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Relationships of the Buddlejaceae s. l. Investigated using Parsimony Jackknife and Branch Support Analysis of Chloroplast *ndhF* and *rbcL* Sequence Data

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ABSTRACT. The systematic positions of genera previously assigned to the Buddlejaceae are investigated using Bremer support and parsimony jackknife analyses of cpDNA *ndhF* and *rbcL* sequence data. The differences between these two methods are discussed: resampling methods such as the jackknife help identify and assess confidence in qualitatively supported groups, whereas Bremer support provides an absolute, quantitative measure of that support. The close relationship of Buddlejaceae s. str. (comprising *Buddleja*, *Nicodemia*, *Emorya*, and *Gomphostigma*) to Scrophulariaceae s. str. is confirmed. Previous suggestions that *Desfontainia* and *Plocosperma* are not related to the Scrophulariales sensu Thorne are corroborated, as are the close relationships of *Sanango* to Gesneriaceae and of *Retzia* to Stilbaceae. Previously unsuspected monophyletic groups revealed include *Androya* and *Myoporium*, together appearing as sister group to the Scrophulariaceae/Buddlejaceae clade, *Nuxia* and the Stilbaceae clade, *Peltanthera* and the *Sanango*/Gesneriaceae clade, and *Polypremum* and *Tetrachondra*. The latter genus is not part of Scrophulariales sensu Thorne, which otherwise is well supported. Oleaceae is resolved as sister group to the rest of the clade. Within the order a gesneriad clade, a stilbacean clade, two 'scroph' clades, Acanthaceae, Bignoniaceae, Lamiaceae, and Verbenaceae occur as distinct lineages. The relationships among these are poorly resolved and the position of *Lindenbergia* is uncertain.

The circumscription and systematic position of Buddlejaceae is a controversial issue in angiosperm systematics. Early classifications included *Buddleja* L. in the Scrophulariaceae (Jussieu 1789), but Benth (1856) moved the genus together with *Polypremum* L., *Gomphostigma* Turcz., *Nuxia* Lam., *Chilianthus* Burch., *Nicodemia* Ten., and *Emorya* Torr. to Loganiaceae in the Loganiaceae. Solereder (1892) distinguished these genera together with *Peltanthera* Benth. as a subfamily within Loganiaceae and further discussed the uncertain position of *Plocosperma* Benth. and *Desfontainia* Ruiz & Pav. Wilhelm (1910) gave Buddlejaceae family status, but he only mentioned *Buddleja* as an included genus. Leenhouts (1963) and Leeuwenberg and Leenhouts (1980) largely follow Solereder, although Leenhouts added two genera described in the 20th century, *Androya* H. Perrier and *Sanango* G. S. Bunting & J. A. Duke, and the controversial Cape genus *Retzia* Thunb. He also moved *Polypremum* to Spigeliaceae within the Loganiaceae. Other recent classifications (Wagenitz 1959; Melchior 1964; Cronquist 1981; Dahlgren 1983; Takhtajan 1987; Thorne 1992) have reinstated a scrophulariacean affinity of the Buddlejaceae, which is supported, for example, by the presence of common parasitic organisms (Mohrbuter 1936), the presence of decarboxylated iridoids (Jensen et

al. 1975; Jensen 1992; Gershenzon and Mabry 1983), the presence of 6-hydroxyluteolin (characteristic of members of the Scrophulariales, Tomas-Barberan et al. 1988), and the presence of verbascoside (Jensen 1992; Scogin 1992 [as acteoside]). It is also supported by palynological characters (Punt 1980), embryological characters (Maldonado de Magnano 1986), wood anatomy (Carlquist 1992), and cpDNA sequences (Bremer et al. 1994; Olmstead and Reeves 1995).

Although evidence for a close relationship with Scrophulariaceae may be overwhelming, the circumscription of Buddlejaceae is still debatable. Olmstead and Reeves (1995) demonstrated gross paraphyly of Scrophulariaceae s. l., with *Buddleja* and *Selago* L. together as sister group to Scrophulariaceae s. str. (represented by *Scrophularia* L., *Verbascum* L., and *Celsia* L.). The *rbcL* sequence study of Bremer et al. (1994) corroborated earlier suggestions (Dahlgren et al. 1979; Carlquist 1986) that *Retzia* is closely related to Stilbaceae. Furthermore, *Desfontainia* is best classified in Dipsacales according to the results of Bremer et al. (1994) and the more extensive *rbcL* study of Backlund and Bremer (1997). Wiehler (1994) argued for the inclusion of *Sanango* in the Gesneriaceae based on the morphological, anatomical, and phytochemical findings of

Norman (1994), Dickson (1994), and Jensen (1994), respectively. This view was further corroborated by Jensen (1996) and a cpDNA *ndhF* study by Smith et al. (1997b), where *Sanango* appears close to *Gesneria* L., with the two nested within the Gesneriaceae. The position of *Polypremum* is also controversial. In contrast to the results obtained for other Buddlejaceae genera already discussed, *Polypremum* has usually been classified with truly Loganiaceae taxa, and Cronquist (1981) expressed some reservations regarding its inclusion in Buddlejaceae. However, Scogin and Romo-Contreras (1992) and Jensen (1992) have presented phytochemical evidence for a position in Scrophulariales, and Jensen (1992) suggested a position in or near Scrophulariaceae or Oleaceae.

This study further explores the phylogenetic positions of the taxa that have been classified in Buddlejaceae using DNA sequences of the chloroplast genes *rbcL* and *ndhF*.

MATERIALS AND METHODS

Choice of Taxa. Representatives of all genera recognized by Leeuwenberg and Leenhouts (1980) as belonging to Loganiaceae, but determined to be placed elsewhere than in Gentianales (Struwe et al. 1994, Backlund et al. in press) were included. Twentyfour *ndhF* sequences and fourteen *rbcL* sequences are reported here for the first time. All GenBank accessions (as of May 1997) of *rbcL* and *ndhF* sequences belonging to Scrophulariales sensu Thorne (1992) were used initially. In addition, selected taxa belonging to Gentianales, Solanales, Boraginales, Hydrangeales, Dipsacales, and Asterales were used as outgroups. Because some groups (e.g., Lamiaceae) are extensively represented in GenBank, a subset was chosen using results from preliminary parsimony runs, with sequence completeness as criterion for inclusion. Experiments with more extensive sampling did not appear to affect in-group results markedly. Taxa used with vouchers and GenBank accession numbers are listed in Table 1.

Most previously unpublished sequences were obtained from small amounts of dried plant material. Ground tissue was incubated in 2% CTAB + 3% mercaptoethanol at 60°C for 1 to 2 hours. DNA was extracted with one volume chloroform:isoamylalcohol (24:1), which was gently shaken for 1 to 2 hours, then centrifuged at 10,000 g for 10 minutes. The supernatant was

then treated with the Qiaquick PCR kit (Qiagen) according to the instructions from the manufacturer. Usually, this yielded DNA suitable for subsequent polymerase chain reactions (PCR) reactions, but for some templates, the durability appeared to be short. PCR primer sequences for *rbcL* were taken from Olmstead et al. (1992). In some cases, the gene was split into two PCR fragments using internal primers designed by G. Zurawski (DNAX Research Institute). PCR of the entire *ndhF* gene was carried out either in a single reaction using the primer pair -47/+606R or in two separate reactions with the primer pairs 1/1350R and 1201/+606R (see Fig. 1 for primer sequences). Amplification products were purified with the Qiaquick PCR kit according to the instructions from the manufacturer. Sequencing reactions were performed with the PRISM Ready Reaction Dye Deoxy Terminator FS kit (Applied Biosystems) and analyzed on a ABI 377 automated sequencer (Applied Biosystems).

