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PHYLOGENY AND BIOGEOGRAPHY OF *OROBANCHACEAE*

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Abstract: *Orobanchaceae*, as it is currently defined, includes all levels of parasitic ability ranging from nonparasitic (*Lindenbergia*) to facultative and obligate hemiparasites to obligate holoparasites. Several genera are of economic importance as crop weeds and have been studied by scientists interested in developing methods of control, but most genera have not been studied in a comparative framework. In this study we have used ITS sequence data to build a phylogenetic framework with which to examine previous systematic hypotheses of relationships among genera, and biogeographic hypotheses of either a Cretaceous, Gondwanan or mid-Tertiary, Laurasian origin of the family. A single-most parsimonious ITS tree was produced from a combined data set of nucleotides and gap characters. Our results support the current classification of *Orobanchaceae* and a hypothesis of a mid-Tertiary, Laurasian origin of the family.

Keywords: Biogeography, ITS, Parasitic plants, Phylogeny

INTRODUCTION

Orobanchaceae has recently been redefined from plastid gene trees to be the "least inclusive clade that contains *Orobanche uniflora*, *Schwalbea americana*, and *Lindenbergia philippinensis*" (YOUNG et al. 1999). The monophyly of parasitic plants within *Lamiales* has been supported by phylogenies based on *rps2*, *rbcL*, and *matK* data (DEPAMPHILIS et al. 1997, WOLFE & DEPAMPHILIS 1998, NICKRENT et al. 1998, OLMSTEAD et al. 2001), resulting in the transfer of 73 parasitic genera of *Scrophulariaceae* plus the non-parasitic genus *Lindenbergia* to *Orobanchaceae*. This brings the total number of genera and species of this redefined family to 87 and ca. 1700, respectively (Table 1). With *Lindenbergia* as the basal lineage of *Orobanchaceae*, all degrees of parasitic ability from non-parasitic to holoparasitic are represented in the family. There is a general consensus that green, leafy, facultative hemiparasites represent the basal condition of parasitism, whereas holoparasitism is derived. Independent origins of holoparasitism throughout *Orobanchaceae* have been demonstrated in previous phylogenetic studies based on plastid nucleotide sequence data (DEPAMPHILIS et al. 1997, WOLFE & DEPAMPHILIS 1998, YOUNG et al. 1999). By comparing the morphological and anatomical transitional series of the evolution of parasitism proposed by BOESHORE (1920) to the topology of the phylogeny generated from plastid sequence data, YOUNG et al. (1999) were able to demonstrate that the transition from hemiparasitism to holoparasitism, and the accompanying reduction of vegetative structures result from ecological convergence.

Members of *Orobanchaceae* are globally distributed, primarily in temperate regions. The majority of genera and species are in the Northern Hemisphere and in Old World regions

(Table 2). Most genera have one to five species with very limited distributions, whereas cosmopolitan genera tend to be large with dozens of species (Fig. 1; Table 1). Five genera have 100 or more species: *Pedicularis* (350+ spp.), *Castilleja* (200+ spp.), *Euphrasia* (170+ spp.), *Orobanche* (ca. 150 spp.) and *Buchnera* (ca. 100 spp.). Three of the largest genera have northern hemisphere distributions with centers of diversity in the Mediterranean, western North America, and the Himalayas (*Orobanche*, *Castilleja*, and *Pedicularis*, respectively; MABBERLEY 1997, PARAN et al. 1997, SCHNEEWEISS et al. 2004). *Euphrasia* has been revised to include 14 sections, most of which are restricted to the southern hemisphere. However, the largest section of *Euphrasia* has a northern hemisphere distribution primarily across the Old World (BARKER 1982, 1986, HONG 1983, HEADS 1994). All but 16 species of *Buchnera* are distributed in tropical or warm temperate regions of the Old World (BARKER 1982, HEADS 1994).

Of the 87 genera currently circumscribed by *Orobanchaceae*, ten have been characterized as having species that are parasitic weeds on food or tree crops (*Aeginetia*, *Alectra*, *Buchnera*, *Christisonia*, *Cistanche*, *Melasma*, *Orobanche*, *Rhamphicarpa*, *Seymeria*, and *Striga*; KING 1966, MUSSELMAN 1980, 1986). The three major parasitic weeds in terms of economic importance are *Alectra*, *Orobanche*, and *Striga* (HEPPER 1963, KING 1966, MUSSELMAN & MANN 1978, MUSSELMAN 1980, 1986, MUSSELMAN & PARKER 1982, ATOKPLE et al. 1995). These economically important parasitic genera have primarily Mediterranean (*Cistanche*, *Orobanche*), Asian (*Aeginetia*, *Alectra*, *Christisonia*, *Cistanche*, *Orobanche*, *Striga*), and African (*Alectra*, *Buchnera*, *Cistanche*, *Melasma*, *Rhamphicarpa*, *Striga*) distributions. Two species of *Seymeria* are restricted to southern North America, one of which (*S. cassioides*) may have an economic impact on pine plantations in the southern United States (MUSSELMAN & MANN 1978, MUSSELMAN 1996).

The primary objectives of this study were to use nuclear rDNA ITS sequence data to infer the phylogeny of *Orobanchaceae* and to compare our results with previous phylogenetic hypotheses generated from plastid nucleotide sequence data (DEPAMPHILIS et al. 1997, WOLFE & DEPAMPHILIS 1998, YOUNG et al. 1999). Using the phylogenetic trees generated from these data, we examine previous biogeographic hypotheses about a Cretaceous, Gondwanan (BARKER 1982, 1986) or Tertiary, Laurasian (RAVEN & AXELROD 1972) origin for some genera, and we present new, phylogenetically-based hypotheses on the origin and radiation of *Orobanchaceae*.

MATERIALS AND METHODS

Nucleotide sequencing

Fifty-nine species representing 32 genera of *Orobanchaceae* were included in this survey along with six species from five outgroup genera (Table 3). Multiple species were included from large genera, or from genera currently under investigation in other studies (e.g., *Hyobanche*), bringing the total number of taxa surveyed to 65.

DNA was extracted from either fresh-frozen or desiccated leaf, calyx or stem material following WOLFE & RANDLE (2001). PCR products were generated for nuclear ribosomal ITS1, 5.8S, and ITS2 using the external primers N-nc18510 and C26A (WEN & ZIMMER 1996). PCR was conducted in 50µl reactions containing 0.5–1 µl purified DNA, 0.2 µM

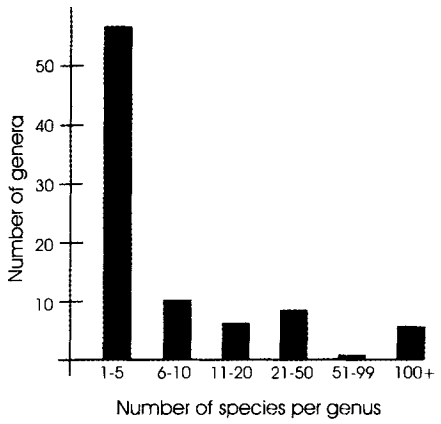

Fig. 1. Number of species per genus for *Orobanchaceae*.

dNTPs, 0.15 mM MgCl2, 0.64 µM each primer, 5% DMSO, 1.25 units *Taq* polymerase (Gibco/BRL, Gaithersburg, MD), and 1× *Taq* polymerase amplification buffer. Reaction conditions were as follows: 1.5 min initial denaturation at 94 °C; 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C, 2 min extension at 72 °C; 1 min denaturation at 94 °C, 1 min annealing at 50 °C, and 10 min extension at 72 °C. PCR products were precipitated from a 10% polyethylene glycol solution and further purified by washing in 85% ethanol.

