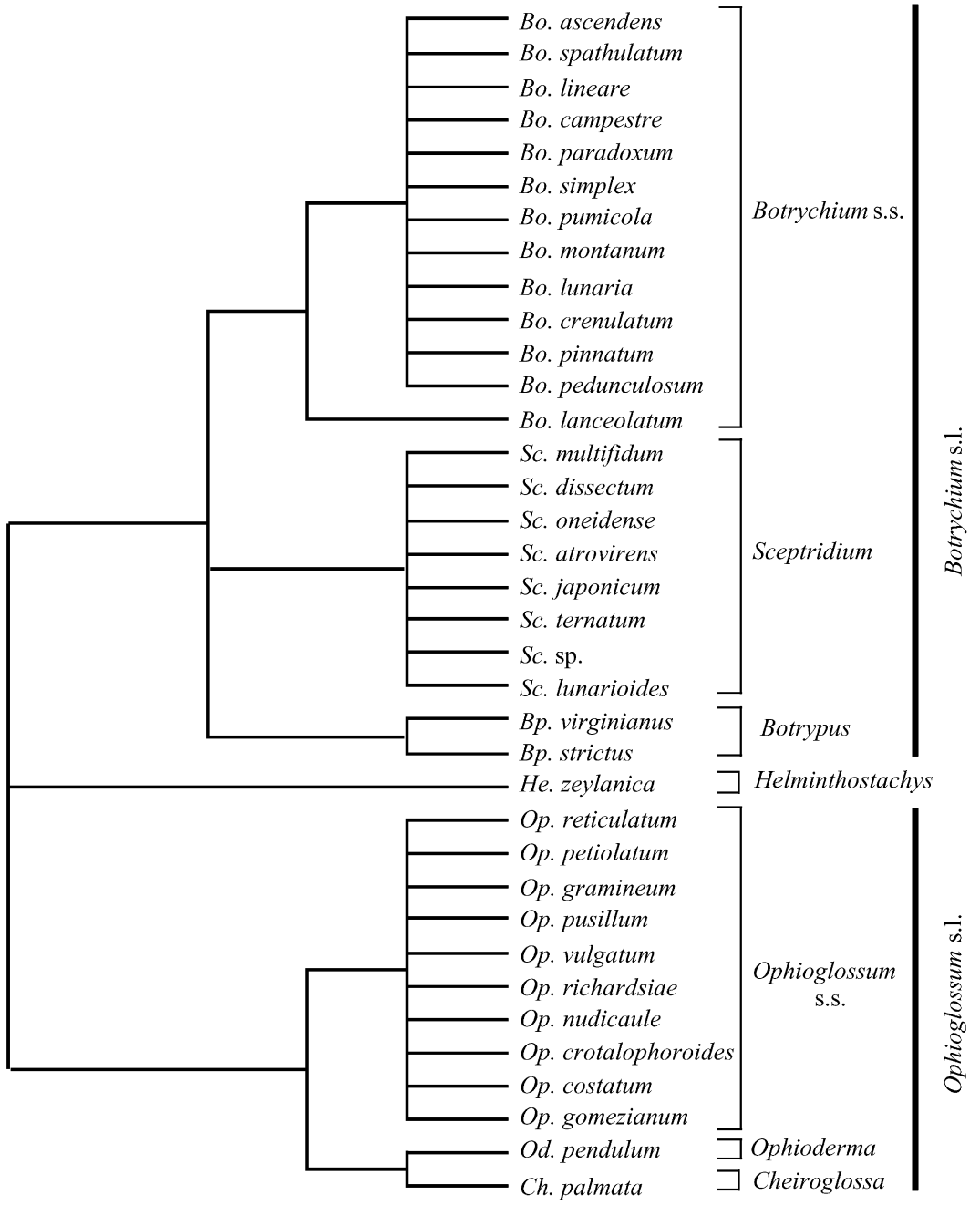

Fig. 3. The strict consensus of the most parsimonious trees (length = 24) produced from analysis of 20 morphological characters of Ophioglossaceae. Midpoint rooting was used for the analysis. *Bo.*, *Botrychium* s.s.; *Sc.*, *Sceptridium*; *Bp.*, *Botrypus*; *He.*, *Helminthostachys*; *Op.*, *Ophioglossum* s.s.; *Od.*, *Ophioderma*; and *Ch.*, *Cheiroglossa*.

primordia are enclosed by the sheathing leaf bases of older, more developed fronds (Bower, 1926; Clausen, 1938; Eames, 1936; Gifford and Foster, 1989; Imaichi, 1989; McAlpin, 1971; Wagner, 1990). *Botrypus* exhibits an incomplete leaf sheath covering, although Imaichi (1989) reported differences between the degree of leaf sheath closure in *Botrypus strictus* and *Botrypus virginianus*. Kato (1987) reported a complete leaf sheath covering with a transversely elongate, small pore in *Japanobotrychium*, *Sceptridium*, *Botrychium*, *Helminthostachys*, and *Ophioglossum* s.s., although the pore is oblique in *Ophioglossum* s.s. and lacking in adult leaves of *Botrychium* s.s. Information on the leaf sheath was not available for *Cheiroglossa* or *Ophioderma*.

(5) Venation—free (0) or anastomosing (1). Venation in *Botrypus*, *Japanobotrychium*, *Sceptridium*, and *Botrychium* is free, whereas anastomosing patterns occur in *Ophioglossum* s.l. *Helminthostachys* exhibits predomi-