Character Coding and Alignment. The *ndhF* sequences were aligned manually to the reading frame starting at position 114,198 (reverse complement) in the *Nicotiana tabacum* L. complete chloroplast genome sequence (Shinozaki et al. 1986, GenBank accession number Z00044). Position 114,168 to 112,096 (corresponding to the *Nicotiana* sequence) of *ndhF* and 57,612 to 59,020 of *rbcL* were used in the analyses. The *rbcL* sequences show no length variation within this range. Indels in *ndhF* were recoded to binary characters using the criteria described in Oxelman et al. (1997). Translation of the GenBank accessions of Gesneriaceae and *Sanango ndhF* sequences revealed early stop codons in many of the sequences. Moreover, in the 3' part of the gene, it became very difficult to align the sequences reliably to any reading frame. The GenBank accession of *Sanango* (U62144) is marked as a pseudogene, and it was suspected that the sequences of Smith et al. (1997a, 1997b) were not homologous with the other sequences in this study. Consequently, they were not used. The sequences of *Streptocarpus* Lindl. and *Nematanthus* Schrad. from the study of Olmstead and Reeves (1995) appeared unproblematic however, and were used to represent Gesneriaceae. The *rbcL* matrix included 82 taxa and 342 potentially informative characters, of which 3.73% were scored as missing or ambiguous. The *ndhF* matrix included 73 taxa and 886 informative characters, of which 5.56% were scored as miss-

TABLE 1. Voucher information for previously unpublished sequences, literature references to those previously published, and GenBank accession numbers for sequences included in the cpDNA sequence survey. Taxa are arranged according to the classification of Thorne (1992). Values refer to the proportion of phylogenetically informative sequence positions scored as missing in the combined (comb.), *ndhF*, and *rbcl* data sets. Names used for representing a certain taxon in the cladograms are in capital letters. Herbarium abbreviations in brackets after voucher citation are according to Holmgren et al. (1990). Literature references: 1 = Kim and Jansen (1995), 2 = Michaels et al. (1993), 3 = Olmstead and Reeves (1995), 4 = Olmstead et al. (1992), 5 = Olmstead et al. (1993a), 6 = Wagstaff and Olmstead (1997), 7 = Gustafsson et al. (1996), 8 = Backlund et al. (in press), 9 = Bremer et al. (1994), 10 = Bremer et al. (1995), 11 = Hedrén et al. (1995), 12 = Scotland et al. (1995), 13 = Albert et al. (1992), 14 = Wagstaff et al. (1997), 15 = Chase et al. (1993), 17 = Olmstead and Sweere (1994), 18 = Olmstead et al. (1993b), 19 = Shikanai et al. (1996), 20 = Bohs and Olmstead (1997), 21 = Endress et al. (1996).

	Voucher / reference	GenBank accession number	comb.	<i>ndhF</i>	<i>rbcl</i>
Asterales					
Asteraceae					
<i>BARNADESIA caryophylla</i> (Vell.) S. F. Blake	1	L39394	0.44	0.56	
	2	L13859			0.00
Boraginales					
Boraginaceae					
<i>BORAGO officinalis</i> L.	3	L36393	5.59	5.30	
	4	L11680			5.26
<i>HÉLIOTROPIUM arborescens</i> L.	5	L14399			2.92
Tetrachodraceae					
<i>TETRACHONDRA patagonica</i> Skottsbo.	Swenson & Martinsson 314 (UPS)	AF027272	2.84	0.11	
<i>T. hamiltonii</i> Petrie	6	U28885			10.23
Campanulales					
Menyanthaceae					
<i>MENYANTHES trifoliata</i> L.	1	L39388	1.69	1.02	
	5	L14006			3.51
Dipsacales					
Adoxaceae					
<i>VIBURNUM rhytidophyllum</i> Hemsl.	Backlund 271 (UPS)	AF027273	1.42	1.69	
	7	X87398			0.29
Caprifoliaceae					
<i>LONICERA orientalis</i> Lam.	Backlund 267 (UPS)	AF027274	1.95	2.37	
	7	X87389			0.29
Gentianales					
Apocynaceae					
<i>ALSTONIA scholaris</i> (L.) R. Br.	Fanning 212 (FTG)	AJ011982	1.69	1.58	
	9	X91760			1.46
Gentianaceae					
<i>EXACUM affine</i> Balf. f.	Bremer 2741 (UPS)	AJ011983	2.93	2.37	
	5	L11684			4.09
Loganiaceae					
<i>GELSEMIUM sempervirens</i> (L.) J. St. Hill	Bremer 3026 (UPS)	AJ011984	2.84	2.82	
	5	L14397			1.75
<i>NEUBURGIA corynocarpa</i> (A. Gray) Leenh.	A. C. Smith 7385 (S)	AF027275	1.86	1.92	
		AJ001755			1.17
<i>PLOCOSPERMA buxifolium</i> Benth.	Salinas 8050 (MEXU)	AJ011985	2.04	1.92	
	21	Z68829			1.46

TABLE 1. Continued.

	Voucher/reference	GenBank accession number	comb.	<i>ndhF</i>	<i>rbcL</i>
<i>POLYPREMUM procumbens</i> L.	Struwe s. n. (UPS)	AJ011986 AJ011989	0.71	0.11	1.75
Rubiaceae					
<i>LUCULIA gratissima</i> (Wall.) Sweet	10 10	AJ011987 X83648	1.06	0.68	1.46
Hydrangeales					
Desfontainiaceae					
<i>DESFONTAINIA spinosa</i> Ruiz & Pav.	Bremer 2739 (UPS) 9	AJ011988 Z29670	1.51	1.13	1.75
Scrophulariales					
Acanthaceae					
<i>ACANTHUS montanus</i> (Nees) T. Anderson	11	L12592			0.58
<i>BARLERIA prionitis</i> L.	12 13	U12653 L01886	11.98	15.24	1.46
<i>BRILLANTAISIA lamium</i> Benth.	12	U12654		4.29	
<i>CRABBEA reticulata</i> C. B. Clarke	12	U12655		3.95	
<i>CROSSANDRA nilotica</i> Oliv.	12	U12656		6.88	
<i>ELYTRARIA cremata</i> Vahl.	12	U12657		14.45	
<i>ERANTHEMUM pulchellum</i> Andrews	12	U12658		14.90	
<i>EREMOMASTAX speciosa</i> (Hochst.) Cufod.	12	U12659		7.90	
<i>HEMIGRAPHIS alternata</i> T. Anderson	12	U12660		11.51	
<i>HYGROPHILA corymbosa</i> Lindau	12	U12661		4.51	
<i>HYPOESTES taeniata</i> Benoist	12	U12662	29.90	40.18	
<i>H. forskaolii</i> R. Br.	13	L12593			1.46
<i>JUSTICIA americana</i> (L.) Vahl.	12	U12663	29.55	23.14	
<i>J. odora</i> (Forssk.) Lam.	5	L01930			0.29
<i>LEPIDAGATHIS villosa</i> Hedrén	11	L12594			0.29
<i>NELSONIA campestris</i> R. Br.	13	L01935			0.29
<i>RUELLIA ciliata</i> Pursh	12	U12664	16.86	22.57	
<i>R. graecizans</i> Backer	5	L12595			0.29
<i>RUTTYA fruticosa</i> (Forssk.) Lam.	13	L02434			0.29
<i>SANCHEZIA speciosa</i> Leonard	12	U12665		14.79	
<i>STROBILANTHES dyeriana</i> Mast.	12	U12666		16.37	
<i>THUNBERGIA alata</i> Bojer ex Sims	12	U12667	4.26	5.19	
<i>T. usambarica</i> Lindau		L12596			0.29
Avicenniaceae					
<i>AVICENNIA germinans</i> (L.) L.	6	U28868			0.58
Bignoniaceae					
<i>CATALPA</i> sp.	3 4	L36397 L11679	9.94	11.51	4.09
<i>MARTINELLA martinii</i> (DC.) Baill. ex K. Schum.	3	L36402 L36444	3.02	3.16	1.46
<i>TABEBUIA heterophylla</i> (DC.) Britton	3 3	L36416 L36451	2.57	3.05	0.29
Buddlejaceae					
<i>ANDROYA decaryi</i> H. Perrier	Phillipson & al.3747 (MO)	AF027276 AJ001756	6.30	1.13	20.18
<i>BUDDLEJA ASIATICA</i> Lour.	Bremer 3500 (UPS)	AF027277 AJ001758	1.24	0.90	1.46

TABLE 1. Continued.