Dideoxy termination cycle-sequencing was performed using the amplification primers and two internal primers, ITS2m (WOLFE & RANDLE 2001) and ITS3 (SANG et al. 1995). Sequencing reactions were done in 5 µl volumes containing 2 µl of Big Dye (ABI), 0.25 µl DMSO, 0.5 µl of primer (2 µM), and 0.5–1.0 µl PCR product. Sequencing reaction conditions were: 25 cycles of 30 s denaturation at 96 °C, 15 s annealing at 50 °C, and 4 min extension at 60 °C. Sequencing products were precipitated in two washes of 85% ethanol, and sequenced using the ABI 310 or 3100 genetic analyzers. Nucleotide sequence fragments from each primer were assembled into contiguous sequences using Sequencher (GeneCodes, Ann Arbor, MI, USA). Reads from both strands were obtained for all ITS sequences.

Phylogenetic analysis

ITS sequences were aligned initially using ClustalX (THOMPSON et al. 1997). The alignment file was imported into Se-Al (RAMBAUT 1996) and adjusted by eye using plant ITS secondary structural motifs as alignment guides. ITS secondary structural motifs, particularly those of ITS2, have been revealed as highly conservative across broad lineages of land plants (HERSHKOVITZ & ZIMMER 1996, MAI & COLEMAN 1997, COLEMAN 2003, GOERTZEN et al. 2003) and can thus serve as anchoring points for sequences from taxa that are more distantly related than species within a genus. Each ITS1 and ITS2 DNA sequence was converted to an RNA sequence and subsequently analyzed in Mfold (ZUKER 2003) using the default settings. The reconstructed secondary structures were evaluated for conserved stem-loop motifs and the corresponding sequences identified in the alignment file. The alignments were adjusted to correspond to structural features found in the molecules (e.g., stems were aligned and expansion/contractions in loops were adjusted where necessary). The 5.8S region was used as the initial anchor point followed by conserved stem-loop regions of ITS2 and ITS1, respectively. A similar protocol for using inferred RNA secondary structure to align divergent ITS sequences has proven useful for species within a genus (DENDUANGBORIPANT & CRONK 2001, RITCHIE et al. 2004), to genera within a family (GOERTZEN et al. 2003), and to families within an order (COLEMAN 2003).

Table 1. Genera, number of species per genera, and distribution of taxa in *Orobanchaceae*. Genera listed in boldface type were included in our phylogenetic studies. Information compiled from MABBERLEY (1997) and International Plant Names Index database (http://www.ipni.org).

Genus	Number of species	Distribution of species
***Aeginetia* L.**	3	Phillipines, Tawain, Japan, India
***Agalinis* RAF.**	40	North and South America
***Alectra* THUNB.**	30–40	Africa, Madagascar, Asia
Ancistrostylis T. YAMAZ	1	Laos
Aureolaria RAF.	11	E United States, Mexico
***Bartsia* L.**	49	North temperate to arctic regions, tropical montane regions; amphi-Atlantic (45 species endemic to Andes of South America)
***Boschniakia* C.A. MEY. ex BONG.**	5	Asia, W North America
Brachystigma PENNELL	1	SW United States
***Buchnera* L.**	100+	Primarily tropical, Old World (16 species are New World ones)
Bungea C.A. MEY.	2	SW and Central Asia, China
Buttonia BENTH.	3	Tropical and southern Africa
***Castilleja* MUTIS ex L.f.**	200+	Primarily western North America, extending to western South America and Eurasia
Centranthera R. BR.	9	China to Australia
Christisonia GARDNER	17	SW China, SE Asia, Indomalaysia
***Cistanche* HOFFMANNS. et LINK**	16	Mediterranean, N Africa, NW China, W India
Clevelandia GREENE	1	Baja California, North America
***Conopholis* WALLR.**	2	North and Central America
***Cordylanthus* NUTT. ex BENTH.**	18	W North America
Cycniopsis ENGL.	3	Tropical NE Africa
***Cycnium* E. MEY. ex BENTH.**	15	Sub-Saharan Africa
Dasistoma RAF.	1	E North America
***Epifagus* NUTT.**	1	E North America
Escobedia RUIZ et PAV.	6	Tropical South America
Esterhazya J.C. MIKAN	5	Bolivia, Brazil
***Euphrasia* L.**	170+	Cosmopolitan
Gerardiina ENGL.	2	Tropical and S Africa
Ghikaea VOLKENS et SCHWEINF.	1	Tropical Africa
Gleadovia GAMBLE et PRAIN	6	W Himalayas, W China
***Graderia* BENTH.**	5	Africa, Socotra
***Harveya* HOOK.**	25–40	Tropical (E) and S Africa
Hedbergia MOLAU	1	Tropical Africa
***Hyobanche* L.**	8	S Africa
Lamourouxia KUNTH	28	Mexico to Peru
***Lathraea* L.**	7	Europe
Leptorhabdos SCHRENK	1	SW and Central Asia, Himalayas
Leucosalpa SCOTT-ELLIOT	3	Madagascar
***Lindenbergia* LEHM.**	15	Himalayas to tropical Asia and Africa
Macranthera TORR. ex BENTH.	1	SE United States
Magdalenaea BRADE	1	SE Brazil
Mannagettaea HARRY SM.	3	E Asia