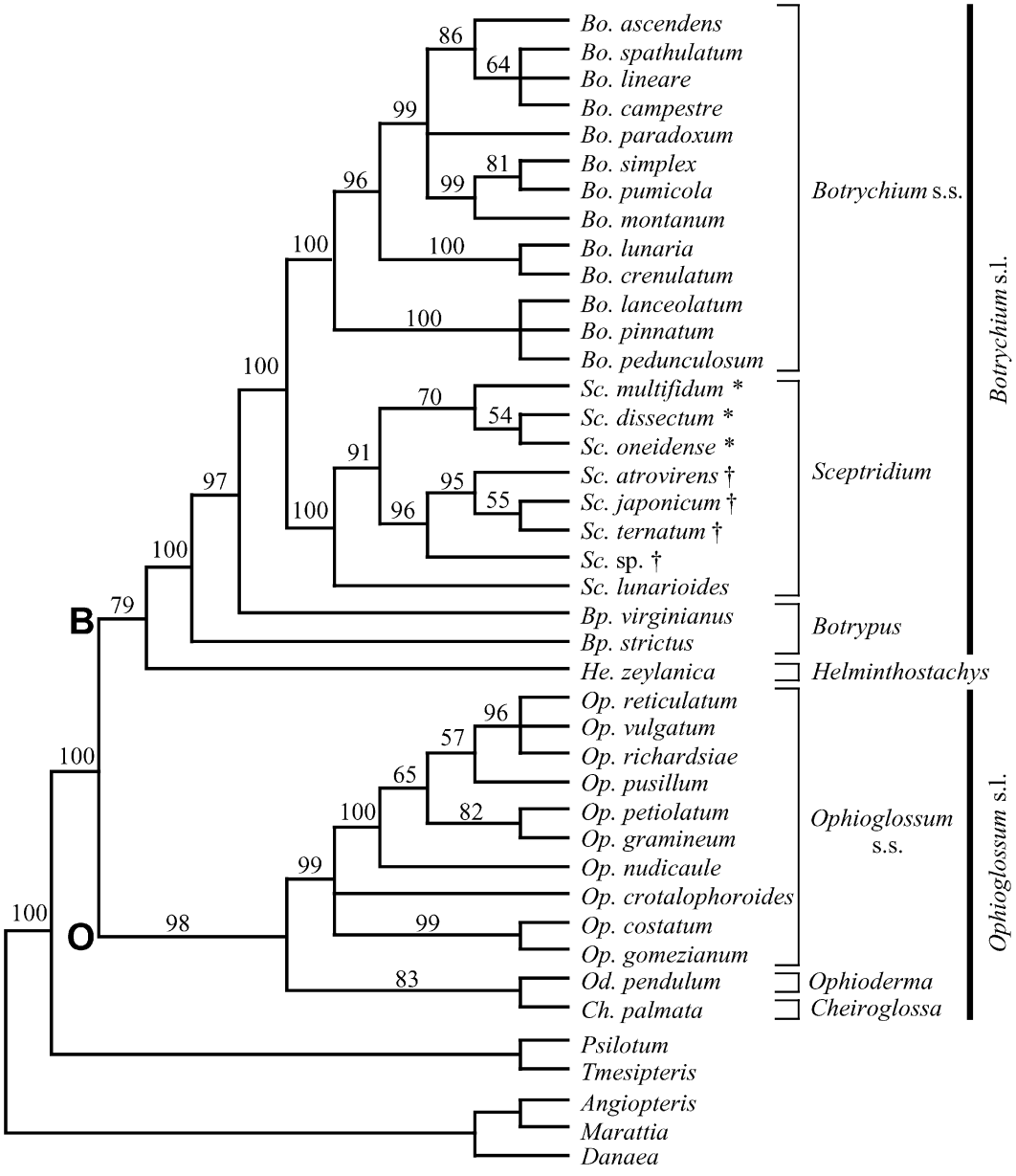

Fig. 4. Strict consensus of 12 equally most parsimonious trees (length = 1529) from analysis of combined *rbcL*, *trnL-F*, and morphological data. Numbers above the branches are bootstrap percentages. Psilotaceae and Marattiaceae were designated as outgroups for the *rbcL* portion of the dataset. Large, bold print "B" and "O" indicate the base of the botrychioid and ophioglossoid lineages, respectively. An asterisk (*) indicates North American collections of *Sceptridium* species and dagger (†) indicates Asian collections. *Bo.*, *Botrychium* s.s.; *Sc.*, *Sceptridium*; *Bp.*, *Botrypus*; *He.*, *Helminthostachys*; *Op.*, *Ophioglossum* s.s.; *Od.*, *Ophioderma*; and *Ch.*, *Cheiroglossa*.

nantly free venation but has anastomosing veins as well (Bhambie and Madan, 1982).

(6) Free included veinlets—absent (0) or present (1). Free included veinlets occur in *Ophioglossum* s.s. (Wagner, 1990) and *Rhizoglossum*. None are reported in *Cheiroglossa* (Britton, 1897; though see illustration in Chrysler, 1941), *Ophioderma* (Wagner, 1990), *Helminthostachys* (Bhambie and Madan, 1982), or *Botrychium* s.l. (Bhambie and Madan, 1982).

(7) Sporophore branching—unbranched (0) or branched (1). The sporophores of *Botrychium* s.l. are branched pinnately, whereas Bower (1926) reported that although sporophore branching can occur in *Helminthostachys*, this is not the norm. The sporophore of *Helminthostachys* bears sporangiophores (Gifford and Foster, 1989), which may represent a reduced form of pinnate branching. However, because *Helminthostachys* does not demonstrate unambiguously a branched sporophore, we coded it as unbranched. The sporophores of *Ophioglossum* s.s.,*Ophioderma*, and *Cheiroglossa* are not branched (although several separate sporophores are borne inserted on trophophores of *Cheiroglossa*).

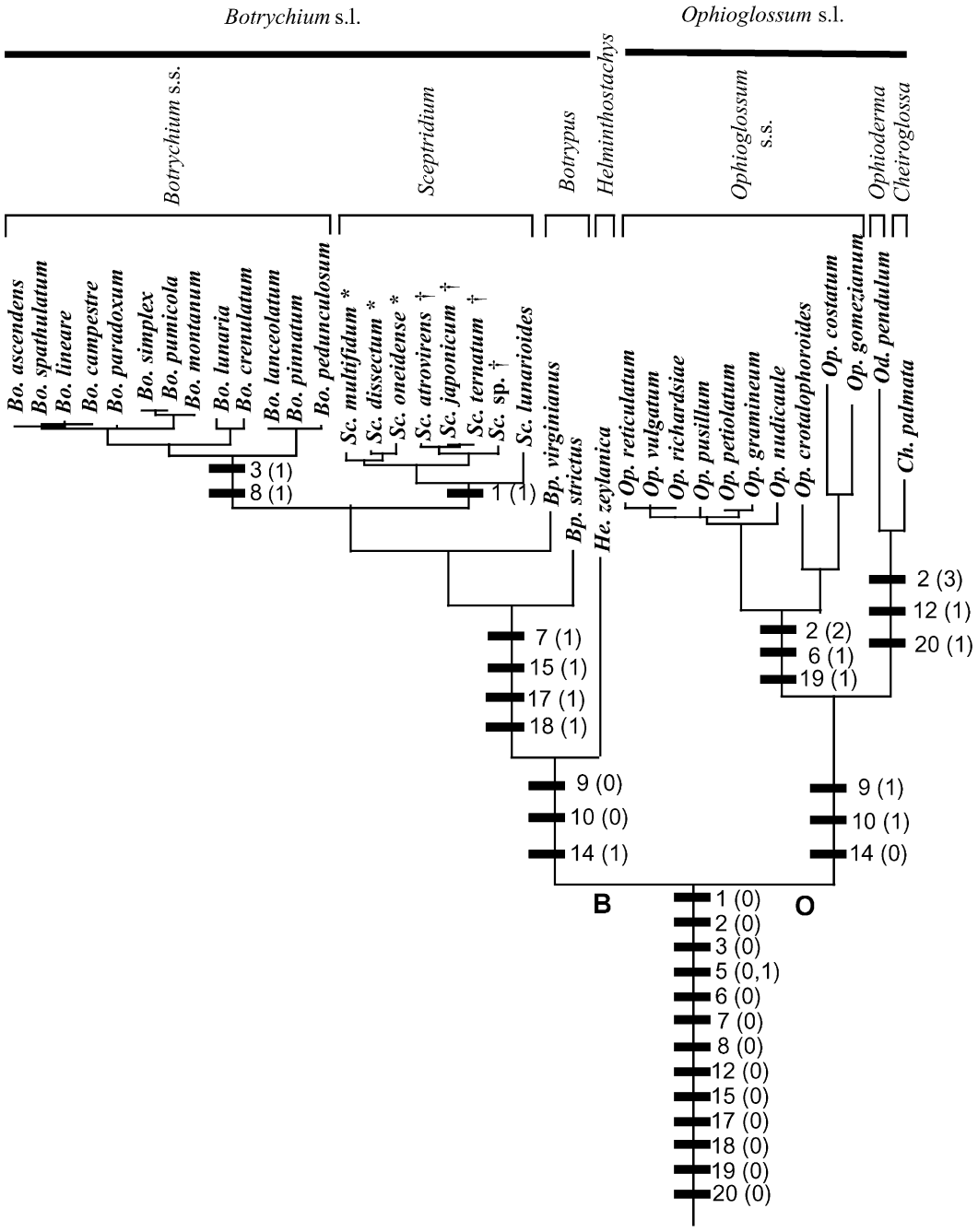

Fig. 5. One of the six equally most parsimonious trees for relationships of Ophioglossaceae generated from analysis of combined *rbcL* and *trnL-F* data. Branch lengths are proportional to amount of change (except for branch extending to outgroups, which are not shown). Only non-homoplastic morphological characters were mapped onto the tree, thus characters 4, 11, 13, and 16 are not shown. Numbers correspond to characters listed in Table 2 and character states are indicated in Table 3. Large, bold "B" and "O" indicate the base of the botrychioid and ophioglossoid lineages, respectively. *Bo.*, *Botrychium* s.s.; *Sc.*, *Sceptridium*; *Bp.*, *Botrypus*; *He.*, *Helminthostachys*; *Op.*, *Ophioglossum* s.s.; *Od.*, *Ophioderma*; and *Ch.*, *Cheiroglossa*.

(8) Sporophore presence—commonly abortive (0) or always present (1). Wagner and Wagner (1993) cited members of *Botrychium* s.s. as the only group within North American *Botrychium* s.l. that always produce a sporophore in conjunction with the trophophore. *Sceptridium* and *Botrypus* are commonly observed without sporophores.