	Voucher / reference	GenBank accession number	comb.	<i>ndhF</i>	<i>rbcL</i>
<i>BUDDLEJA DAVIDII</i> Franch.	3	L36394	3.19	3.84	
	Bremer 3315 (UPS)	AJ001757			0.29
<i>EMORYA suaveolens</i> Torr.	Villarreal 6995 (TEX)	AF027278	0.62	0.79	
		AJ001759			0.00
<i>GOMPHOSTIGMA scoparioides</i> Turcz.	Örtendahl 606 (UPS)	AF027279	1.86	1.69	
		AJ001760			1.46
<i>NICODEMIA diversifolia</i> (Vahl) Tenore	3	L36405	3.90	4.06	
	5	L24413			2.05
<i>NUXIA</i> sp.	Hedberg 4731 (UPS)	AF027280	0.80	0.34	
		AJ001761			1.46
<i>PELTANTHERA floribunda</i> Benth.	Hammel 19855 (MO)	AF027281	0.89	0.56	
		AJ001762			1.46
<i>SANANGO</i> sp.	Bremer 3352 (UPS)	AF027283	1.15	0.56	
		AJ001763			2.05
Callitrichaceae					
<i>CALLITRICHE hermaphroditica</i> L.	3	L36441			11.11
Gesneriaceae					
<i>NEMATANTHUS hirsutus</i> (Mart.) Wiehler	3	L36404	3.28	3.27	
		L36446			2.05
<i>STREPTOCARPUS holstii</i> Engl.	3	L36415	4.35	3.84	
	5	L14409			4.97
Globulariaceae					
<i>GLOBULARIA cordifolia</i> L.	Bremer 3865 (UPS)	AF027282	10.12	0.90	
		AJ001764			36.26
Hippuridaceae					
<i>HIPPURIS vulgaris</i> L.	3	L36401	13.84	14.67	
	3	L36443			10.53
Lamiaceae					
<i>AJUGA reptans</i> L.	3	L36391	5.50	6.66	
	3	U32163			1.46
<i>CARYOPTERIS bicolor</i> (Roxb.) Mabb.	14	U78680	3.11	3.84	
		U78711			0.29
<i>TECTONA grandis</i> L.	Bremer 3086 (UPS)	AF027284	0.62	0.56	
		AJ001765			0.29
Lentibulariaceae					
<i>PINGUICULA caerulea</i> Walter	13	L01942			0.29
<i>UTRICULARIA biflora</i> Lam.	15	L13190			21.93
Martyniaceae					
<i>PROBOSCIDEA louisianica</i> (Mill.) Thell.	13	L01946			0.29
Myoporaceae					
<i>MYOPORUM mauritianum</i> DC.	3	L36403	6.03	3.84	
		L36445			11.11
Nesogenaceae					
<i>CYCLOCHEILON somaliense</i> Oliv.	6	U28871			1.17
Oleaceae					
<i>JASMINUM suavisimum</i> L.	13	L01929			0.29
<i>LIGUSTRUM vulgare</i> L.	4	L11686			3.22

TABLE 1. Continued.

	Voucher/reference	GenBank accession number	comb.	<i>ndhF</i>	<i>rbcL</i>
<i>NYCTANTHES arbor-tristis</i> L.	14	U78708	3.90	4.29	
	6	U28877			1.46
<i>OLEA europaea</i> L.	Å. Dahl 703 (GB)	AF027288	1.24	1.35	
		AJ001766			0.29
Pedaliaceae					
<i>HARPAGOPHYTUM granidieri</i> Baill.	13	L01923			0.29
<i>SESAMUM indicum</i> L.	3	L36413	6.21	6.09	
	5	L14408			5.85
Phrymataceae					
<i>PHRYMA leptostachya</i> L.	6	U28881			0.29
Plantaginaceae					
<i>PLANTAGO lanceolata</i> L.	3	L36408	10.74	10.72	
	3	L36454			10.23
Scrophulariaceae					
<i>ANTIRRHINUM majus</i> L.	3	L36392	3.90	3.39	
	4	L11688			4.68
<i>DIGITALIS grandiflora</i> Mill.	3	L36399	3.11	3.50	
<i>D. purpurea</i> L.	5	L01902			1.17
<i>HEBENSTRETIA dentata</i> L.	Bremer 3303 (UPS)	AF027285	0.71	0.68	
		AJ001767			0.29
<i>LINDENBERGIA</i> sp.	Thulin 8079 (UPS)	AF027286	3.37	0.68	
		AJ001768			11.70
<i>PAULOWNIA tomentosa</i> L.	3	L36447			2.92
<i>SCHLEGELIA parviflora</i> (Oerst.) Monach.	3	L36410	2.93	3.05	
<i>SCROPHULARIA</i> sp.	3	L36448			1.46
		L36411	8.52	7.34	
		L36449			10.53
<i>SELAGO thunbergii</i> Choisy	3	L36412	7.81	6.32	
		L36450			10.82
<i>VERBASCUM thapsus</i> L.	3	L36417	4.08	4.63	
		L36452			1.46
<i>VERONICA catenata</i> Pennell	3	L36419	9.05	7.45	
		L36453			11.99
Stilbaceae					
<i>EUTHYSTACHYS abbreviata</i> (E. Mey.) A. DC.	9	Z29671			0.29
<i>RETZIA capensis</i> Thunb.	Källersjö 0401191 (BOL)	AF027289	1.51	1.35	
	9	Z29669			1.46
<i>STILBE albiflora</i> E. Mey.	Goldblatt 10464 (MO)	AF027287	0.44	0.23	
<i>S. vestita</i> Berg.		Z68827			0.58
Symphoremataceae					
<i>CONGEA tomentosa</i> Roxb.	14	U78689	8.61	5.08	
	6	U28870			18.13
Verbenaceae					
<i>BOUCHEA fluminensis</i> (Vell.) Moldenke	6	U32162			0.58
<i>PETREA RACEMOSA</i> Kunth	6	U28879			0.29
<i>PETREA VOLUBILIS</i> L.	6	U28880			0.29
<i>RHAPHITHAMNUS spinosus</i> (Jussieu) Moldenke	3	L36409	4.35	3.95	
	6	U32160			4.68

TABLE 1. Continued.

	Voucher/reference	GenBank accession number	comb.	<i>ndhF</i>	<i>rbcl</i>
<i>STACHYTARPHETA dichotoma</i>	3	L36414	6.03	7.34	
(Ruiz & Pav.) Vahl.	6	U32161			0.88
<i>VERBENA bracteata</i> Lag. & J. D. Rodriguez	3	L36418	10.20	11.96	
<i>V. bonariensis</i> L.	6	L14412			4.09
<i>VITEX agnus-castus</i> L.	14	U78707	5.15	3.05	
	14	U78716			10.23
Solanales					
Solanaceae					
<i>JUANULLOA aurintiaca</i> Otto & Dietr.	17	U08919	4.08	4.51	
		U08612			1.46
<i>LYCOPERSICON esculentum</i> Mill.	17	U08921	3.90	4.40	
	5	L14403			1.75
<i>NICOTIANA tabacum</i> L.	18	L14953	1.42	1.35	
<i>N. debneyi</i> Domin	19	D70815			1.17
<i>SOLANUM luteoalbum</i> Pers.	20	U72749		4.74	
Total			5.06	5.56	3.73

ing or ambiguous. The combined matrix included 62 taxa and 1127 informative characters, of which 5.06% were scored as missing or ambiguous. Details on the proportion of missing entries for each sequence can be found in table 1. Complete alignments are available upon request.

Phylogenetic Analysis. We used three different strategies to infer phylogenetic relationships.