Genus	Number of species	Distribution of species
***Melampyrum* L.**	35	Palaearctic (1 species in North America)
***Melasma* P.J. BERGIUS**	5	Africa and South America
Micrargeria BENTH.	5	Tropical Africa and India
Micrargeriella R.E. FR.	1	Tropical E Africa
Monochasma MAXIM. ex FRANCH. et SAV.	4	E Asia
Necranthus GILLI	1	Turkey
Nothobartsia BOLLIGER et MOLAU	2	Mediterranean
Nothochilus RADLK.	1	Brazil
Odontites LUDW.	30	W Mediterranean to Central Asia
Omphalotrix MAXIM.	1	NE Asia
Ophiocephalus WIGGINS	1	Baja California, North America
***Orobanche* L.**	150	North America, Mediterranean, E Europe to SW Asia
***Orthocarpus* NUTT.**	9	W North America
Parastriga MILDBR.	1	Tropical Africa
***Parentucellia* VIV.**	2	Europe, Mediterranean, SW Asia
***Pedicularis* L.**	350+	N Hemisphere with most species in Central Asia; 1 species in Andes of South America
Petitmenginia BONATI	2	S China, SE Asia
Phacellanthus SIEBOLD et ZUCC.	2	E Asia
Phelypaea L.	4	Mediterranean
Phtheirospermum BUNGE	7	E Asia
Physocalyx POHL	2	Brazil
Platypholis MAXIM.	1	Japan
Pseudobartsia D.Y. HONG	1	China
Pseudomelasma EB. FISCH.	1	Madagascar
Pseudosopubia ENGL.	7	Tropical Africa
Pseudostriga BONATI	1	SE Asia
Pterygiella OLIV.	3	China
Radamaea BENTH.	5	Madagascar
Rhamphicarpa BENTH.	6	Tropical and S Africa, India, Tropical Australia
***Rhinanthus* L.**	45	N Hemisphere, mainly Palearctic of Europe
***Rhynchocorys* GRISEB.**	6	S Europe to Iran
***Schwalbea* L.**	1	SE United States
***Seymeria* PURSH.**	25	S North America
Sieversandreas EB. FISCH.	1	Madagascar
Silviella PENNELL	2	Mexico
Siphonostegia BENTH.	3	E Mediterranean to E Asia
***Sopubia* BUCH.-HAM. ex D. DON**	60	Tropical Old World to S Africa, Madagascar
Spirostegia IVANINA	1	Baja California, North America
***Striga* LOUR.**	40	Tropical Old World to S Africa, S and Central Asia
Tetraspidium BAKER	1	Madagascar
Thunbergianthus ENGL.	2	W Africa, Tropical E Africa
Tienmuia HU	1	E China
***Tozzia* L.**	1	European Alps
Triphysaria FISCH. et C.A. MEY	5	W North America
Vellosiella BAILL.	3	Brazil
Xizangia D.Y. HONG	2	China
Xylocalyx BALF. f.	5	Somalia

All gaps in the sequence alignment were coded as separate characters using the "complex" method of SIMMONS & OCHOTERENA (2000). Gaps within the matrix were treated as missing data, and 41 indel characters were concatenated onto the nucleotide matrix, resulting in a total of 711 characters. Of these indel characters, sixteen were treated as ordered characters. Step matrices for the ordered indel characters were constructed in MacClade 3.05 (MADDISON & MADDISON 1992). Fifteen of the sixteen ordered indel characters were coded using asymmetric step matrices (rules 4, 5, and 6 of SIMMONS & OCHOTERENA 2000).

Phylogenetic analyses with maximum parsimony as an optimality criterion were performed in PAUP*4.10b (SWOFFORD 2002). Characters ordered using asymmetric step matrices are implicitly polar; alternate directions of character state change may result in differing numbers of steps on a tree (i.e., the choice of root affects the length of the tree). In tree searches using PAUP*, characters ordered using asymmetric step matrices are optimized to result in the fewest character state changes, globally. In previous studies, *Orobanchaceae* have been shown to be monophyletic using *rps2*, *matK*, and *rbcL* data (WOLFE & DEPAMPHILIS 1998, YOUNG et al. 1999, OLMSTEAD et al. 2001). Because the choice of the root of a tree affects tree length with asymmetrically-ordered characters a constraint tree preserving the monophyly of *Orobanchaceae* was implemented in the combined data tree searches and when assessing bootstrap support. The outgroup consisted of the non-parasitic plants excluded from *Orobanchaceae*.

Heuristic tree searches were conducted for nucleotide characters alone, indel characters alone, and the concatenated nucleotide/indel character set. All searches were conducted using 100 random sequence additions, holding five trees per step with TBR branch swapping. Bootstrap support was calculated using a full heuristic search of 100 replicates using one random sequence addition each, holding five trees per step with TBR branch swapping.

A maximum likelihood analysis was conducted using PHYLIP 3.6 (FELSENSTEIN 2004). An HKY85 (HASEGAWA et al. 1985) model was used with a transition/transversion ratio of 2.0 and constant rate variation among sites. A bootstrap analysis with 100 replicates was conducted to measure relative support of nodes.

Estimation of sequence divergence times and test of a molecular clock

The ITS maximum parsimony tree obtained from our PAUP* analysis was used to estimate sequence divergence times and rates of substitution using r8s 1.50 (SANDERSON 2003) with the Langley and Fitch, and TN settings. Because there are no fossils for *Orobanchaceae* with which to set a reference date, an internal reference of "1" was given for the root node and relative divergence dates calculated from this reference. Divergence time estimates have been calculated for *Lamiales* based on sequence data (71–74 mybp; WIKSTRÖM et al. 2001) or fossil data (37 mybp for *Oleacaceae*; MAGALLÓN et al. 1999). We took an average of the oldest and youngest estimates from these two studies to set the reference node time as 55.5 mybp. An estimate of sequence divergence dates were based on calculations of the relative divergence dates multiplied by 55.5 mybp.