(9) Sporangium initiation—single superficial cell (0) or sporangiogenic band (1). Burlingame (1907) reported that the sporangia of *Botrychium* s.l. and *Helmintho-*

Table 2
Characters, character states, and coding for 20 characters utilized in construction of morphological dataset of Ophioglossaceae

Character	(0)	(1)	(2)	(3)
1. Wintergreen phenology	Absent	Present		
2. Trophophore architecture	Ternate	Lobed/pinnate	Simple/entire	Bifurcating/entire
3. Leaf primordia pubescence	Present	Absent		
4. Leaf sheath covering	Complete	Incomplete		
5. Venation	Free	Anastomosing		
6. Free included veinlets	Absent	Present		
7. Sporophore branching	Unbranched	Branched		
8. Sporophore presence	Commonly abortive	Always present		
9. Sporangium initiation	Single cell	Sporangiogenic band		
10. Sporangium attachment	Stalked	Embedded		
11. Sporangium dehiscence	Transverse	Longitudinal/subtransverse		
12. Rhizome apex scales	Absent	Present		
13. Vascular branching	Extra-marginal	Marginal		
14. Axillary buds	Absent	Present		
15. Vascular cambium	Absent	Present		
16. Suspensor	Absent	Present		
17. Gametophyte symmetry	Axial	Dorsiventral		
18. Gametophyte branching	Branched	Unbranched		
19. Leaf stomata position	Hypostomatic	Amphistomatic		
20. Stomata construction	Absent/anomocytic	Cyclocytic		

See Table 3 for data matrix.

Table 3
List of morphological characters and coded character states for species or species groups within Ophioglossaceae

Character	BO	BOL	SC	SCL	BP	BS	HS	OP	OD	CH
1. Wintergreen phenology	0	0	1	1	0	0	0	0	0	0
2. Trophophore architecture	1	0	0	0	0	0	0	2	3	3
3. Leaf primordia pubescence	1	1	0	0	0	0	–	–	–	–
4. Leaf sheath covering	0	0	0	0	1	1	0	0	0	0
5. Venation	0	0	0	0	0	0	0 and 1	1	1	1
6. Free included veinlets	0	0	0	0	0	0	0	1	0	0
7. Sporophore branching	1	1	1	1	1	1	0	0	0	0
8. Sporophore presence	1	1	0	0	0	0	0	0	0	0
9. Sporangium initiation	0	0	0	0	0	0	0	1	1	1
10. Sporangium attachment	0	0	0	0	0	0	0	1	1	1
11. Sporangium dehiscence	0	0	0	0	1	1	0	0	0	0
12. Rhizome apex scales	–	–	–	–	–	–	–	0	1	1
13. Vascular branching	1	1	0 and 1	0 and 1	0	0	0	1	1	1
14. Axillary buds	1	1	1	1	1	1	1	0	0	0
15. Vascular cambium	1	1	1	1	1	1	0	0	0	0
16. Suspensor	0	0	0 and 1	1	0	0	1	0	–	0
17. Gametophyte symmetry	1	1	1	1	1	1	0	0	0	0
18. Gametophyte branching	1	1	1	1	1	1	0	0	0	0
19. Leaf stomata position	0	0	0	0	0	0	0	1	0	0
20. Stomata construction	0	0	0	0	0	0	0	0	1	1

Table 2 details character states and coding. BO, *Botrychium* s.s.; BOL, *Botrychium lanceolatum*; SC, *Sceptridium*; SCL, *Sceptridium lunarioides*; BP, *Botrypus virginianus*; BS, *Botrypus strictus*; HS, *Helminthostachys*; OP, *Ophioglossum* s.s.; OD, *Ophioderma pendulum*; and CH, *Cheiroglossa palmata*. "–," missing data.

stachys develop primarily from single superficial cells, whereas the sporangia of *Ophioglossum* s.s and *Ophioderma* originate from 1 to 2 superficial cells with a much larger contribution from surrounding sterile tissues. Chrysler (1941) described sporangium development of *Cheiroglossa* as involving divisions within the sporangiogenic band.

(10) Sporangium attachment—stalked sporangia (0) or embedded sporangia (1). All species of *Ophioglossum* s.s., *Ophioderma*, and *Cheiroglossa* have two rows of sporangia sunken into and surrounded by laminar tissues, the whole of which is referred to as a spike (Bower, 1926). *Botrychium* s.l. and *Helminthostachys* have sporangia borne on stalks, although in the latter the stalks are reduced (Wagner, 1990).

(11) Sporangium dehiscence—transverse (0) or longitudinal/subtransverse (1). *Helminthostachys*, *Japanobotrychium*, *Sceptridium*, and *Botrychium* s.s. possess

transverse sporangial dehiscence, whereas *Botrypus* exhibits longitudinal/subtransverse dehiscence (Kato, 1978, 1982, 1983). Sporangial dehiscence in *Ophioglossum*, *Ophioderma*, and *Cheiroglossa* is also transverse (Kato, 1987).

(12) Rhizome apex scales—absent (0) or present (1). Tryon and Tryon (1982) reported that *Ophioglossum* and *Rhizoglossum* have glabrous rhizome apices, whereas *Cheiroglossa* and *Ophioderma* posses scales or hairs. Data for *Botrychium* s.l. were not available.

(13) Vascular branching in leaf axis—extra-marginal (0) or marginal (1). Bower (1923) recognized marginal and extra-marginal as the two basic types of vascular trace branching patterns in fern leaves and reported marginal departure for *Ophioglossum* s.s., *Cheiroglossa*, *Ophioderma* (presumably), and most species of *Botrychium* s.s. Kato (1978, 1987) reported extra-marginal branching in *Botrypus*, *Japanobotrychium*, and *Helminthostachys* and marginal branching in *Ophioglossum* s.l. and *Botrychium* s.s. Because both types of branching occur in *Sceptridium* (Kato, 1978), vascular branching in *Sceptridium* was coded as polymorphic.

(14) Axillary buds—absent (0) or present (1). Lang (1913) and Petry (1915) reported axillary buds in *Botrychium* s.s., *Sceptridium*, and *Botrypus*. Lang (1915) and Kato et al. (1988) documented axillary buds in *Helminthostachys*. *Ophioglossum* s.s., *Ophioderma*, and *Cheiroglossa* do not exhibit buds in leaf axils. Because axillary buds are otherwise known in extant Filicopsida only in Hymenophyllaceae and onocleoid ferns, each of these axillary branching systems is probably derived independently (Kato, 1987).

(15) Vascular cambium—absent (0) or present (1). Ophioglossaceae are the only extant group of pteridophytes exhibiting secondary growth (Gifford and Foster, 1989). Limited secondary growth occurs in *Botrypus*, *Japanobotrychium*, *Sceptridium*, and *Botrychium* s.s., but not in *Helminthostachys* or *Ophioglossum* s.l. (Kato, 1987; Petry, 1915).

(16) Suspensor—absent (0) or present (1). An embryo bearing a suspensor is present in a variety of plant groups including *Lycopodium*, *Selaginella*, *Phylloglossum*, gymnosperms, and angiosperms (Gifford and Foster, 1989). The absence of a suspensor in embryo development is observed in relatively few plants (e.g., *Isoetes*). Ophioglossaceae and Marattiaceae represent two of the few plant families for which variable occurrence of the suspensor is known (Gifford and Foster, 1989). Although early embryogeny is not fully investigated in Ophioglossaceae (Kato, 1987), *Botrychium* s.s., *Botrypus*, *Japanobotrychium*, *Ophioglossum* s.s., and *Cheiroglossa* putatively do not have a suspensor, whereas *Helminthostachys* and certain species of *Sceptridium* are reported to possess a suspensor (Bierhorst, 1971; Lyon, 1905; Nishida, 1955; Nishida and Imaichi, 1971). Kato (1987) suggested that the presence of a suspensor in certain groups of Ophioglossaceae is likely an advanced state.

(17) Gametophyte symmetry—axial (0) or dorsiventral (1). *Botrychium* s.s., *Sceptridium*, *Botrypus*, and presumably *Japanobotrychium* possess dorsiventral adult gametophytes (Bower, 1926). The gametophytes of *Ophioglossum* s.s., *Ophioderma*, and *Cheiroglossa* are fundamentally radial in construction (Mesler, 1975), although a degree of flattening can be observed in some *Ophioderma* and *Cheiroglossa* specimens. Bierhorst (1971) reported axial organization for gametophytes of *Helminthostachys*. Mesler (1975) reported that the gametophytes of *Cheiroglossa palmata* are cylindrical and branched and resemble those of *Ophioderma pendulum*.

