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SYSTEMATICS AND EVOLUTION OF TRIBE SINNINGIEAE (GESNERIACEAE): EVIDENCE FROM PHYLOGENETIC ANALYSES OF SIX PLASTID DNA REGIONS AND NUCLEAR *NCPGS*[1]

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For nearly all species in the three genera of tribe Sinningieae (Gesneriaceae), *Sinningia*, *Paliavana*, and *Vanhouttea* (mostly in southeastern Brazil) plus 10 outgroups, we have sequenced six non-coding DNA regions (i.e., plastid intergenic spacers *trnT-trnL*, *trnL-trnF*, *trnS-trnG*, *atpB-rbcL*, and introns in the *trnL* and *rpl16* genes) and four introns in nuclear plastid-expressed glutamine synthetase gene (*ncpGS*). Separate and combined analyses of these data sets using maximum parsimony supported the monophyly of Sinningieae, but the genera *Paliavana* and *Vanhouttea* were found embedded within *Sinningia*; therefore a new infrageneric classification is here proposed. Mapping of pollination syndromes on the DNA-based trees supported multiple origins of hummingbird and bee syndromes and derivation of moth and bat syndromes from hummingbird flowers. Perennial tubers were derived from perennial stems in non-tuberous plants.

Key words: linalool; non-coding plastid DNA; *Paliavana*; plastid-expressed glutamine synthetase; pollination syndrome; *Sinningia*; species-level phylogenetics; *Vanhouttea*.

Taxonomy and nomenclature of species included in tribe Sinningieae Fritsch (Gesneriaceae) are particularly complex, and more than 300 names, including various generic and tribal arrangements, are documented in the literature (see review in Hanstein, 1864; Fritsch, 1894; Wiehler, 1983; Chautems, 1990). Such confusion and the extensive synonymy arose as a result of their extensive variation in habit, inflorescence form, and corolla shape, which in turn exemplify their morphological diversification in the Neotropics. The genera *Sinningia* Nees (which includes the commonly cultivated florist gloxinia), *Vanhouttea* Lemaire, and *Paliavana* Vandelli were first included in Gloxinieae by Wiehler (1983) together with 17 other genera. However, the lack of rhizomes and the presence of a tuber in most *Sinningia* distinguish the former three genera from the rest of the tribe (Wiehler, 1983; Boggan, 1991). Then, based on molecular data, Smith et al. (1997) and Zimmer et al. (2002) proposed to remove *Sinningia*, *Vanhouttea*, and *Paliavana* from Gloxinieae and include them in a redefined Sinningieae. Major progress in generic circumscription has also been made based on hybridization experiments, which has led to the inclusion of *Rechsteineria* Regel and *Lietzia* Regel & Schmidt in *Sinningia* (Clayberg, 1968; Moore, 1973; Wiehler and Chautems, 1995). Finally, 61 species of *Sinningia*, four of *Paliavana* and four of *Vanhouttea* are currently recognized (Wiehler, 1983, 1984; Chautems, 1990, 1991, 1995; Chautems and Weber, 1999; Chautems et al., 2000), but six new taxa still await formal publication (A. Chautems, unpublished manuscript). These species are distributed from Central America to northern Argentina, with the highest diversity occurring in southeastern Brazil. Habit ranges from herbaceous species with a perennial tuber in most *Sinningia* species to woody shrubs without tubers in *Paliavana* and *Vanhouttea*. Stems are annual in most *Sinningia* species with new shoots growing every year from the tuber or perennial in *Paliavana*, *Vanhouttea*, and nine species of *Sinningia* (Chautems and Weber, 1999). They occur mostly on rocky outcrops within the Atlantic rain forest between sea level and 2000 m a.s.l. (e.g., Meirelles et al., 1999; Safford and Martinelli, 2000), but some are epiphytic or terrestrial (Chautems and Weber, 1999). Floral shapes, colors, nectar sugar composition, and fragrance indicate adaptations to various pollinators including hummingbirds, bees, bats, and hawk moths (Vogel [1969] under *Gesneria* and *Lietzia*; Silberbauer-Gottsberger and Gottsberger, 1975; Proctor et al. [1996] under *Rechsteineria*; Perret et al., 2001).