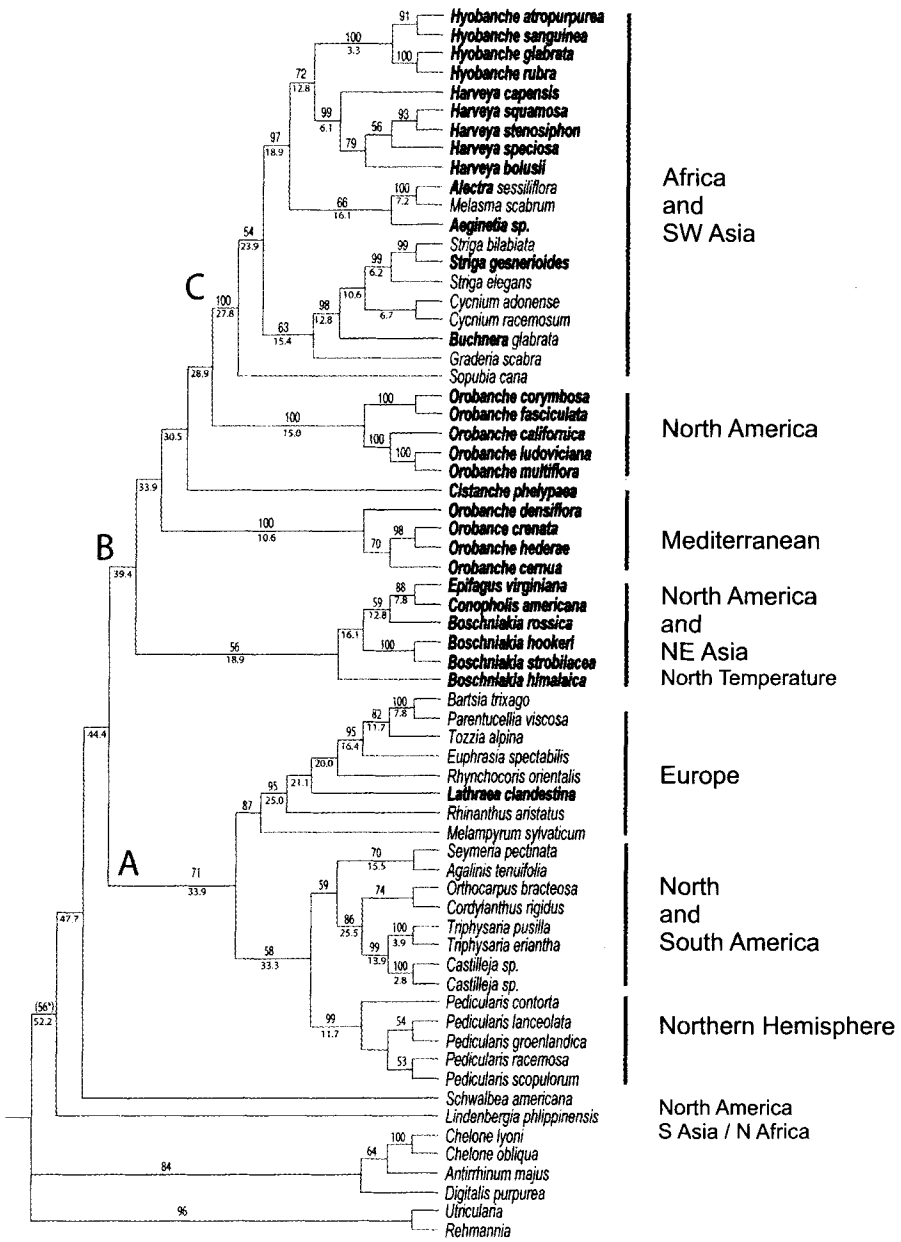

Fig. 2. Single most parsimonious tree from ITS nucleotide plus gap characters data. Taxa in boldface represent holoparasitic species or indicate genera that include holoparasitic species when the species included in the study was not holoparasitic. Bootstrap values above 50 are shown (* = bootstrap calculated on unconstrained tree from nucleotide data only) above the nodes. General distributions of genera are listed. Letters A, B, and C refer to major clades discussed in the text. Numbers shown below the nodes indicate the mean time in mybp since divergence estimates calculated from ITS sequences.

Table 2. Summary of distribution patterns for genera and species of *Orobanchaceae*. Number of species estimated from literature.

	New World Only	Old World Only	Cosmopolitan	Northern Hemisphere	Southern Hemisphere	Both Hemispheres
No. of genera	23	54	10	53	23	11
No. of species	163	1103	1031	1031	188	480
No. of species per genus	7.1	20.4	110	19.2	8.2	43.6

RESULTS

ITS sequences ranged in length from 535 to 592 bp (mean = 572) and had an aligned nucleotide sequence length (including 5.8S) of 670 bp resulting in 388 informative characters. Gap coding resulted in 41 additional informative characters, bringing the total number of informative characters for 65 taxa to 429. Heuristic searches were conducted for nucleotide characters alone, gap characters alone, and for the entire data set.

The heuristic search of nucleotide characters alone resulted in three most parsimonious trees of 2503 steps (CI = 0.367; RI = 0.691). A heuristic search of gap characters alone resulted in 22 most parsimonious trees of 296 steps (CI = 0.115; RI = 0.688), and the combined search yielded a single most parsimonious tree of length 2818 (CI = 0.338; RI = 0.688; Fig. 2).

Lindenbergia is sister to the parasitic genera of *Orobanchaceae* in the single most parsimonious tree (Fig. 2). Because we imposed outgroup constraints for the bootstrap analysis, we were unable to calculate a bootstrap on the basal node. However, in the nucleotide characters only analysis, no constraints were used and the bootstrap value for the node showing *Lindenbergia* as sister to the parasitic genera was 56. *Schwalbea*, a shrubby hemiparasite of southeastern United States appears at the base of the parasite clade in the most parsimonious tree, but with low relative nodal support. In general, the strongest bootstrap support is for terminal clades. Clades of species within genera generally had bootstrap support values above 90, with the exception in the single most parsimonious tree of *Boschniakia*, which formed a grade with *Conopholis* and *Epifagus*, and species of *Orobanche*, which fell into two clades separated by *Cistanche*.

The maximum likelihood tree (not shown) was similar to the single most parsimonious tree (Fig. 2) in the terminal clades with three exceptions: (1) *Schwalbea* and *Lindenbergia* appear as sister taxa (bootstrap value < 50), (2) *Graderia* and *Sopubia* are placed as basal members of the *Alectra/Harveya/Hyobanche* clade (bootstrap value << 50), and (3) *Cistanche* is sister to a monophyletic *Orobanche* clade (bootstrap value = 51). The backbone of both phylogeny reconstructions collapses with bootstrap values hovering around 50. Because the topological differences between these phylogeny reconstructions are minor and without relative nodal support where differences are noted, we present only the parsimony analysis as representative of our phylogenetic hypotheses.

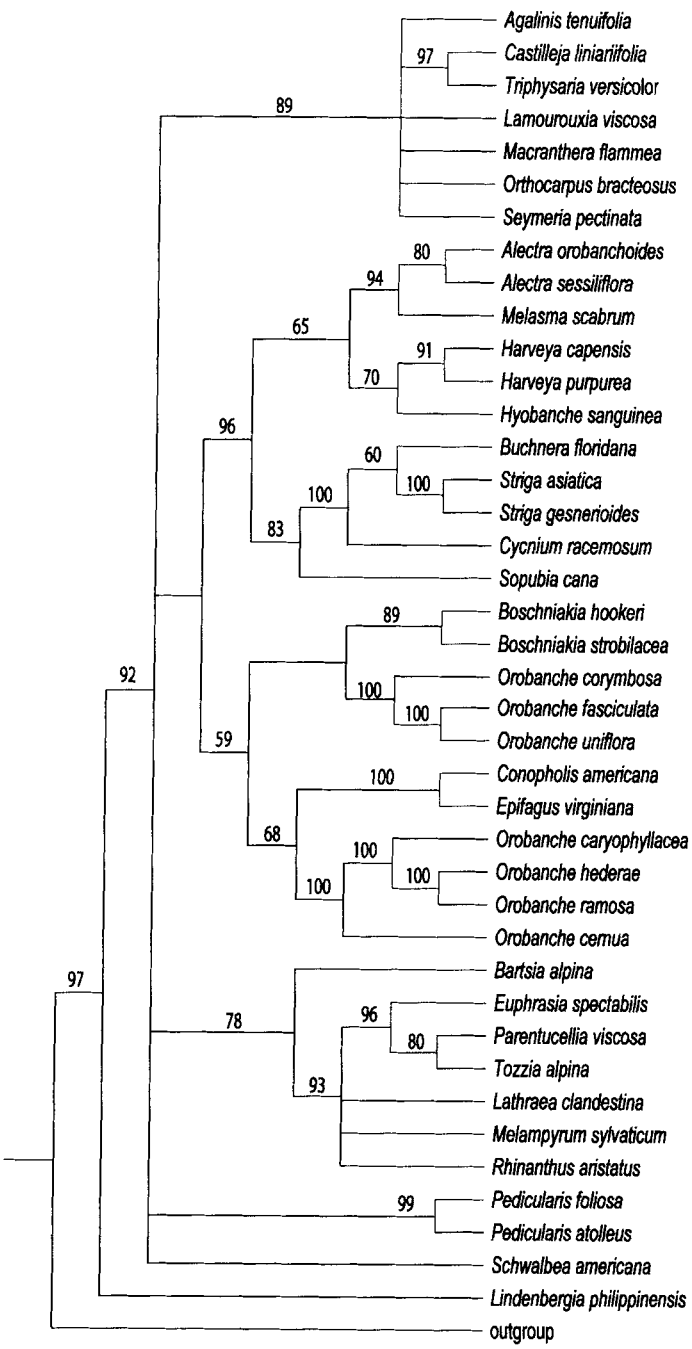

Fig. 3. Plastid phylogeny (strict consensus tree from combined *rps2* and *matK* data) reconstructed from YOUNG et al. (1999).