(18) Gametophyte branching—branched (0) or unbranched (1). Eames (1936) reported that the gametophytes of *Ophioglossum* and *Helminthostachys* are [usually] branched, whereas those of *Botrychium* s.l. are typically unbranched. Mesler (1975) reported branched gametophytes in *Cheiroglossa*, as did Lang (1901) in *Ophioderma*.

(19) Leaf stomata position—hypostomatic (0) or amphistomatic (1). Pant and Khare (1969) reported that *Botrypus* and *Helminthostachys* are hypostomatic, whereas *Ophioglossum* s.s. is amphistomatic. Van Cotthem (1970) described *Botrychium* s.s., *Sceptridium*, *Cheiroglossa*, and *Ophioderma* as hypostomatic and concurred that *Ophioglossum* s.s. is amphistomatic.

(20) Stomata construction—anomocytic/absent (0) or cyclocytic (1), *Cheiroglossa* and *Ophioderma* have cyclocytic organization of subsidiary cells (Maróti, 1965; Mesler, 1975; Van Cotthem, 1970). All other members of the family lack subsidiary cells (Inamdar, 1970; Pant and Khare, 1969), although Van Cotthem (1970) reported some development of subsidiary cells in all groups except *Ophioglossum* s.s.

2.4. List of autapomorphic morphological characters

Ophioglossaceae are replete with species exhibiting unique characters, but these characters are not useful for phylogenetic reconstruction. Wagner (1990) regarded autapomorphic characters as particularly important in Ophioglossaceae and stressed the number of distinctive characters separating species or species groups.

(1) Rhizome structure—radial (0) or dorsiventral (1). The *Helminthostachys* rhizome is dorsiventral in construction (Bower, 1926; Farmer and Freeman, 1899). Rhizomes of other members of Ophioglossaceae are radial: *Botrypus*, *Japanobotrychium*, *Sceptridium*, *Botrychium* s.s., and *Ophioglossum* s.l. (Kato, 1987).

(2) Pubescent leaves—*J. lanuginosum* is the only species in Ophioglossaceae that has pubescent adult leaves (Wagner, 1990). Although most members of *Botrychium* s.l. exhibit pilose leaf primordia, the hairs are apparently lost during maturation.

(3) Tracheidal idioblasts—Arnott (1960) observed unusually large cells with helical or scalariform thickenings in the common stalk of leaves of *Sceptridium lunarioides*. The function of these tracheidal idioblasts is unknown (Wagner, 1990).

(4) Insertion of sporophore on rachis of trophophore—*J. lanuginosum* is the sole species of Ophioglossaceae in which the sporophore is inserted above the trophophore base, making the sporophore appear as a modified, single pinna (Chrysler, 1925).

(5) Creeping rhizome—*Helminthostachys* possesses a horizontal rhizome with two rows of leaves on the dorsal side (Farmer and Freeman, 1899), whereas all other species in Ophioglossaceae have vertical rhizomes (Wagner, 1990).

(6) Sterile projections on sporophore axis—Sporangia in *Helminthostachys* are borne on short stalks. Interspersed among the sporangial stalks are sterile projections for which the origin and function are not known (Bierhorst, 1971; Wagner, 1990).

(7) Anomalous gametophytes—Gametophytes of *O. crotalophoroides* are lens-shaped and dorsiventral, whereas gametophytes of other species of *Ophioglossum* s.s. are globose or nearly cylindrical (Mesler, 1976).

(8) Basal attachment of sporophore—*Ophioglossum bergianum* (not sampled for this study) has sporophores and trophophores "separate to the base" (Clausen, 1938).

3. Results

3.1. Analysis of rbcL

A total of 1321 bp of the *rbcL* gene (1351 minus the 30 bp of the forward primer) was obtained for each of 36 ingroup species. No insertions or deletions were detected. Of the 1321 characters, 903 (68%) were constant, 77 (6%) were variable but parsimony-uninformative, and 341 (26%) were variable and potentially parsimony-informative. There were 690 transitions and 290 transversions resulting in a transition/transversion (ts/tv) ratio of 3.4 with CI (consistency index) = 0.42 and 0.74 and retention index (RI; Farris, 1989) = 0.82 and 0.88, respectively. Thus, although the CI for the more numerous transitions was lower, they performed nearly as well, as measured by RI. The *rbcL* analysis (Fig. 1) included the 36 Ophioglossaceae sequences and outgroup taxa from Psilotaceae (*Psilotum* and *Tmesipteris*) and Marattiaceae (*Angiopteris*, *Danaea*, and *Marattia*). Four equally most parsimonious trees of 889 steps were produced with CI [here excluding uninformative characters] = 0.54 and RI = 0.85. Fig. 1 shows the strict consensus of the four trees with bootstrap percentages (BP).

Two major clades were identified in the *rbcL* parsimony analysis: the ophioglossoid (BP = 98) and the botrychioid clades (BP = 75, Fig. 1). Three subclades composed the former, one contained *C. palmata* + *Ophioderma pendulum* (BP = 88), a second included *O. costatum* + *O. gomezianum* (BP = 99), and a third contained all other species of *Ophioglossum* s.s. (BP = 76; *O. reticulatum*, *O. vulgatum*, *O. richardsiae*, *O. petiolatum*, *O. gramineum*, *O. pusillum*, *O. nudicaule*, and *O. crotalophoroides*). The botrychioid clade contained two well-supported subclades corresponding to *Botrychium* s.s. (BP = 100) and *Sceptridium* (BP = 81). *Botrypus*, however, was paraphyletic. *Bp. virginianus* was sister to the *Botrychium* s.s. + *Sceptridium* clade (BP = 98), and *Bp. strictus* was sister to all three (BP = 100). *Helminthostachys* was sister to all species of *Botrychium* s.l. (*Botrychium* s.s. + *Sceptridium* + *Botrypus*).

Within *Sceptridium*, most collections were separated into a well-supported (BP = 96) Asian group and a less well-supported (BP = 61) North American group. *S. lunarioides* was sister to all other *Sceptridium* species. Within *Botrychium* s.s., three major subclades were supported (for additional information on species–level relationships in *Botrychium* s.s. see Hauk, 1995).

3.2. Analysis of trnL-F

Among the 34 species of Ophioglossaceae for which we obtained *trnL-F* sequences (the sequence for *Ophioderma pendulum* could not be aligned, and no sequence of *O. nudicaule* was recovered), size variants for the *trnL-F* spacer ranged between 238 and 368 bp. After computer alignment and manual adjustments, the *trnL-F* matrix had a total of 377 bp and 18 scored indels. Of these 395 characters (including the 18 indels), 90 (23%) were constant, 80 (20%) were variable but parsimony-uninformative, and 225 (57%) were variable and potentially parsimony-informative. There were 358 transitions and 220 transversions for a ts/tv ratio of 1.6 with CI = 0.69 and 0.75 and RI = 0.89 and 0.91, respectively. Thus, the performance of transitions and transversions was more consistent as measured by CI and RI than that observed in the *rbcL* data set. Although the variable sites in *rbcL* and *trnL-F* were changing at similar rates (2.61 and 2.69 times each, respectively), the frequency of variable sites within the short length of the *trnL-F* intergenic spacer was nearly twice that for *rbcL*. Pair-wise sequence divergence for *trnL-F* was two to five times greater than those found for *rbcL* (results not shown). Analysis of the *trnL-F* matrix produced 277 equally most parsimonious trees of 605 steps with CI (excluding uninformative characters) = 0.73 and RI = 0.90. Fig. 2 shows the strict consensus of the 277 trees with the arrangement of the clades obtained by mid-point rooting and bootstrap percentages indicated.

Ophioglossum s.s. was well supported (BP = 90). Within the botrychioid clade, *Botrychium* s.s. and *Sceptridium* were monophyletic (BP = 98, 96, respec-

tively). As in the *rbcL* tree, *Botrypus* was paraphyletic. Sister–group relationships among *Helminthostachys*, *Botrypus*, *Sceptridium*, and *Botrychium* s.s. were consistent with those produced by the *rbcL* analysis, with minor differences in resolution, bootstrap percentages, and placement of individual species within *Sceptridium* and *Botrychium* s.s. In general, species–level relationships were more resolved in the *rbcL* tree (Fig. 1), especially within *Botrychium* s.s and *Ophioglossum* s.s. However, within *Sceptridium* relationships determined by *rbcL* and the *trnL-F* spacer differed. Both analyses placed *S. lunarioides* sister to all other *Sceptridium* species, but the *trnL-F* spacer dataset did not support separate North American and Asian clades, as did the *rbcL* analysis.