1) PAUP* 4.0d64 (D. L. Swofford unpublished) was used to find the most parsimonious trees from individual *rbcl* and *ndhF* matrices, as well as the combined matrix, using the following search strategy. First, 500 replicates with random sequence addition and NNI swapping and

MULPARS off were performed. The shortest tree from each replicate was saved, even if it was not optimal over all replicates. These trees were used as starting trees for a new search with MULPARS on and TBR branch swapping. Consistency index (CI), retention index (RI) and the strict consensus tree were calculated from the most parsimonious trees found. This strategy was used to find groups qualitatively supported by the *ndhF/rbcl* data.

2) In order to assess the degree of support, Bremer support (BS) values (Bremer 1988, 1994) on the strict consensus trees were calculated. A problem with BS values is that they will be over-

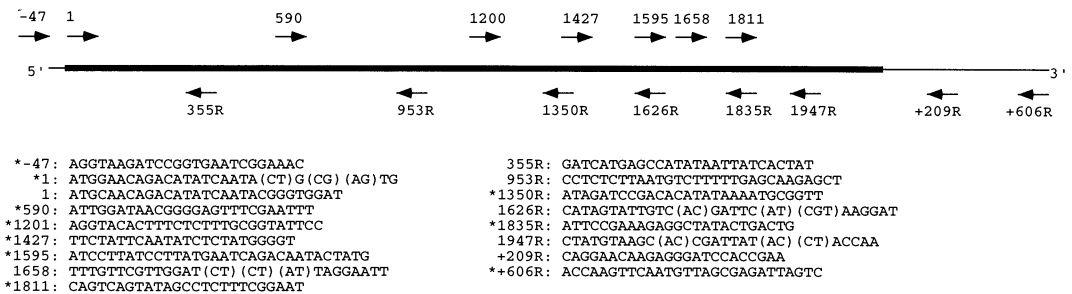


FIG. 1. Map of the chloroplast gene *ndhF* with primer sites indicated. Coding strand (forward) primers are indicated above the line, and reverse (indicated by an R) are below the line. Numbers indicate the 5' end of primer relative to the position in the tobacco sequence (Shinozaki 1986, GenBank accession number Z00044), - denotes a position upstream from the 5' end, + denotes a position downstream from the 3' end of the gene. Primer sequences denoted with an asterisk (*) were kindly shared by Robert K. Jansen and Richard G. Olmstead.

estimated when the heuristic methods used fail to find the optimal solution. Several strategies for calculating BS values have been proposed and evaluated (e.g., Davis 1995, Morgan 1997). We chose the reverse constraint method, originally described by Eernisse and Kluge (1993), which Morgan (1997) found to be the most efficient. Still, there are many different heuristic search strategies to choose from. Considering the potentially large number of nodes to evaluate, it was impractical to use a strategy as thorough as the one described in 1. Here, we compare the results of two less thorough strategies. In the ST strategy, each of the constrained searches was made using one of the most parsimonious trees from the unconstrained searches as starting tree, TBR swapping and a maximum of 25 trees saved (nchuck=25). The other strategy, RA, was to perform 25 random additions to each constrained search, and to apply TBR swapping with MULPARS off. Batch command files were generated using AutoDecay 2.9.10 (Eriksson 1997). In order to assess the efficiency of these two strategies, we randomly selected eight groups in the *rbcl* matrix where we used the search methods described in strategy 1. In the combined matrix, the RA and ST strategies were used with the STEEPEST DESCENT option in effect for comparison.

3) Another way to measure support is by using resampling methods. The most widely used resampling method used in phylogenetics is the bootstrap (Felsenstein 1985). In bootstrapping, characters are resampled with replacement, and the frequency by which individual groups occur in the pseudoreplicates are taken as measurement of support. In jackknifing, a specified number, N , of characters is randomly deleted in each pseudoreplicate. When $N = e^{-1}$ (about 36%) the two methods coincide if there is no homoplasy in the data, and if an infinite number of invariant characters are added before bootstrapping (Harshman 1994, Farris et al. 1996, Farris 1998). Here, we use jackknifing, as implemented in the program *xac*, version 2.1 (J. S. Farris, unpublished,) which is an enhanced variant of the *jac* program described by Farris et al. (1996). Among other things, it avoids (or at least diminishes) underestimation errors due to inefficient search algorithms (Farris et al. unpubl. data) by invoking multiple random additions and a global branch swapping algorithm. To test for the effect of variable numbers of random additions,

1, 5, 10, 25, 100, and 500 random additions were used for the combined matrix. The 95% confidence interval for each resampled frequency was calculated using the binomial distribution (Hedges 1992). Only the increase from one to five random additions gave differences in resampling frequencies that on average were higher than these confidence intervals. Thus, five random additions and branch swapping appeared to be sufficient to give reasonably precise results in this case, so these settings were used for the other two analyses. 1,000 jackknife replicates were performed throughout.

The two sequence regions used in this study are linked together in the haploid chloroplast genome, thus their histories are linked (Doyle 1992). However, their patterns of evolution may be different. For example, Gaut et al. (1997) showed that rates of nonsynonymous substitutions are not correlated between *rbcl* and *ndhF* in the grass family (Poaceae). In order to check for heterogeneity between the *rbcl* and *ndhF* matrices, we performed an Incongruence Length Difference test (ILD, Farris et al. 1994) between the two regions using the program *xarn*, version 1.6 (J.S. Farris unpublished) with 999 randomized replicates in addition to the original partitions. Each replicate matrix was subjected to five random additions and a global branch swapper. All uninformative characters were excluded before performing ILD tests (see Cunningham 1997).

RESULTS

rbcl. The heuristic parsimony search produced 480 minimal-length trees of 1,934 steps (CI = 0.30, RI = 0.50). The strict consensus tree with BS and jackknife values can be found in Fig. 2. Strong support (i.e., jackknife $\geq 95\%$ and BS ≥ 5) was found for 14 clades of the 67 clades. The ST strategy gave more accurate (i.e., lower) BS values in 40 cases, RA was better in five cases, and in 22 cases there was no difference between the two strategies. For two clades, the minimum branch length was shorter than the BS value found by either strategy. For seven groups, the RA strategy did not find BS values lower than the respective minimum branch length. For the eight nodes subjected to a more elaborate search strategy, two nodes had lower BS than either of the ST or the RA strategy could find.

ndhF. The alignment of the *ndhF* sequences

required several insertions/deletions, of which 12 were potentially informative. The heuristic search produced 384 minimal-length trees of 5,284 steps (CI = 0.35, RI = 0.59). The strict consensus tree with BS and jackknife values can be found in Fig. 3. The *ndhF* data revealed more groups with strong support (i.e., jackknife $\geq 95\%$ and BS ≥ 5) than the *rbcL* data, 32 nodes of 57 are strongly supported. In sharp contrast to the *rbcL* matrix, the RA strategy was more efficient in finding low BS values. In 14 cases, the RA strategy found a lower BS value than the ST strategy, and in the remaining 43 cases there were no difference between the two strategies. In three cases, the ST strategy failed to find values lower than the minimum branch length. All the values were on long branches with very high BS values: Solanaceae (branch length: 85; ST: 138), Scrophulariales except Oleaceae and Tetradymnaceae (branch length: 21; ST: 23), and *Rhapthamnus/Verbena* in the Verbenaceae (branch length: 9; ST: 16).