Table 3. DNA accession and voucher specimen data for taxa included in this study.

Family/Species	Collector, Accession code, Herbarium acronym	GenBank Accession Number
Gesneriaceae		
Rehmannia sp.	M. SINGER *s.n.* [LL]	AY911206
Lentibulariaceae		
Utricularia sp.	D. LES *s.n.* [CONN]	AY911207
Orobanchaceae		
Aeginetia sp.	C. DEPAMPHILIS *95.21* [PAC]	AY911208
Agalinus tenuifolia RAF.	A. COLWELL *s.n.* [WTU]	AY911209
Alectra sessiliflora KUNTZE	K. STEINER *3261* [NBG]	AY911210
Bartsia trixago L.	K. STEINER *3473* [CAS]	AY911211
Boschniakia himalaica HOOK.f. et THOMSON	A. COLWELL *s.n.* [GH]	AY911212
Boschniakia hookeri WALP.	A.WOLFE *588* [OS]	AY911213
Boschniakia rossica B. FEDTSCH.	A. COLWELL *s.n.* [WTU]	AY911214
Boschniakia strobilacea A. GRAY	A. WOLFE *530* [OS]	AY911215
Buchnera glabrata BENTH.	C. RANDLE *107* [OS]	AY911216
Castilleja sp.	A. WOLFE *532* [OS]	AY911217
Castilleja sp.	S. DATWYLER *78* [OS]	AY911218
Cistanche phelypaea (L.) COUT.	D. NICKRENT *4064* [ORO]	AY911219
Conopholis americana WALLR.	A. COLWELL *s.n.* [MO]	AY911220
Cordylanthus rigidus JEPS.	C. DEPAMPHILIS *90.02* [PAC]	AY911221
Cycnium adonense E. MEY. ex BENTH.	K. STEINER *3262* [NBG]	AY911222
Cycnium racemosum BENTH.	K. STEINER *3276* [NBG]	AY911223
Epifagus virginiana (L.) W.P.C. BARTON	D. NICKRENT *3016* [SIU]	AY911224
Euphrasia spectabilis PHIL.	C. DEPAMPHILIS *93.39* [PAC]	AY911225
Graderia scabra BENTH.	A. WOLFE *905* [OS]	AY911226
Harveya bolusii KUNTZE	K. STEINER *2440* [PAC]	AY911227
Harveya capensis HOOK.	A. WOLFE *715* [OS]	AF120224
Harveya speciosa BERNH.	K. STEINER *3267* [OS]	AY911228
Harveya squamosa STEUD.	A. WOLFE *716* [OS]	AF120225
Harveya stenosiphon HIERN	K. STEINER *3254* [OS]	AY911229
Hyobanche atropurpurea BOLUS	A.WOLFE *716* [OS]	AF120213
Hyobanche glabrata HIERN	A. WOLFE *721* [OS]	AF120217
Hyobanche rubra N.E. BR.	A. WOLFE *735* [OS]	AF120218
Hyobanche sanguinea L.	A. WOLFE *714* [OS]	AF120223
Lathraea clandestina L.	M. CHASE *2605* [K]	AY911230
Lindenbergia philipinensis BENTH.	J. ARMSTRONG *1163* [PAC]	AY911231
Melampyrum sylvaticum L.	W. WETSCHNIG *s.n.* [PAC]	AY911232
Melasma scabrum P.J. BERGIUS	K. STEINER *3496* [CAS]	AY911233
Orobanche californica CHAM. et SCHLTDL.	A. COLWELL *97CABLO-1* [WTU]	AY911234
Orobanche cernua LOEFL.	L.J. MUSSELMAN *s.n.* [PAC]	AY911235
Orobanche corymbosa (RYDB.) FERRIS	A. WOLFE *664* [OS]	AY911236
Orobanche crenata FORSK.	D. NICKRENT *4074* [ORO]	AY911237
Orobanche densiflora SALZM. ex REUT.	D. NICKRENT *4061* [ORO]	AY911238
Orobanche fasciculata NUTT.	A. WOLFE 663 [OS]	AY911239
Orobanche hederae DUBY	cultivated at Kew *2836* [K]	AY911240
Orobanche ludoviciana NUTT.	A. COLWELL *s.n.* [MO]	AY911241
Orobanche multiflora NUTT.	A. COLWELL *s.n.* [MO]	AY911242
Orthocarpus bracteosa BENTH.	A. WOLFE *571* [OS]	AY911243

Family/Species	Collector, Accession code, Herbarium acronym	GenBank Accession Number
Parentucellia viscosa (L.) CARUEL	C. DEPAMPHILIS *90.89* [PAC]	AY911244
Pedicularis contorta BENTH.	A. WOLFE *542* [OS]	AY911245
Pedicularis groenlandica RETZ.	A. COLWELL *CoMRS4* [WTU]	AY911246
Pedicularis lanceolata MICHX.	C. RANDLE *90* [OS]	AY911247
Pedicularis racemosa DOUGLAS et HOOK.	A. COLWELL *CoMRS5* [WTU]	AY911248
Pedicularis scopulorum GRAY	A. COLWELL *CoMRS7* [WTU]	AY911249
Rhinanthus aristatus ČELAK.	W. WETSCHNIG *s.n.* [PAC]	AY911250
Rhynchocorys orientalis (L.) BENTH.	D. ALBACH & SCHNEEWEIß *s.n.* [WU]	AY911251
Schwalbea americana L.	K. KIRKMAN *s.n.* [PAC]	AY911252
Seymeria pectinata PURSH.	J. ALLISON *4260b* [PAC]	AY911253
Sopubia cana HARV.	K. STEINER *2473* [PAC]	AY911254
Striga bilabiata KUNTZE	K. STEINER *3273* [NBG]	AY911255
Striga elegans BENTH.	K. STEINER *2476* [PAC]	AY911256
Striga gesnerioides (WILLD.) VATKE	K. SEINER *3263* [NBG]	AY911257
Tozzia alpina L.	W. WETSCHNIG *s.n.* [PAC]	AY911258
Triphysaria eriantha (BENTH.) T.I. CHUANG et HECKARD	A. COLWELL *s.n.* [WTU]	AY911259
Triphysaria pusilla (BENTH.) T.I. CHUANG et HECKARD	A. COLWELL *s.n.* [WTU]	AY911260
Scrophulariaceae		
Antirrhinum majus L.	A. WOLFE *s.n.* [OS]	AF375150
Chelone lyonii PURSH	A. NELSON *131* [OKL]	AF375163
Chelone obliqua L.	A. WOLFE *586* [OS]	AF375164
Digitalis purpurea L.	A. WOLFE *511* [OS]	AY911261

Calculation of substitution rates and estimates of a molecular clock

A molecular clock can not be strictly enforced on these data due to significant rate heterogeneity observed in the r8s analysis. Clades with accelerated rates of nucleotide substitution were those containing *Alectra*, *Melasma*, *Aeginetia*, *Cordylanthus*, and several holoparasitic lineages (Fig. 2). Increased substitution rates were also observed in the plastid genome of some genera of *Orobanchaceae* included in this study (*Buchnera*, *Cycnium*, *Conopholis*, *Epifagus*, *Euphrasia*, *Orobanche*, *Parentucellia*, and *Striga*; DEPAMPHILIS et al. 1997). Increased rates of nucleotide substitution have been demonstrated for rDNA molecules from the nuclear and plastid genomes of other lineages of parasitic plants as well (NICKRENT & STARR 1994, NICKRENT et al. 1997).