3.3. Morphological analyses

The 20 morphological characters (Tables 2 and 3) were analyzed to determine relationships among the six presumably monophyletic groups cited by Kato (1987) (i.e., *Ophioglossum* s.l., *Helminthostachys*, *Botrypus*, *Japanobotrychium*, *Sceptridium*, and *Botrychium* s.s.). The 141,879 equally most parsimonious trees of 24 steps produced a CI = 0.92 and RI = 0.99. The strict consensus of the trees is shown in Fig. 3. *Botrychium* s.s., *Sceptridium*, *Botrypus*, and *Ophioglossum* s.s. were monophyletic. Within *Botrychium* s.s., *Bo. lanceolatum* was sister to all other species. Relationships among *Sceptridium*, *Botrypus*, and *Botrychium* s.s. were not resolved, and *Helminthostachys* formed a polytomy with *Ophioglossum* s.l. and *Botrychium* s.l. (*Botrypus* + *Sceptridium* + *Botrychium* s.s). The 10 *Ophioglossum* s.s. species formed a polytomy, which was sister to *Ophioderma* + *Cheiroglossa*.

3.4. Combined analysis

Because both molecular matrices produced nearly perfectly congruent results with regards to the major species groups and the morphological analysis did not contradict those relationships, the three matrices were combined for a comprehensive analysis. The combination of *rbcL*, *trnL-F*, and morphological data yielded a total of 1736 characters, 586 of which were potentially parsimony informative. Twelve equally most parsimonious trees of 1529 steps were produced with CI (excluding uninformative characters) = 0.64 and RI = 0.87. The strict consensus tree (Fig. 4) resembled closely those of *rbcL* and *trnL-F* (Figs. 1 and 2).

Differences in topology among the *rbcL*, *trnL-F*, and combined analyses were limited to species-level relationships (i.e., within genera), none of which received BP >50 in any of the individual or combined analyses. For example, among *Sceptridium* species, the *rbcL* and *trnL-F* datasets produced slightly different hypotheses of relationship (Figs. 1 and 2), but the combined analysis (Fig. 4) produced a topology most similar to that of the *rbcL* analysis. Node by node inspection of the *rbcL* (Fig. 1), *trnL-F* (Fig. 2), and combined analysis (Fig. 4) indicated that BP at nearly every node increased in the combined analysis relative to the individual analyses. An exception to this was the node below *Helminthostachys* and *Botrychium* s.l. where BP = 75 for *rbcL*, BP = 100 for *trnL-F*, and BP = 79 for the combined analysis. Nodes in the ophioglossoid clade were difficult to assess because of the absence of *trnL-F* sequences for *Ophioderma* and *Ophioglossum nudicaule*.

4. Discussion

4.1. Comparison of sequence divergence between coding and non-coding regions of plastid DNA

In most land plants the *rbcL* gene is 1428 bp (Chase et al., 1993; Hasebe et al., 1995; Manhart, 1994), whereas the *trnL-F* spacer region generally ranges from 358 to 497 bp (Taberlet et al., 1991), approximately one-third the size of *rbcL*. Another difference between the *rbcL* and *trnL-F* datasets was the presence of indels in the *trnL-F* intergenic spacer (non-coding DNA), and these were distributed throughout the region. The presence of indels in DNA sequences has necessitated appropriate methods of incorporating them into phylogenetic analyses (Simmonds and Ochoterena, 2000). Addition of indels as presence/absence characters to the *trnL-F* data matrix reduced the number of equally most parsimonious trees, and they had less homoplasy than the substitutions as estimated by CI (0.79) and RI (0.96). Thus, indels in the *trnL-F* of Ophioglossaceae provided phylogenetic information and increased internal support.

4.2. The monophyly of Ophioglossaceae

Ophioglossaceae possess a combination of morphological characters that collectively distinguishes this family from other pteridophyte lineages (Bower, 1926). These features include: (1) non-circinate fronds composed of distinct fertile (sporophore) and sterile (trophophore) segments, with the production of only one or a few fronds per year; (2) clasping leaf bases that sheath successive leaf primordia; (3) homosporous spores produced in eusporangia; (4) subterranean, non-chlorophyllous gametophytes; (5) the formation of presumably obligatory mycorrhizal relationships in both sporophytic and gametophytic generations; (6) eustelic vascular organization, (7) circular bordered pits, (8) collateral leaf traces, and (9) the absence of root hairs and sclerenchyma. Spore exine structure and surface ornamentation further differentiate Ophioglossaceae from other known pteridophytes (Tryon and Lugardon,

1991; Tryon and Tryon, 1982). Thus, there are a substantial number of morphological characters to support the monophyly of Ophioglossaceae (Bierhorst, 1971; Bower, 1926; Campbell, 1905; Wagner, 1990).

Molecular studies of *rbcL* (Hasebe et al., 1995; Hauk and Chase, 1991; Manhart, 1994), 18s rDNA (Wolf, 1995), and *atpB* + *rps*4 + *rbcL* + nuclear small-unit rDNA (Pryer et al., 2001a) have provided additional confirmation of the monophyly of Ophioglossaceae, but the sampling from Ophioglossaceae in each of these studies was limited to two or only a few species. The 36 species sampled for this study are the first broad sample of species diversity in Ophioglossaceae and provide further molecular evidence of the monophyly of the family. A more extensive analysis including 30 sequences of leptosporangiate ferns (analysis not shown) also supported the monophyly of the family. Thus, morphological and molecular data collectively support the monophyly of Ophioglossaceae.

4.3. Relationships among major lineages within Ophioglossaceae

Separate analysis of *rbcL*, *trnL-F*, and morphological data provided consistent hypotheses of relationship among major monophyletic lineages within Ophioglossaceae (Figs. 1–3). Both molecular datasets (Figs. 1 and 2) and their combination (Figs. 4 and 5) identified two major clades within the family: ophioglossoid and botrychioid. The ophioglossoid clade corresponds to the traditional concept of *Ophioglossum* s.l. However, in the outgroup analysis (*rbcL*), the botrychioid clade includes *Helminthostachys* and clearly aligns it sister to *Botrychium* s.l. Unrooted and midpoint rooted analyses of *trnL-F* and morphological data are consistent with this result. Most morphological studies have considered *Helminthostachys* as "intermediate" between *Ophioglossum* s.l. and *Botrychium* s.l. (Campbell, 1905; Bower, 1926; Clausen, 1938; Eames, 1936). Once the root was placed in this morphological dataset via comparison to the *rbcL* and combined analyses, the sister group status of *Helminthostachys* and *Botrychium* s.l. was supported by the synapomorphies of stalked sporangia, single cell initiation of sporangia, and axillary buds (Fig. 5).

4.4. The monophyly of Ophioglossum s.l.

Previously, *Ophioglossum* s.l. has included four subgenera: *Ophioglossum* s.s., *Cheiroglossa*, *Ophioderma*, and *Rhizoglossum*. However, Wagner (1990) suggested that *Ophioglossum palmatum* and *Ophioglossum pendulum* could be recognized as distinct genera, *Cheiroglossa* and *Ophioderma*, respectively, following the system of Presl (1845). In the *rbcL* tree (Fig. 1), all species of *Ophioglossum* s.l. formed a well-supported clade (BP 98). Although the analyses did not include *Rhizoglossum* (= *Ophioglossum bergianum*), based on morphology this species is likely sister to or a member of *Ophioglossum* s.s., and thus it should fall well within the limits of *Ophioglossum* s.l. Morphological characters such as embedded sporangia and sporangia arising from a sporangiogenic band support the monophyly of *Ophioglossum* s.l. (Fig. 5).