Combined Analysis. The heuristic search of the combined *rbcL/ndhF* data sets produced 36 minimal-length trees of 6,268 steps (CI = 0.36, RI = 0.53). The strict consensus tree with branch support and jackknife values can be found in Fig. 4. Twenty-seven of the 54 nodes are strongly supported (i.e., jackknife $\geq 95\%$ and BS ≥ 5). All except four of these nodes were strongly supported by the *ndhF* data alone. The ST strategy gave more accurate (i.e., lower) BS values in four cases, RA was better in 11 cases, and in 39 cases there was no difference between the two strategies. In one case, the minimum length of the node was shorter than the ST strategy could find. When the steepest descent option was invoked, the minimal BS value was always found, but the searches required 9 to 29 times as many branch swappings. There were detectable differences between the *rbcL* and the *ndhF* matrices

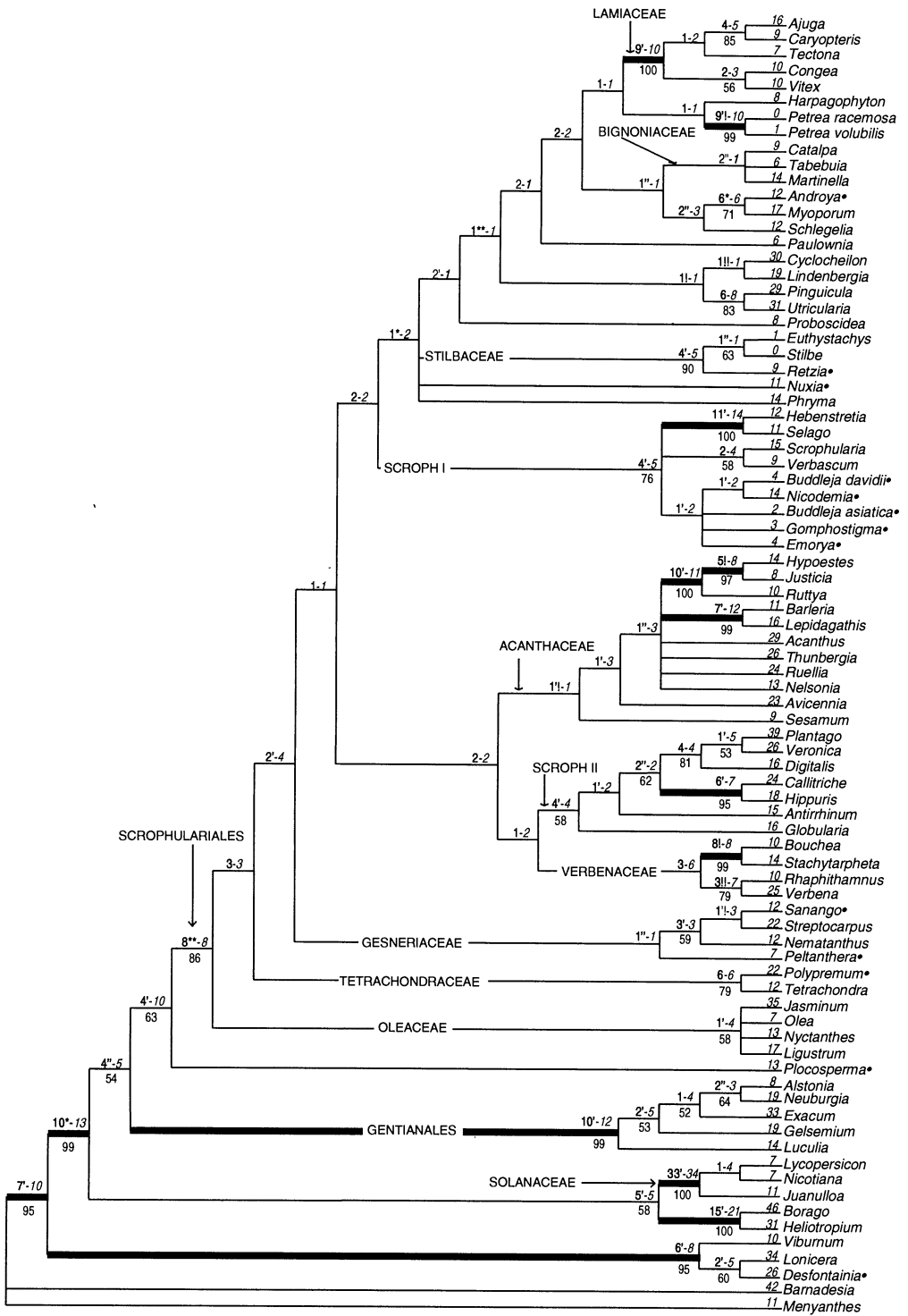
($\alpha = 19/1000$). Inspection of Fig. 2 and Fig. 3 reveals incongruent positions of *Desfontainia* and *Plocosperma* with relatively high support. The *rbcL* data reveal *Desfontainia* as sister group to *Viburnum/Lonicera* (jackknife = 95%, BS = 6), whereas the *ndhF* data reveals this group as paraphyletic (jackknife = 89%, BS = 7). In the *rbcL* tree, *Plocosperma* is sister taxon to Scrophulariales (jackknife = 86%, BS = 8), whereas *ndhF* groups it with *Borago* (jackknife = 74%, BS = 5). Another possible explanation for the significant incongruence found could be that nine terminal taxa are represented by different organisms for the two genes. Exclusion of these nine taxa rather emphasized the incongruence as measured by the ILD test ($\alpha = 1/1000$). In contrast, removal of *Desfontainia* ($\alpha = 63/1000$) and *Plocosperma* ($\alpha = 58/1000$), respectively, decreased incongruence. Removing both these taxa simultaneously increased α even further ($\alpha = 130/1000$). Application of 95% confidence intervals from the binomial distribution (Hedges 1992) revealed each of these removal effects as significant, even after a Bonferroni correction for multiple simultaneous tests (Rice 1989).

DISCUSSION

Branch Support, Combination of Data, and Phylogenetic Utility of Different Chloroplast DNA Loci. Recently, a tendency among systematists has been to focus on the degree of 'support', or 'stability', of particular groups, rather than to merely assess which groups are most parsimoniously supported. Resampling statistics (bootstrap, jackknife) and Bremer support are among the most common methods used. However, few have addressed what is actually meant by support. Olmstead and Sweere (1994) proposed that bootstrap and BS values estimate the same parameter. The parameter estimated by

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FIG. 2. Strict consensus tree from 480 minimal-length trees resulting from *rbcL* sequence data matrix. Dot after a name indicates membership in Buddlejaceae fide Leeuwenberg and Leenhouts (1980). Numbers below branches denote jackknife frequencies (if higher than 50%), numbers above branches denote Bremer support (BS) values (in boldface) and minimum branch lengths (in italics) from one arbitrarily chosen tree. An asterisk (*) denotes that the RA strategy found the smallest BS value, ' denotes that the ST strategy found the smallest. Double characters indicate that the other method failed to find a value as low as the minimum branch length. BS values at branches where a more thorough strategy was employed are denoted by !. Double exclamation marks (!!) denote that both the RA and the ST strategy were unable to find such a low BS value. Branches with jackknife support $\geq 95\%$ and BS ≥ 5 are indicated by thick lines. Taxon names on internal branches are given to enhance readability, and should be considered as tentative.