Rate heterogeneity is not a problem in estimating times since divergence in r8s (SANDERSON 2003). However, not having a fossil for *Orobanchaceae*, and calculating divergence times based on an average estimated age of *Lamiales* calculated from WIKSTRÖM et al. (2001) and MAGALLÓN et al. (1999) means that our inference of divergence times should be considered as a baseline for future studies. We are presenting these results as a starting framework in assessing the biogeographic hypotheses proposed for *Orobanchaceae* (RAVEN & AXELROD 1972, BARKER 1982, 1986).

Starting with an average estimated age for *Lamiales* as 55.5 mybp, *Orobanchaceae* is estimated to have originated 52.2 mybp (Fig. 2). The backbone of the ITS tree is not supported in the bootstrap analysis and, thus, estimating divergence dates are problematic. The age estimates for backbone nodes of this tree labeled in Fig. 2 range from 47.7 to 28.9 mybp. Age estimates for interior nodes with bootstrap values above 70 range from 33.9 to 7.8 mybp for clades containing two or more genera (clades including *Pedicularis* and *Epifagus*, respectively). Age estimates for monophyletic genera in the ITS tree range from 11.7 to 3.3 mybp (*Pedicularis* and *Hyobanche*, respectively).

DISCUSSION

Phylogeny

In parsimony analyses of ITS and plastid gene sequences (Fig. 3), the non-parasitic genus *Lindenbergia* is resolved as sister to the parasitic genera. The plastid tree showed strong nodal support for this relationship (bootstrap = 97; Fig. 3), whereas the ITS tree produced from nucleotide sequence data without the gap coding had a bootstrap of 56 for this node (Fig. 2). The maximum likelihood tree of ITS data (not shown) placed *Lindenbergia* as sister to *Schwalbea*. Previous classifications had placed *Lindenbergia* in tribe *Gerardieae* in affinity with *Sopubia* or *Euphrasia*, or as a member of *Gratioleae* (*Scrophulariaceae* s.l.; HJERTSON 1995). Our sampling did not include members of *Gratioleae*, but YOUNG et al. (1999) did include members of this tribe in their survey and found that *Lindenbergia* was not closely related to this group. YOUNG et al. (1999) placed *Lindenbergia* as the basal member of *Orobanchaceae*, and our unconstrained analyses of nucleotide only characters support this classification. Although *Lindenbergia* shows no evidence of a parasitic life history strategy (HJERTSON 1995), it has many characteristics (iridoid glycosides, rhinanthoid floral aestivation, stomata that do not close) in common with parasitic plants of *Orobanchaceae* (HJERTSON 1995; J. ARMSTRONG, Illinois State University, pers. comm.).

The single most parsimonious ITS tree (Fig. 2) unites the remaining parasitic *Orobanchaceae* into a single clade, although with no bootstrap support. *Schwalbea* is placed as sister to the other parasitic taxa in the ITS tree (Fig. 2), but in an unresolved position in the plastid tree (Fig. 3), and as a sister taxon to *Lindenbergia* in the ITS maximum likelihood tree (not shown). PENNELL (1935) hypothesized that *Schwalbea* was the basal member of the North American parasites of *Euphrasieae* and that it was a member of the Palaearctic Flora. The basal position of *Schwalbea* in the ITS tree lends support to PENNELL's (1935) statements about its possession of plesiomorphic characters (e.g., presence of a posterior sepal, septicidal capsule dehiscence, and the presence of two bractlets subtending the flower).

The ITS tree (Fig. 2) also supports the hypothesis that leafy, facultative parasites of *Orobanchaceae* represent the plesiomorphic condition with obligate parasitic and holoparasitic plants as derived. With the exception of the holoparasite *Lathraea* and cryptic hemiparasite *Tozzia* in clade A (Fig. 2), all other genera in this clade are leafy and primarily facultative hemiparasites (MUSSELMAN & MANN 1978, BARKER 1982). Most of the genera in clade B (Fig. 2) are either holoparasites or genera polymorphic for either hemi- or holoparasitism (e.g., *Alectra* and *Striga*). WEBER (1980) examined the haustoria of *Alectra*, *Bartsia*, *Euphrasia*, *Hyobanche*, *Parentucellia*, *Pedicularis*, *Rhinanthus*, and *Striga*, and

concluded that genera such as *Euphrasia* and *Parentucellia* represent the simplest form of parasitism in the group, whereas the haustoria of *Striga*, *Alectra*, and *Hyobanche* were the most "advanced". Holoparasitic genera and genera with hemi-and holoparasitic species occur in clade C of the ITS tree (Fig. 2). With the basal members of clade B consisting of holoparasitic genera only, it would appear from our results that the degree of parasitism was maintained as a variable character over a long period of the evolutionary history of this group. A reversal from holoparasitism to hemiparasitism is unlikely given what is known about the loss of photosynthesis genes in holoparasitic *Orobanchaceae* (DEPAMPHILIS & PALMER 1990, DEPAMPHILIS et al. 1997, WOLFE & DEPAMPHILIS 1997, 1998, WOLFE & RANDLE 2001).

The plastid phylogeny (Fig. 3) shows three separate clades (*Pedicularis* clade, *Agalinis* clade, *Bartsia* clade) collapsed to the base of the parasite clade, whereas the ITS tree shows resolution among these taxa (clade A, Fig. 2). The relationship among genera is generally consistent within the terminal clades of both trees (Fig. 2–3; e.g., *Agalinis*, *Seymeria*, *Castilleja* and *Orthocarpus* in one clade; *Bartsia*, *Euphrasia*, *Parentucellia*, *Tozzia*, *Melampyrum* and *Rhinanthus* in another clade). Whereas the plastid phylogeny shows no resolution among taxa in the *Agalinis* clade (Fig. 3), the ITS tree shows a sister relationship for *Seymeria* and *Agalinis* (bootstrap = 70), and a clade of *Castilleja*, *Triphysaria*, *Orthocarpus*, and *Cordylanthus* (bootstrap = 86). However, the relative nodal support for the clade including all these taxa is weak. In the *Bartsia* clade, there is disagreement between the two trees in the placement of *Bartsia* (Figs. 2–3), which may have resulted from the sampling of different species of *Bartsia* from different geographic regions for the two studies. The general structure of the ITS tree is better resolved with higher measures of nodal support than is the plastid phylogeny. Further sampling of taxa is needed before making systematic recommendations of relationships among these genera. However, one can note in the ITS tree that the clades are mainly defined by geography. For example, in clade A there are two groups, one of which includes *Agalinis* and *Pedicularis* (Fig. 2) and consists primarily of American and Asian taxa, whereas the group containing *Parentucellia* (Fig. 2) includes genera with predominantly European distributions.