4.5. The monophyly of Ophioglossum s.s.

The combined analysis strongly supported the monophyly of *Ophioglossum* s.s. (BP = 98), although *rbcL* and *trnL-F* showed individually BP = 56 and 90, respectively. However, removal of Psilotaceae (results not shown) from the *rbcL* dataset results in a paraphyletic *Ophioglossum* s.s., in which *Ophioglossum costatum* + *Ophioglossum gomezianum* is sister to *Cheiroglossa* + *Ophioderma*. When Marattiaceae were removed (results not shown), relationships among *Cheiroglossa* + *Ophioderma*, *O. costatum* + *O. gomezianum*, and all other species of *Ophioglossum* s.s. were not well resolved. Thus, in the *rbcL* dataset there was general instability among the earliest diverging lineages of *Ophioglossum* s.l. In either case of removing outgroup taxa, addition of the *trnL-F* data to the *rbcL* data restored the monophyly of *Ophioglossum* s.s., and the morphological data supported monophyly as well.

Morphologically, *Ophioglossum costatum* and *Ophioglossum gomezianum* resemble species of *Ophioglossum* s.s. and have previously been classified with them, but our *rbcL* (Fig. 1) and combined analysis (Fig. 4) indicate that they represent a lineage distinct from other members of *Ophioglossum* s.s. To our knowledge, *O. costatum* and *O. gomezianum* have never been proposed as a distinct, divergent clade within *Ophioglossum* s.s., although both species possess globose rhizomes, in contrast to the more irregular to cylindrical rhizomes of most *Ophioglossum* s.s. species. *Ophioglossum crotalophoroides* is a New World species that also has a globose rhizome, and in all molecular analyses it too was a divergent and relatively isolated member of *Ophioglossum* s.s. *Ophioglossum nudicaule* is reported to have an irregular rhizome (Lellinger, 1985). In the combined analysis (Fig. 4), *O. nudicaule* was sister to most other *Ophioglossum* s.s. species (except *O. crotalophoroides*, *O. costatum*, and *O. gomezianum*), but the monophyly of the species with irregular to cylindrical rhizomes was not strongly supported (BP = 65). Thus, the earliest diverging species of *Ophioglossum* s.s. apparently had globose rhizomes, and this may represent the ancestral state for members of *Ophioglossum* s.s.

Webb (1981) reported one-trace unilacunar nodal anatomy in *O. petiolatum* (cylindrical rhizome) and two-trace unilacunar nodal anatomy in *O. crotalophoroides* (globose rhizome) and, on this basis, suggested that *Ophioglossum* s.s. is an unnatural group composed of at

least two fundamentally different species lineages. Mesler (1976) noted that the embryogeny of *O. crotalophoroides* differs from that of other *Ophioglossum* s.s. species. Detailed morphological and developmental studies of many *Ophioglossum* s.s. species are necessary to evaluate relationships within this morphologically depauperate yet sequence-divergent species assemblage. If *O. costatum* and *O. gomezianum* possess two-trace unilacunar nodal anatomy and anomalous embryogeny, there will be clear evidence for a close relationship to *O. crotalophoroides* and perhaps the existence of a species clade corresponding to Prantl's (1884) *Macrorrhiza* group. Our analyses indicated a distinction between species with globose and irregular to cylindrical rhizomes, but the former are strongly supported as paraphyletic to the latter. At least three factors may influence this: (1) the *trnL-F* dataset did not include a sequence from *Ophioderma pendulum* because the small product that amplified could not be aligned with other *trnL-F* sequences, (2) no *trnL-F* sequence was obtained from *Ophioglossum nudicaule*, and (3) no sequences of *Rhizoglossum* were included in our analyses because material was not available. Because of the general instability among basal lineages within *Ophioglossum* s.l. when different outgroups are used for analysis, a strongly supported evaluation of the monophyly of *Ophioglossum* s.s. will require additional data, including those from *Ophioderma*, *Rhizoglossum*, and other species of *Ophioglossum* s.s.

4.6. The position of Cheiroglossa and Ophioderma

Cheiroglossa palmata and *Ophioderma pendulum* are two mostly epiphytic species of Ophioglossaceae, and both species possess elongate, bifurcating trophophores (Wagner, 1990). Most authors apparently did not regard *Cheiroglossa* and *Ophioderma* as particularly closely related (Copeland, 1947; Nishida, 1952; Prantl, 1884; Tryon and Tryon, 1982; Wagner, 1990), perhaps because they regarded similarities between the two as plesiomorphic rather than synapomorphic (see Bower, 1926; Clausen, 1938; Gifford and Foster, 1989). Nakai (1925, 1926), however, classified them as closely related, although without citing an explicit rationale. Mesler (1975) described the gametophytes of *Cheiroglossa* and supported sister-taxon status for *Cheiroglossa* and *Ophioderma* by documenting fundamental similarities in gametophyte structure. The *rbcL* analyses (Fig. 1) placed *O. pendulum* and *C. palmata* as sister taxa (BP = 88) in a clade sister to *Ophioglossum* s.s.

Wagner (1990) suggested that *Cheiroglossa* and *Ophioderma* could be recognized as separate genera (after Presl, 1845) rather than as subgenera (after Clausen, 1938). Wagner and Wagner (1993) recognized *Cheiroglossa* as a genus but did not address directly the ranking of *Ophioderma* because the classification they published treated only North American species. The *rbcL* and combined analyses (Figs. 1 and 4) do not contradict this change of rank for *Cheiroglossa*, unless *Ophioderma* were to remain in *Ophioglossum*. If *Ophioderma* is not treated as a separate genus parallel to *Cheiroglossa*, or if both are not subsumed into a single genus, then *Ophioglossum* would be paraphyletic. Thus, the *rbcL* and combined analyses support recognition of *Ophioderma* as a taxon of generic rank along with *Cheiroglossa* or as congeneric with *Cheiroglossa*.

Two species not available for molecular analyses may provide additional information to clarify the relationship between *Ophioderma* and *Cheiroglossa*. Clausen (1938) placed two other species, *Ophioglossum simplex* and *Ophioglossum intermedium* in *Ophioderma* with *O. pendulum*. *Ophioderma* (*Ophioglossum*) *simplex* is known from only the type locality in Sumatra, and *Ophioderma intermedium* is also restricted geographically (Sumatra and Sarawak; Clausen, 1938). Bower (1926) hypothesized that *O. pendulum*, *O. intermedium*, and *O. simplex* form a closely related group. However, Clausen (1938) suggested that *O. simplex* and *O. intermedium* represent anomalous, reduced forms of *O. pendulum* growing in suboptimal habitats. If *O. intermedium*, *O. simplex*, and *O. pendulum* form a clade sister to *Cheiroglossa*, then recognizing *Ophioderma* and *Cheiroglossa* as separate taxa can be perhaps justified. However, if *O. intermedium* or *O. simplex* is sister to *Cheiroglossa*, then *Ophioderma* would not be monophyletic, and the current distinctions between *Ophioderma* and *Cheiroglossa* would require revision. Because no recent collections of *O. simplex* and *O. intermedium* were available for this study, evaluation of the status of these species must await additional collections.

4.7. The monophyly of Botrychium s.l.

In most classifications (Bower, 1926; Clausen, 1938), *Botrychium* s.l. was composed of three subgenera, *Botrychium* s.s, *Sceptridium*, and *Osmundopteris* (= *Botrypus*). Kato and Sahashi (1977) recognized a fourth subgenus, *Japanobotrychium*, based on different leaf sheath development, sporophore attachment, and sporangial features. Historically, researchers have proposed several different hypotheses of relationship among the four *Botrychium* s.l. species groups (Bower, 1926; Clausen, 1938; Copeland, 1947; Eames, 1936; Kato, 1987; Presl, 1845; Wagner, 1990; Wagner and Wagner, 1993). Our molecular, morphological, and combined analyses produced trees that most closely approximate the taxonomic system of Bower (1926), who considered *Botrychium* s.l. a natural group, although he proposed *Sceptridium*, not *Botrypus* (= *Osmundopteris*), as the earliest diverging species group of *Botrychium* s.l. based primarily on endoscopic embryogeny.