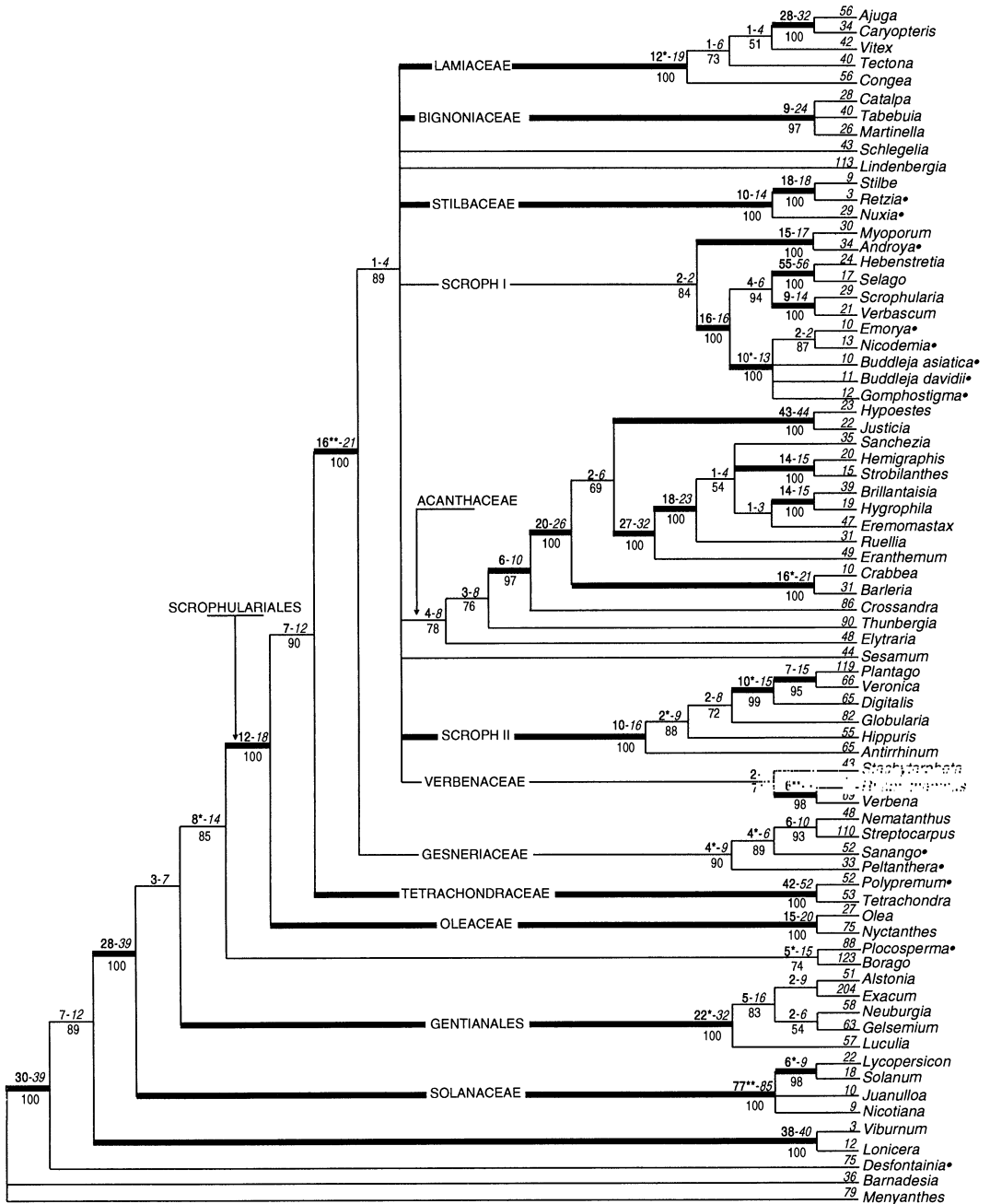


FIG. 3. Strict consensus tree from 384 minimal-length trees resulting from *ndhF* sequence data matrix. Dot after a name indicates membership in Buddlejeae fide Leeuwenberg and Leenhouts (1980). Numbers below branches denote jackknife frequencies (if higher than 50%), numbers above branches denote Bremer support (BS) values (in boldface) and minimum branch lengths (in italics) from one arbitrarily chosen tree. An asterisk (*) denotes that the RA strategy found the smallest BS value. Double asterisks indicate that the ST method failed to find a value as low as the minimum branch length. Branches with jackknife support $\geq 95\%$ and BS ≥ 5 are indicated by thick lines. Taxon names on internal branches are given to enhance readability, and should be considered as tentative.

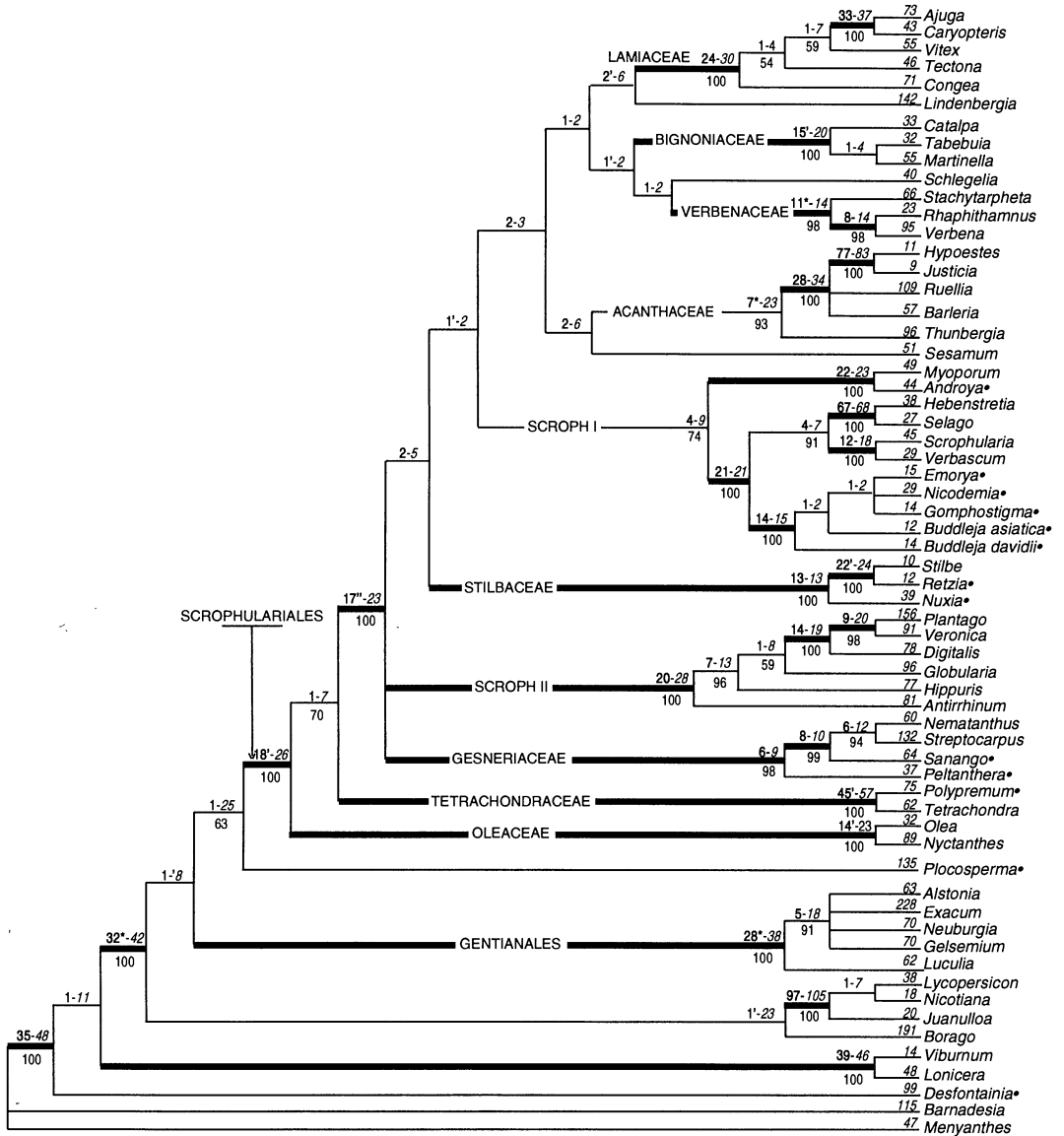


FIG. 4. Strict consensus tree from 36 minimal-length trees resulting from the combined data matrix. Dot after a name indicates membership in Buddlejaceae fide Leeuwenberg and Leenhouts (1980). Numbers below branches denote jackknife frequencies (if higher than 50%), numbers above branches denote Bremer support (BS) values (in boldface) and minimum branch lengths (in italics) from one arbitrarily chosen tree. An asterisk (*) denotes that the RA strategy found the smallest BS value, ' denotes that the ST strategy found the smallest. Branches with jackknife support $\geq 95\%$ and BS ≥ 5 are indicated by thick lines. Taxon names on internal branches are given to enhance readability, and should be considered as tentative.

bootstrap when used in cladistics is the topology of the tree (Efron et al. 1996). Assuming random sampling of characters, a possible definition of a particular group's resampling frequency is the probability that the group should be found in a

different, random, sample of characters of the same size from the same population of characters. One basic assumption is that the characters are drawn independently from a common probability distribution (e.g., Felsenstein 1985). This

assumption is usually violated in biological systematics. Another problematic underlying assumption is that the number of characters is very large (Felsenstein 1985), such that each value occurs in the same proportion as in the parameter distribution. It is not clear how close the data used in this study match this assumption, but the sometimes drastic differences between the two sequence regions suggest that this effect could be considerable.