Our results place the species of *Boschniakia* in a grade containing *Conopholis* and *Epifagus* (Fig. 2). These results disagree with the plastid gene tree (Fig. 3) where *B. hookeri* and *B. strobilacea* were sister to a clade of North American species of *Orobanche*, which in turn was sister to a clade showing *Epifagus* and *Conopholis* as sister taxa in a clade with European species of *Orobanche*. Neither of the two tree topologies group these taxa with high relative nodal support. However, the ITS tree can be interpreted in terms of biogeographic pattern in such a way that the grade exhibited in Fig. 2 may make some sense. The distribution of *Boschniakia* and relatives is from the Himalayas (*B. himalaica*) through eastern Asia (*B. rossica*) across Beringia (*B. rossica*) into western North America (*B. rossica*, *B. strobilacea*, *B. hookeri*, *Conopholis mexicana*) and eastern North America (*C. americana* and *Epifagus*). *Conopholis* and *B. strobilacea* and *B. hookeri* share a habit of short, squamous stems; *B. himalaica*, *B. rossica*, and *Epifagus* have taller and successively slender stems. All the taxa in this clade except *Epifagus* are perennials with robust tuberous haustoria. One possibility is that the grade depicted in Fig. 2 represents a single genus with an origin in Asia

and subsequent migration across Beringia into North America. *Epifagus* would display an extreme reduction in stem and inflorescence size correlated to the transition to an annual habit. Alternatively, the species of *Boschniakia*, as currently circumscribed, may represent more than one genus as proposed by BECK (1930). Further studies will be necessary to test these hypotheses.

The classification of *Orobanche* has been under consideration since BECK's (1930) monograph. The genus has at various times been segregated into four genera with the New World and Old World taxa falling into separate genera (HOLUB 1990); alternatively, all taxa have been merged into *Orobanche* and the differences among species were used as the basis for sectional classification (BECK 1930). Our most parsimonious tree shows a New World clade segregated from the Old World taxa with each separate clade having strong bootstrap support (Fig. 2). A similar pattern of segregation was revealed in the plastid gene tree (Fig. 3), but with different topological patterns for other genera of *Orobanchaceae*. For example, the plastid tree (Fig. 3) placed the two clades of *Orobanche* with *Boschniakia* (New World taxa) or with *Epifagus* and *Conopholis* (Old World taxa). A broader sampling of *Orobanche* for an ITS phylogeny was unable to resolve the taxonomic boundaries of this large and diverse genus (SCHNEEWEISS et al. 2004). Our results support the separation of New World and Old World species of *Orobanche* into separate taxa, but the sampling in this study is insufficient to make definite recommendations.

Relationships among *Buchnera*, *Cycnium*, and *Striga* were investigated by BACKLUND et al. (1993) in their revision of *Cycniopsis*. The hierarchical key displayed in their Fig. 1 showed the same general structure as our results in Fig. 2, but differs from the plastid tree topology (Fig. 3). *Buchnera*, *Cycnium*, and *Striga* share the morphological character of equal, monothecous anthers, and the latter two share a floral character (two upper corolla lobes fused; BACKLUND et al. 1993). Our results support a sister relationship for *Harveya* and *Hyobanche* (Fig. 2), which has also been supported in plastid *rps2* and *matK* gene trees (Fig. 3; DEPAMPHILIS et al. 1997, YOUNG et al. 1999), but not in the *rbcL* tree presented in WOLFE & DEPAMPHILIS (1998). The latter result may reflect excessive sequence divergence in the *rbcL* pseudogenes observed for every species of *Hyobanche* (WOLFE & DEPAMPHILIS 1998, WOLFE & RANDLE 2001). The strong support for a primarily African clade of parasitic genera (bootstrap = 100 for ITS clade C; Fig. 2) is also in agreement with the plastid gene tree (bootstrap = 96; Fig. 4 in YOUNG et al. 1999).

An interesting result from our study is the placement of *Aeginetia* in the clade containing *Alectra* and *Melasma* (Fig. 2). The sister relationship exhibited by the latter two genera (bootstrap = 100) was also revealed in the plastid gene tree (bootstrap = 94; Fig. 3). However, *Aeginetia* was not included in the plastid gene survey. The morphology of *Aeginetia* differs greatly from *Alectra* and *Melasma*, with the former being a holoparasite producing only flowering stems above ground, whereas the latter two are leafy hemiparasites. *Aeginetia* has a distinctive anther morphology similar to *Harveya* and *Hyobanche* where the stamens bear a single fertile anther lobe with curved spurs at the tip of the anther sac (BOESHORE 1920). *Alectra* and *Melasma* have two fertile anthers with the same spur morphology, but one anther sac is reduced in size (FISCHER 1996). KUIJT (1969) suggested that the anther morphology trends shared among these taxa may indicate a close relationship with *Aeginetia* perhaps

being a derivative of *Harveya*. The relationships among genera in this clade is currently under investigation (J. MORAWETZ & A.D. WOLFE, Ohio State University, unpubl. data).

Biogeography

Of the 87 genera currently recognized for *Orobanchaceae* (Table 1), 23 are New World endemics, 54 are Old World endemics and 10 are distributed across the globe (Table 2). Also, 53 genera are found in the Northern Hemisphere only, 23 in the Southern Hemisphere only, and 11 across both regions (Table 2). The general distributions of taxa in this survey are shown in Fig. 2.

Very few studies of genera of *Orobanchaceae* have focused on biogeography. The primary exception is *Euphrasia*, for which the Australian species were monographed (BARKER 1982). HONG (1983) also included *Euphrasia* and several other genera of *Orobanchaceae* in his biogeographic analysis of *Scrophulariaceae*. *Euphrasia* has an unusual distribution with separate regions of diversity in the northern and southern hemispheres. BARKER (1982, 1986) hypothesized that *Euphrasia* is an ancient Gondwanan genus that originated during the Cretaceous, and that its unusual distribution represents vicariance following the breakup of Gondwanaland. BARKER (1982, 1986) based this conclusion on the lack of dispersal mechanisms that would allow for long distance dispersal and on Southern hemisphere species having morphological characters that were proposed to be basal in the genus. HONG (1983) proposed that these genera also have a Gondwanan affinity. However, RAVEN & AXELROD (1972, 1974) proposed that *Euphrasia* is much younger and attained its distribution from a northern origin since the Neogene (ca. 27 mya). There are no well-documented fossils for *Orobanchaceae* or genera closely affiliated with the family prior to the Eocene (56 mya), and most families in *Lamiales* date to the Miocene (23 mya) or more recently (BARKER 1982). Our age estimates (Fig. 2) suggest a divergence of 16.4 mybp for the clade including *Euphrasia*, *Tozzia*, *Parentucellia* and *Bartsia* (Fig. 2). This supports an hypothesis of a much more recent origin than Cretaceous for *Euphrasia*, and is consistent with what little is known about *Lamiales* from fossil data. Our study was not designed to examine the biogeography of *Euphrasia*. However, the placement of *Euphrasia* and *Bartsia*, another genus with hypothesized Gondwanan affinities (BARKER 1986), as non-basal members of the clade to which they belong (Fig. 2) does not support the hypothesis of a Gondwanan origin for these taxa (BARKER 1982, 1986, HONG 1983) given that none of the other genera in this clade have a Gondwanan distribution pattern.