Kato (1987) analyzed 13 morphological and developmental characters for six presumably monophyletic groups within Ophioglossaceae and concluded that *Botrychium* s.l. is paraphyletic. Kato (1987) placed *Helminthostachys* as sister to *Sceptridium* based on trophophyll size and the presence of a suspensor. *Ophioglossum* s.l. and *Botrychium* s.s. were considered sister groups on the basis of trophophyll size and marginal branching in leaf axes. After recoding and reanalyzing Kato's morphological characters with additional characters, we found no support for the paraphyly of *Botrychium* s.l. Our morphological analysis (Fig. 3) placed *Botrychium* s.s., *Sceptridium*, and *Botrypus* in an internally unresolved clade. Four morphological characters supported the monophyly of *Botrychium* s.l.: branched sporophores, secondary growth, and unbranched and dorsiventral gametophytes (Fig. 5). The *rbcL* analysis (Fig. 1) provided strong support (BP = 100) for *Botrychium* s.l., and the *trnL-F* analysis (Fig. 2) was consistent with this result. Thus, all molecular, morphological, and combined analyses are consistent with the monophyly of *Botrychium* s.l. (Figs. 1–4).

4.8. The paraphyly of Botrypus (= Osmundopteris)

Historically, *Botrypus* included three species, circumtemperate to subtropical *Bp. virginianus*, temperate/subtropical *Bp. strictus* of China and Japan, and *Bp. chamaeconium* of Cameroons and Uganda (Clausen, 1938). Degree of leaf dissection, overall size, and leaf sheath characters were used to support a close relationship among these species, although sporophore organization in the three species is distinctive (Clausen, 1938). *Bp. virginianus* has a highly branched sporophore with a relatively long stalk, whereas the sporophore of *Bp. strictus* has a relatively short, stalked sporophore with short, ascending, sporangia-bearing divisions (Clausen, 1938). In a detailed examination of leaf sheath development, Imaichi (1989) reported that *Bp. virginianus* and *Bp. strictus* differed in the degree of leaf sheath closure. Differences in leaf sheath structure and development, in addition to differences in sporophore construction, led Imaichi (1989) to conclude that *Bp. strictus* and *Bp. virginianus* represent distinct lineages that may deserve separate subgeneric/generic status. The *rbcL* and *trnL-F* datasets clearly support the hypothesis of Imaichi (1989). In the *rbcL* trees, the position of *Bp. strictus* had BP = 100, and the position of *Bp. virginianus* had BP = 98 (Fig. 1). The *trnL-F* trees showed BP = 100 and 71, respectively. In the *rbcL*, *trnL-F*, and combined trees (Figs. 1, 2, 4, and 5), *Bp. strictus* was sister to all other species of *Botrychium* s.l. However, in the morphological analysis (Fig. 3) two characters supported *Botrypus* as monophyletic (4, 11). Although character 4, leaf sheath covering, was coded as incomplete for both *Bp. strictus* and *Bp. virginianus*, developmental differences in degree of closure exist (Imaichi, 1989). However, because discrete character states in degree of leaf sheath closure were not apparent, the "synapomorphy" is an artifact of the difficulty of coding developmental states in cladistic analyses. Closer examination of character 11, sporangial dehiscence, may reveal subtle developmental differences that would accord with the molecular data and support the paraphyly of *Botrypus*, or this character may be plesiomorphic for *Botrychium* s.l.

4.9. The position of Japanobotrychium

Japanobotrychium is unique in Ophioglossaceae because the sporophore is inserted laterally on the trophophore (Chrysler, 1925; Kato and Sahashi, 1977). Although aligned with *Botrychium* s.l. by possession of stalked sporangia, axillary buds, a branched sporophore, vascular cambium, and dorsiventral and unbranched gametophytes, its position among *Botrypus*, *Sceptridium*, and *Botrychium* s.s. is not clear. The highly dissected trophophore and extra-marginal vascular bundles of the sporophore point to a close relationship to *Botrypus*, but differences in sheathing leaf bases, pinnation patterns, attachment, and dehiscence of sporangia, and spore features argue against this relationship (Kato and Sahashi, 1977). *Japanobotrychium* shares a sheathing leaf base structure similar to that of *Sceptridium* and *Botrychium* s.s. (Kato and Sahashi, 1977). *Sceptridium* and *Japanobotrychium* possess similar trophophore pinnation patterns (triangular pinnae with the basal pinnules the largest) and sporangia attachment (adnate at base and perpendicular to branch), and this may indicate recent shared ancestry (Kato and Sahashi, 1977). Thus, this interpretation of morphology indicates that *Japanobotrychium* diverged after *Botrypus* but before *Sceptridium* and *Botrychium* s.s. separated, and if included in molecular analyses it would likely be sister to *Sceptridium* + *Botrychium* s.s. Because of the lack of fresh material for DNA extraction and the difficulty of coding many pertinent morphological characters, *Japanobotrychium* was not included in this study. However, the work of Kato and Sahashi (1977) provides a substantive hypothesis for the placement of *Japanobotrychium*.

4.10. The monophyly of Sceptridium

Species of *Sceptridium* form a natural group based mainly on phenology, i.e., the wintergreen nature of the leaves (Fig. 5). *Sceptridium* is the only group within Ophioglossaceae in which the fronds emerge in midspring to summer and persist through the winter. Both the *rbcL* and *trnL-F* analyses supported the monophyly of *Sceptridium*, although to different degrees. The *rbcL*

analysis had BP = 81 for the monophyly of *Sceptridium* (Fig. 1), the *trnL-F* analysis (Fig. 2) had BP = 96, and the combined analysis (Fig. 4) showed BP = 100. Thus, all data, individually and in concert, supported the monophyly of *Sceptridium*. Within *Sceptridium*, both individual molecular analyses and the combined analysis placed *S. lunarioides* (*S.* section *Hiemobotrychium*; Wagner and Wagner, 1993) sister to all other *Sceptridium* species, as was suggested by Wagner and Wagner (1993). This sister–group relationship is consistent with the ideas of Clausen (1938). Among species of *Sceptridium* (except *S. lunarioides*), the *rbcL* and combined analyses provided weak support (BP = 61 and 70, respectively) for a clade of North American species (*Sceptridium multifidum*, *Sceptridium oneidense*, and *Sceptridium dissectum*) and strong support (BP = 96 and 96, respectively) for an Asian clade (*Sceptridium atrovirens*, *Sceptridium japonicum*, *Sceptridium ternatum*, and *Sceptridium* sp.).

4.11. The monophyly of Botrychium s.s.

Species of *Botrychium* s.s. possess a suite of morphological characters distinguishing them collectively from other groups within *Botrychium* s.l. (Bower, 1926; Clausen, 1938; Tryon and Tryon, 1982; Wagner, 1990; Wagner and Wagner, 1993). The small size, relatively pale color, fleshy leaves, pinnate/pinnatifid trophophore (except *Bo. lanceolatum*, see below), invariable presence of a fertile segment, and ephemeral leaves support the monophyly of *Botrychium* s.s. Micromorphological characters such as similar spore architecture (Tryon and Lugardon, 1991), glabrous leaf primordia (Wagner, 1990), and a leaf sheath covering with a fused slit (Kato, 1987) have been reported as synapomorphies for the group. Reports of hybrids among many species have documented the close genetic relationship among species (Wagner, 1980, 1991; Wagner and Wagner, 1988; Wagner et al., 1985). Despite the apparent number of distinct morphological attributes distinguishing *Botrychium* s.s., the difficulty of coding many of these characters for cladistic analysis precluded their inclusion in the morphological analysis. Thus, in our analysis only two morphological synapomorphies supported the monophyly of *Botrychium* s.s. However, the *rbcL* and *trnL-F* analyses provided, respectively, BP = 100 and 98 for the monophyly of *Botrychium* s.s., and the combined analysis had BP = 100. The monophyly of *Botrychium* s.s. was clearly supported by all available evidence. Among *Botrychium* s.s. species, diploid *Bo. lanceolatum* and its polyploid derivatives (here *Botrychium pinnatum* and *Botrychium pedunculosum*) were sister to all other species (see Hauk, 1995). Of all the groups sampled in Ophioglossaceae, *Botrychium* s.s. showed more resolution among species than any other (see Hauk, 1995).