The resampling frequencies can be viewed as measurements of specific properties of the data at hand. For example, bootstrap frequencies can be viewed as measurements of how sensitive the data are to differential weighting of the characters (Davis 1995). Similarly, jackknife frequencies measure the sensitivity of data to removal of a certain proportion of characters. It seems clear that bootstrapping and jackknifing are conceptually closely related, although it is unclear how resampling frequencies using different deletion percentages in the jackknife relates to bootstrap frequencies. Under some circumstances, they will converge to the same result (Farris 1998).

In contrast to resampling statistics, BS values are integers that simply indicate the number of extra steps required to collapse a clade. Therefore, they cannot be viewed as probabilistic estimates. Often, high BS values are accompanied by high resampling frequencies, and indeed the correlation between jackknife and BS values in this study is high ($R^2 = 0.73$ for nodes with jackknife values higher than 50% but less than 100%). BS values of 12 or more appear to saturate jackknife values (i.e., 100%). Thus, BS values are more sensitive to quantitative differences among clades. On the other hand, jackknife values are more complex and sensitive to the structure in the entire data matrix. For example, a subclade in a clade supported by a large number of characters will have fewer alternative places in the resampled replicates than it would if the containing clade had only a few supporting characters. BS values are not affected by the support for sub- or superordinate clades.

Equating resampling frequencies with the degree of support is somewhat misleading, because they rather measure confidence in qualitative support. A matrix with two characters supporting a group and one contradicting it has an expected bootstrap frequency of about 60% (Harshman 1994), and *xac* gives a jackknifing frequency of 58%. Keeping the ratio of support-

ing and contradicting characters constant, an increase in the absolute number of characters will increase the bootstrap/jackknife value. For example, a group with 20 supporting and 10 contradicting characters receives a jackknife value of 98%. Thus, although the number of supporting characters has increased, the ratio supportive/contradictive characters is the same and it is actually the confidence in qualitative support that has increased. By contrast, BS values will measure only the difference between the number of supporting and contradicting characters. For example, a matrix with 5 uncontradicted characters give a jackknife value of 99% for that group, whereas a matrix with 105 supporting and 100 contradicting characters gives a jackknife frequency of 66%. Although the BS value is the same, there is less confidence in support in the latter case. These examples clearly illustrate that resampling frequencies and BS values measure different properties of the data. Resampling statistics measure confidence of support, whereas BS values measure the degree of support in terms of the number of extra changes required to collapse a group. One way to incorporate confidence information without invoking resampling methods is to accompany the BS value with the minimum possible branch length. However, the method would still suffer from the lack of a standardized scale, making comparisons between matrices difficult.

Both resampling frequencies and BS values may suffer from the problem that parsimony cannot be calculated exactly for matrix sizes as large as those in this study. For jackknife values, there may be cases where one particular group is harder to find than another in the resampled replicates, although their 'true' values are equal. However, when branch swapping is invoked in *xac*, increasing the number of random stepwise additions to more than five for each pseudoreplicate did not affect the jackknife values significantly in this study, and five appears to be enough in many cases (Farris et al., unpubl.). Varying the number of random additions per resampled replicate may serve as a check for the precision of the performance of jackknifing for particular matrices. In this respect, jackknife values appear to provide a more exact value (given the data) than BS, where the degree of error can only be guessed. Checking BS values against minimum branch lengths may improve accuracy, and add confidence information, but it is uncer-

tain how close these BS values are to the true value when heuristic approaches have been used. The ST method was more efficient than the RA method for the *rbcL* and the combined data matrices in this study. For the *ndhF* data the RA method was more efficient, albeit more time-consuming. However, the ST approach could be improved. PAUP* automatically discharges all trees not satisfying the negative constraint, except the first one, if the steepest descent option is not invoked (D. L. Swofford, pers. comm.). Invoking steepest descent means that other trees found during the search will also be saved, making that strategy impractical for large data matrices. Alternative strategies could be to perform several ST searches with different starting trees or to remove the negatively constrained group from the starting trees. Accuracy assessments of the BS values could be employed in similar fashion as was done here for the jackknife. However, the more exhaustive procedures used for some groups in this study found shorter trees in several cases, indicating that such assessments would be laborious and time-consuming. Further studies of the behavior of different search strategies for finding accurate BS values are therefore desirable.

The present study, as well as several other recent studies, have used multiple cpDNA loci. It may be argued that the chloroplast genome behaves as a single locus (Doyle 1992). Nevertheless, different parts of the genome may be under different selective regimes and have properties that make them more or less suitable for phylogenetic studies. In agreement with previous studies, *ndhF* had a higher proportion of informative positions than *rbcL* (35.8% vs 20.6%, data from the combined matrix). Since the sequence also is longer, the absolute number of informative sites is nearly three times greater for *ndhF*. The degree of homoplasy is slightly lower for *ndhF* (CI = 0.35 vs. 0.30). Although there is no direct relation between homoplasy and support, the *ndhF* data produced results with more support for the individual branches. In all but two cases, reasonably well supported (jackknife value $\geq 65\%$) branches by one data set were not contradicted by well supported branches in the other. The most striking case involves the position of *Desfontainia* (Figs. 2, 3). The other case concerns the relationship of *Borago* to *Plocosperma*, which is supported by a 74% jackknife value in the *ndhF* tree, but is contradicted by the *rbcL*

tree, in which *Borago* (together with *Heliotropium*) is resolved as the sister group of Solanaceae. This incongruence could be attributed to the differences in taxon sampling in the *rbcL* and *ndhF* matrices. However, the same pattern emerges when the *ndhF* and *rbcL* portions of the combined matrix are analyzed separately (results not shown). Significant ILD values could have several explanations. The data sets may actually be the result of incongruent phylogenies or, alternatively, the incongruence may be due to conflicting patterns caused by other processes. In practice, it may be hard to distinguish between these. For instance, conflicting phylogenies may be observed when one of the organisms representing a terminal taxon has been misidentified in one data set. Removing the taxa where different organisms had been used for acquiring the *rbcL* and *ndhF* sequences in this study did not improve congruence. By contrast, removing *Plocosperma* and *Desfontainia* had demonstrable effects. Both of these taxa belong to the outgroup, where taxon sampling is sparse. Future, more extensive taxon sampling may resolve the problem. In some cases, support values were lower with the combined data than with the individual data sets alone. One such example is the resolution within Gentianales (Figs. 2–4). Both the resolved topologies are incongruent with the topology found by Backlund et al. (in press) and this occurrence is probably best explained by the much denser sampling in that study. The long terminal branches and the relatively large differences between BS and branch lengths may be cautionary indications.

The ILD analyses performed in this study also suffer from the problem that exact solutions cannot be guaranteed on the resampled partitions, due to the relatively high number of taxa. A particular problem (pointed out by an anonymous referee of this paper) is that the results are sensitive to how difficult it is to find the shortest tree length of the original partitions relative to the resampled data.

Buddlejaceae. The remote relationship of the Buddlejaceae to the Loganiaceae is further emphasized by the results in this study. It is evident that the Buddlejeae (fide Leeuwenberg and Leenhouts 1980) is an unnatural assemblage of taxa. Many taxa classified there clearly belong elsewhere, leaving only a small group of taxa, albeit well supported, in Buddlejaceae. In the

following text the fates of the genera previously classified in Buddlejaceae are discussed.