Lindenbergia and *Schwalbea* represent the basal lineages of *Orobanchaceae* in the ITS tree (Fig. 2). The 12 species of *Lindenbergia* have a distribution from northeastern Africa to southeastern Asia with a center of diversity in the Himalayan range (HJERTSON 1995). Furthermore, previous studies have suggested an origin for the genus in the Himalayas with subsequent migration and diversification from east to west (HJERTSON 1995). *Schwalbea* is monotypic with a distribution in the southeastern region of the United States. PENNELL (1935) hypothesized that the closest relative to *Schwalbea* is *Siphonostegia*, which has three species distributed from the eastern Mediterranean region to eastern Asia (Table 1). PENNELL (1935) also proposed these taxa represent primitive Palaearctic genera. Our age estimates for the basal nodes of the tree are 52.2 and 47.7 mybp (Fig. 2), which would support a hypothesis of

a late Tethyan Seaway affinity for origin/dispersal of the basal lineages of *Orobanchaceae* (AXELROD 1975).

Several clades in the ITS tree have Eurasian/North American distribution patterns that would match a Madrean-Tethyan or Beringian dispersal/diversification route with subsequent migration to and diversification in South America. Clade A of the ITS tree (Fig. 2) has subclades of genera that are predominantly distributed either in northern temperate regions with an emphasis in Eurasia or in the New World. Estimates of divergence times separating the two major groups in this clade range from 33.3 to 33.9 mybp (Fig. 2). Some genera are widespread (*Pedicularis*, *Bartsia*, *Euphrasia*), whereas others have species that are concentrated in one region such as western North America (*Castilleja*, *Orthocarpus*) or in southern North America and into South America (*Agalinis*, *Seymeria*). *Melampyrum* is a genus of ca. 35 species with a single species in North America and the rest concentrated in the Mediterranean (HONG 1983). *Pedicularis* is the largest genus of *Orobanchaceae* and it has a center of diversity in the eastern Himalayas (PENNELL 1935, HONG 1983). Our study does not include sufficient sampling of genera in this clade to propose definite hypotheses, but it is possible that diversification and dispersal routes for genera in Clade A (Fig. 2) proceeded in several directions radiating from a point of origin north of the Tethys Sea.

The two groups of *Orobanche* in the ITS tree exhibit a Mediterranean or North American pattern of distribution (Fig. 2), with a center of diversity in the Mediterranean region (PARAN et al. 1997). Divergence time estimates among members of these separate clades range from 15 to 10.6 mybp, and for the grade containing these taxa from 33.9 to 28.9 mybp (Fig. 2). *Cistanche* has species distributed from the Mediterranean and Northern Africa east to Asia (Table 1). Given the general east-west pattern of distribution in the context of time since divergence for *Orobanche*, and the pattern of monophyly for the North American *vs.* Old World species of *Orobanche*, diversification across the region fits AXELROD's (1975) Madrean-Tethyan hypothesis.

Two clades in the ITS tree do not show a Tethyan Seaway affinity. The *Boschniakia* clade was discussed in the previous section, and it seems most probable that dispersal was from eastern Asia across Beringia into North America in the mid- to late Tertiary. The remaining clade C (Fig. 2) has genera with predominantly African or paleotropical distribution patterns (Table 1). The age estimate for clade C is 27.8 mybp (Fig. 2). *Sopubia* lies at the base of clade C (Fig. 2) and has a distribution that spans the range of most other taxa in the clade from Old World tropics to southern Africa and Madagascar. Most of the genera in this clade have their center of diversity and majority of species in south or eastern Africa (e.g., *Alectra*, *Cycnium*, *Graderia*, *Harveya*, *Hyobanche*, *Striga*), and *Buchnera* and *Melasma* have a small minority of their species distributed in the New World. *Striga* has a secondary center of diversity in India with species from Africa and India sharing distributions in the southern part of the Arabian peninsula (MUSSELMAN & HEPPER 1988, MOHAMED et al. 2001). *Graderia* also has a species distributed in Socotra (Table 1), an island southeast of the Arabian Peninsula. *Hyobanche* is unique in the clade having species entirely restricted to the southern tip of the African continent. Species of *Striga*, *Alectra*, and *Buchnera* are often associated with inselbergs in eastern Africa, especially toward the south (SEINE et al. 1995), and species of *Harveya* and *Hyobanche* are often concentrated in high elevation zones in southern Africa

(pers. observ.). Given the general distributions of the taxa in the clade as a whole with several species spanning the southern Arabian peninsula, the trend for high species numbers and diversity in eastern and southern Africa, and the association of several genera with high elevation habitats, it seems probable that diversification of genera began in the Old World tropics and spread down the eastern arid corridor of Africa that formed starting in the mid-Tertiary (FJELDSA & LOVETT 1997).

CONCLUSIONS

Orobanchaceae as a whole have been understudied with the exception of economically important agricultural weeds. Our study included less than half of the described genera, many of which are monotypic and difficult to collect. Few systematic studies of genera within *Orobanchaceae* have examined morphological or anatomical characters of entire plants, primarily because many preserved herbarium specimens are of partial plants or are so poorly preserved as to make comparative studies difficult at best. Even fewer studies have made morphological or anatomical comparisons among genera. While molecular data offer characters to resolve relationships among some taxa, it is clear from a comparison of Figs. 2–3 that more studies including more genera and morphological/anatomical characters are needed to better resolve the phylogeny of *Orobanchaceae*. In addition, it would be good to examine the sources of conflict between the nuclear and plastid gene trees.

Even with the coarse divergence time estimates gleaned from this study, it would appear that an origin of the family during the Cretaceous is unlikely. More probable is a mid-Tertiary origin north of the Tethys Sea, possibly in eastern Asia as proposed by HONG (1983), followed by subsequent radiation and diversification to the west and northeast for most of the genera included in the study, with the terminal clade taxa diversifying and spreading through the highlands of eastern Africa to the southern tip of the continent. If we assume an origin of *Orobanchaceae* near the Eocene-Oligocene boundary 40 to 50 million years ago, the continental plates would have been situated for the diversification routes described here. The spread of the family from Eurasia to North America could have occurred westward along the diminishing Tethyan Seaway route and/or northeastward over Beringia. As *Orobanchaceae* diversified, the spread of taxa across northern Africa into South America, from North America into South America, and from the paleotropics to arid regions of Africa and into Australasia would have likely occurred in successive waves of diversification as the continental plates shifted into their current configuration. The topology of the ITS tree (Fig. 2) supports the early east-west diversification in the Northern Hemisphere and later spread through the paleotropics and southern hemisphere.

The biogeography of *Euphrasia* and *Bartsia* is unusual for the family and further elucidation of their evolution awaits robust phylogenetic studies, as do the evolutionary details for the large genera (*Pedicularis*, *Orobanche*, etc.) of *Orobanchaceae*. Our study serves as a preliminary framework for future research on the phylogenetics and biogeography of the family, but we can clearly support the clade definition of *Orobanchaceae* sensu YOUNG et al. (1999) and hypothesize a mid-Tertiary Laurasian origin of the family.

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