4.12. The position of Helminthostachys

Throughout much of the systematic history of Ophioglossaceae, *Helminthostachys* has been interpreted as a group "intermediate" between *Botrychium* s.l. and *Ophioglossum* s.l. (Clausen, 1938; Eames, 1936; Farmer and Freeman, 1899) or as an "isolated type" within the family (Bower, 1926). Hypotheses of the lineage to which *Helminthostachys* is most closely allied have not been explicit. The *rbcL* analysis (with outgroups) placed *Helminthostachys* sister to *Botrychium* s.l. (BP = 75; Fig. 1), and the *trnL-F* analysis was consistent with this (BP = 100; Fig. 2). The morphological analysis (Fig. 3) did not resolve the relationship of *Helminthostachys* to *Botrychium* s.l. and *Ophioglossum* s.l., but sporophore branching, vascular branching, gametophyte symmetry, and gametophyte branching differentiated *Botrychium* s.l. from *Helminthostachys* (Fig. 5; Table 3). However, the combined analysis BP = 79 supported the placement of *Helminthostachys* sister to *Botrychium* s.l., and this was not strongly supported in the *rbcL* analysis (BP = 75), well below that of the *trnL-F* (BP = 100). For almost every other node in the analyses, BPs in the combined analysis were higher than that observed in either individual analysis. Because of this, there may be some degree of ambiguity in the *rbcL* data, although both the individual and combined analyses were consistent with *Helminthostachys* sister to *Botrychium* s.l. Farmer and Freeman (1899) reported similarities between *Helminthostachys* and *Botrychium* in stipular appendages enclosing leaf primordia, but this character needs further investigation and clarification of character states. Thus, in our analyses, three morphological synapomorphies (i.e., single cell sporangium initiation, stalked sporangia, and axillary buds; Fig. 5) lend strength to the argument that this sister–group relationship is indeed valid. Therefore, all available evidence supports the hypothesis that *Helminthostachys* is a member of the botrychioid clade and not closely aligned to *Ophioglossum* s.l.

4.13. A role for molecular phylogenies: reconstruction of the primitive ancestor?

In our analyses, molecular and morphological data yielded similar estimates of relationships of major groups within Ophioglossaceae. To interpret character evolution in the family, we mapped unambiguous morphological character changes onto one of six equally most parsimonious trees generated from a combined analysis of *rbcL* and *trnL-F* data (Fig. 5). This exercise provided some insight into questions about character evolution in Ophioglossaceae, and use of any of the other five trees (or the combined all data trees) did not change interpretations of character evolution. In Fig. 5, the ophioglossoid and botrychioid clades diverged

relatively early in the history of the family. Based on this tree topology, we deduced hypotheses of characters/character states present in the common ancestors of these two principal lineages (i.e., plesiomorphies) by examining the distribution of characters/character states on the combined *rbcL* + *trnL-F* tree (Fig. 5).

Synapomorphies that likely occurred in the common ancestor of the ophioglossoid clade include sporangia derived from a sporangiogenic band (#9) that are embedded in tissues around the sporophore axis (#10). The hypothetical ophioglossoid ancestor should have lacked axillary buds (#14), stalked sporangia (#10), and sporangia initiated from a single cell (#9) because these appear as synapomorphies for the botrychioid lineage. *Helminthostachys* represents the earliest diverging species of the botrychioid lineage (Figs. 1, 2, 4, and 5). The placement of *Helminthostachys* sister to *Botrychium* s.l. was critical to assessments of primitive character states in *Botrychium* s.l. Synapomorphies that occurred in the hypothetical botrychioid ancestor (Fig. 5) were sporangia derived from a single superficial cell (#9), sporangia borne on stalks (#10), and axillary buds (#14). A branched sporophore (#7), a vascular cambium (#15), and dorsiventral (#17), unbranched gametophytes (#18) are synapomorphies for *Botrychium* s.l. and thus not representative of the entire botrychioid lineage.

Character states shared among the earliest diverging lineages (or all lineages) of Ophioglossaceae provide some insight into what characters the common ancestor of Ophioglossaceae may have exhibited. The division of the leaf into trophophore and sporophore almost certainly occurred in the common ancestor. Because *Ophioglossum* s.s. and *Helminthostachys* + *Botrychium* s.l. are terrestrial, the common ancestor was most likely terrestrial as well. The epiphytic habit likely evolved later as a series of changes in the common ancestor of the sister taxa *Cheiroglossa* and *Ophioderma*. The leaf probably had a complete leaf sheath covering (#4) because in the family only *Botrypus* deviates from this condition (Table 3). The leaf of the common ancestor was not likely wintergreen (#1), cyclocytic (#20), and probably lacked amphistomatic leaves (#19) with free included veinlets (#6) because these characters are restricted to *Sceptridium*, *Cheiroglossa* + *Ophioderma*, and *Ophioglossum* s.s., respectively. Although nearly all other putatively ancient fern families exhibit free venation, the presence of anastomosing veins in *Ophioglossum* s.l. and *Helminthostachys* (partly) implies that anastomosing veins could have been the ancestral state for the family, or, alternatively, there may have been a mixture of free and anastomosing veins similar to that found in *Helminthostachys*. Thus, either a reversal to free venation must have occurred in the ancestor of *Botrychium* s.l., or free venation is a plesiomorphy retained from a common pteridophyte ancestor. The sporophore of the common ancestor was probably unbranched (#7) and present variably (#8), as is the case in *Ophioglossum* s.l. and *Helminthostachys*. Sporangium dehiscence (#11) was likely transverse in the common ancestor because this is present in all groups except *Botrypus*. The presence of a vascular cambium in *Botrychium* s.l. (#15), and not at the base of the botrychioid lineage, implies that this is an independently derived state and likely not a synapomorphy with the seed plant lineage, confirming a similar hypothesis proposed by Chau (1986) based on morphological studies. Character states shared between *Ophioglossum* s.l. and *Helminthostachys* also indicate that gametophytes of the common ancestor should have been branched (#18) with axial symmetry (#17) and a suspensor in the embryo (#16) was not likely.

Combining molecular and morphological characters into a single cladistic analysis provides the first opportunity to assess the problematic and confusing distribution of morphological characters found in Ophioglossaceae. The comparison of hypothetical ophioglossoid and botrychioid ancestors produces basic hypotheses concerning the nature of the primitive ancestor of all Ophioglossaceae species and addresses fundamental questions regarding the distribution of some historically problematic characters. However, for some characters (e.g., sporangium initiation and attachment, and axillary buds), opposing character states in the ophioglossoid and botrychioid clades create a conundrum as to the ancestral states present in the common ancestor. In many groups of organisms, comparison to an appropriate outgroup is used to resolve ancestral character states in a lineage. For certain characters in Ophioglossaceae, this may not be a valid strategy because Psilotaceae are the sister group based on molecular analyses (Pryer et al., 2001a). The lack of homology between morphological features of Psilotaceae and Ophioglossaceae precludes insight into ancestral states of Ophioglossaceae for all characters of the trophophore (e.g., venation, stomata, architecture, primordia pubescence), sporophore (e.g., branching), and perhaps characters associated with the sporangium (e.g., attachment and initiation). Even if a more morphologically complex outgroup were identified, the unique structure and enigmatic origin of the ophioglossaceous "leaf" would make direct comparisons to outgroups problematic (Chrysler, 1945; Clausen, 1938; however, see Nishida, 1957). Certainly inclusion of the newly described *Mankyua* (Sun et al., 2001) could provide some insights if it diverged early in the evolution of the ophioglossoid lineage (and this appears likely based on its morphology), but until material is available for study the placement of *Mankyua* will remain undetermined. Unless new extant or fossil taxa that diverged at the base of either the ophioglossoid or botrychioid lineages are discovered, our ability to resolve many questions of character evolution will remain limited even with well-resolved and well-supported molecular phylogenies.

Acknowledgments

The authors thank J.E. Burrows, D. Farrar, F. Lang, S. Leonard, R. Moran, J. Nekola, R. Peet, N. Sahashi, J. Sharpe, P. Madhusoodanan, W.H. Wagner, F.S. Wagner, E. Watkins, and K. Wurdack for generously providing leaf material, and H.G. Hills, K.A. Kron, and Y.-L. Qiu for technical advice and assistance to WDH. We also thank P. Wolf and the anonymous reviewers for comments helpful for improving the manuscript. Funding for this study was provided by Sigma Xi Grants in Aid of Research, the Department of Biology, University of North Carolina at Chapel Hill, and from a National Science Foundation Doctoral Dissertation Improvement grant for the research of WDH (BSR-9224287).

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