Desfontainia. Backlund and Donoghue (1996) and Backlund and Bremer (1997) found moderate support based on morphological characters and *rbcL* sequences, respectively, for a sister-group relationship between *Columellia* Ruiz & Pav. and *Desfontainia* (already suggested by Hallier 1910) within the Dipsacales. In this study, with very restricted sampling, *rbcL* sequences gave a 95% jackknife support for a monophyletic Dipsacales (*Lonicera*, *Viburnum* and *Desfontainia*), whereas the more extensive *rbcL* study of Backlund and Bremer (1997) did not get bootstrap or jackknife values above 50% for a monophyletic Dipsacales. In this study, however, the evidence from *ndhF* sequences strongly disagrees with a monophyletic Dipsacales including *Desfontainia*. Keeping Dipsacales monophyletic in the *ndhF* matrix requires 9 extra steps. The combined matrix leaves the issue virtually unresolved (BS = 1).

Plocosperma. The inclusion of *Plocosperma* in Loganiaceae (i.e., Thorne 1992) is clearly inappropriate. A position outside the Gentianales appears evident (Endress et al. 1996; Backlund et al. in press), as is a position outside the Scrophulariales. Further studies focusing on this problem are needed, perhaps using alternative data sources, but more importantly, involving more extensive sampling of Solanales/Boraginales/Dipsacales.

Polypremum and Tetrachondra. The strong relationship found between these two taxa is unexpected. In fact, it is the third most strongly supported clade in the combined matrix. *Tetrachondra* Petrie has an Antarctic distribution and comprises two small aquatic species. Due to the presence of a gynobasic style, relationships to Lamiaceae and Boraginaceae have been proposed (Skottsberg 1913). Moore (1948) noted that the embryogeny of *Polypremum* is different from that of Loganiaceae and Rubiaceae in having cellular endosperm formation, which is typical for Scrophulariales. However, differences concerning endosperm haustoria, chromosome number, and the early development of the proembryo caused Moore to conclude that the relationship with *Buddleja* is remote. The embryology of *Tetrachondra* appears to be poorly known, although Skottsberg's (1913) observations do not contradict a relationship with *Polypremum*. The thickening of the embryo sac in the

micropylar end, observed by Skottsberg, may be homologous to the extension of the embryo sac to the surface of the ovule in *Polypremum* (Moore 1948). Jensen (1992) found phytochemical support for a position of *Polypremum* near Scrophulariaceae or Oleaceae, and recent investigations (S. R. Jensen, unpubl. data) further support the relationship between *Polypremum* and *Tetrachondra* found in this study.

Buddlejaceae s. str. The results of this study leave only *Buddleja*, *Nicodemia*, *Emorya*, and *Gomphostigma* in a well supported Buddlejaceae. This result is further corroborated by *rps16* intron sequences (B. Oxelman et al., unpubl. data). However, it has been suggested that *Nicodemia* (Leeuwenberg and Leenhouts 1980) and *Emorya* (Bisset et al. 1980) should be included in *Buddleja*. The *rbcL* and *ndhF* data are not conclusive in this respect, but using denser sampling and faster-evolving loci such as *rps16* introns and nrDNA ITS sequences may resolve this problem.

Androya and Myoporum. The *rbcL* and *ndhF* sequences strongly support a close relationship between *Androya* and *Myoporum*. *Androya* was originally described as a member of the Oleaceae (Perrier de la Bâthe 1952), but was moved to Buddlejaceae by Leenhouts (1963). The morphological features are poorly known, but the presence of confluent anther cells of *Androya* supports a relationship with Myoporaceae. Menega (1980) noted that the wood anatomy of *Androya* did not fit well with other Buddlejeae. Nevertheless, she interpreted *Androya* as a phylogenetic derivative of *Buddleja*, and Carlquist (1996) found no reason to exclude *Androya* from Buddlejaceae on the basis of wood anatomical features. Punt (1980) noted palynological similarities between *Androya* and *Nicodemia madagascariensis* (Lam.) R. Parker. Analysis of *rps16* intron sequence variation does not support such a relationship (B. Oxelman et al., unpubl. data). Rather, the affinity of *Androya* with *Myoporum* is further emphasized.

Nuxia and Retziaceae. *Nuxia* is morphologically similar to *Buddleja* sect. *Chilianthus* (Burch.) Leeuwenberg, but it differs in having confluent anther cells. Bentham (1836) included *Chilianthus* in *Nuxia*, but Leeuwenberg and Leenhouts (1980) apparently considered the discrete anther cells as a character important enough to support the placement of *Chilianthus* within *Buddleja*. Anther confluence is a rare condition in this group, and optimization of this character on the molec-

ular tree reveals that discrete anther cells is probably a plesiomorphic character state in this group. Unfortunately, no material of *Chilianthus* has been available for sequencing. The strong sister-group relationship between *Nuxia* and Stilbaceae is another unexpected result of this study. However, Jensen et al. (1998) recently found derivatives of the rare iridoid unedoside to be present in *Nuxia* and Stilbaceae, but not in other Buddlejaceae. The *ndhF* sequence for another species of *Nuxia* (*N. floribunda* Benth.) confirmed the relationship of this genus to Stilbaceae (results not shown).

Sanango, Peltanthera and Gesneriaceae. Recently, strong evidence has been presented for a close relationship between Gesneriaceae and *Sanango* (Wiehler 1994; Norman 1994; Dickison 1994; Jensen 1994, 1996; Smith et al. 1997a). Although Bisset et al. (1980) recognized strong similarities between *Sanango* and *Peltanthera* in pollen morphology and inflorescence architecture, none of these authors appears to have seriously considered *Peltanthera*. The results presented here strongly indicate that *Peltanthera* is also related to Gesneriaceae. Again, recent phytochemical investigations of S. R. Jensen (unpubl. data) has corroborated this finding.

Other Findings. The monophyly of Scrophulariales sensu Thorne (1992) is strongly supported by the *rbcl* and *ndhF* sequences, with the exception that *Tetrachondra* should be included. This corroborates the conclusions of Jensen (1992) and Scogin (1992), which they based on the shared presence of verbascoside (acteoside) within the order. However, the relationship of Scrophulariales to other taxa remains unclear. Preliminary experiments with a more complete sampling of Asteridae do not appear to resolve the issue (results not shown). Further character sampling may thus be more fruitful than expanding the taxon sample.

Oleaceae appears as sister taxon to the rest of the order. This position is in contrast to the results of Wagstaff and Olmstead (1997), in which *Tetrachondra* occupies that position. In this study, *Polypremum* and *Tetrachondra* have a well supported sister-group relation to the rest of the order, with the exception of Oleaceae. The reason for this discrepancy could be that Wagstaff and Olmstead did not include *Polypremum* in their study.

Most elements of Scrophulariales examined in this study fall with groups recognizable as

Acanthaceae, Bignoniaceae, Lamiaceae s.l., Verbenaceae s.s, a gesneriad clade, a stilbacean clade, and two nonsister 'scrophulariad' clades. Relationships among these lineages are poorly resolved, however. The polyphyly of Scrophulariaceae discovered by Olmstead and Reeves (1995) is further substantiated here. *Lindenbergia* occupies an uncertain position in the order, it appears not to be part of either the 'Scroph I' or the 'Scroph II' clades. The relationship of *Lindenbergia* to *Cyclocheilon* Oliv. and Lentibulariaceae, as suggested by the *rbcl* data, is surprising but weakly supported. The strong relationship between *Selago* and *Hebenstretia* L. in the 'Scroph I' clade and the strongly supported inclusion of *Globularia* L. in the 'Scroph II' clade support the continued recognition of Selaginaceae and Globulariaceae as distinct taxa (in contrast to the view of Cronquist 1981).

Although it would improper to give too much attention to weakly supported branches, the topological distance between the two representatives of Pedaliaceae (*Harpagophytum* DC. and *Sesamum* L.) in the *rbcl* tree called for closer attention. Forcing monophyly of this pair (with a positive constraint) required two extra steps.

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