At lower taxonomic levels, molecular phylogenetic analyses are the most powerful tool to identify parallelisms/reversals, character correlations, and factors that may promote speciation (e.g., see Harvey and Purvis, 1991; Larson and Losos, 1996; Givnish, 1997; Barraclough et al., 1999; Barraclough and Nee, 2001). In plants, the use of species-level phylogenetic studies has provided insight to our understanding of the evolutionary pathways leading to the diversification of traits associated with flower evolution and life history (e.g., Armbruster, 1993; Silvertown and Dodd, 1997; Givnish et al., 2000; Möller and Cronk, 2001).

Here, we have used a portion of the nuclear plastid-expressed glutamine synthetase gene (*ncpGS*, introns 7–10), as well as four plastid intergenic spacers (*trnT-trnL*, *trnL-trnF*, *trnS-trnG*, *atpB-rbcL*) and two introns (within *trnL* and *rpl16*) to produce a nearly complete species-level phylogenetic tree for the members of Sinningieae. We sampled all known species (Wiehler, 1983, 1984; Chautems, 1990, 1991, 1995; Chautems and Weber, 1999; Chautems et al., 2000) with the excep-

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tion of six taxa for which we could not obtain suitable material (i.e., *S. helleri*, *S. schomburkiana*, *S. sulcata*, and three unpublished new species). Phylogenetic utility of the *ncpGS* introns was described by Emshwiller and Doyle (1999), whereas the *trnL-trnF* region, the *atpB-rbcL* spacer, and the *rpl16* intron have been widely used to assess infrafamilial relationships (e.g., see Taberlet et al., 1991; Manen et al., 1994a; Jordan et al., 1996; Kelchner and Clark, 1997; Samuel et al., 1997; MacDade and Moody, 1999). The *trnT-trnL* spacer has been used less often for phylogenetic analyses (but see Böhle et al., 1996), whereas the *trnS-trnG* spacer was suggested to be more useful at the population level (Hamilton, 1999). Based on separate and simultaneous analyses, we have compared congruence and phylogenetic usefulness of these DNA regions. We also propose a new taxonomic treatment for the tribe as well as scenarios for diversification in pollination syndromes and habits.

MATERIALS AND METHODS

Sampling and DNA sequencing—We produced DNA sequences for 95 accessions from 62 species of *Sinningia*, six species of *Paliavana*, and eight species of *Vanhouttea*, plus 10 taxa used as outgroups; complete list of taxa and voucher have been archived at the Botanical Society of America website (http://ajbsupp.botany.org/). For nine species, two accessions from distinct geographical origins were analyzed. Outgroup species were selected from Gloxinieae, Epicieae, and Gesneriae, all representing closely related tribes based on a molecular phylogenetic analysis of the whole family (Smith et al., 1997). Tissues were mostly sampled from cultivated specimens held in the Geneva Botanical Garden (Switzerland), the Gesneriad Research Foundation (USA), or at the Instituto Plantarum Ltda and associated private collections (Brazil).

Total genomic DNA was isolated from silica gel dried leaf tissue using the cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) with the addition of 1% polyphenol polyvinylpyrolidon in the extraction buffer to bind phenolic compounds that might inhibit DNA amplification (Savolainen et al., 1995). DNA was then purified using silica particles according to the manufacturer's protocol (Prep-A-Gene; Bio-Rad Laboratories, Hercules, California, USA). Double-stranded DNA was amplified via the polymerase chain reaction (PCR); each reaction contained 10 μL of GeneAmp 10× reaction buffer (100 mmol/L Tris-HCl, pH 8.3 at 25°C, 500 mmol/L KCl), 10 μL of 25 mmol/L $MgCl_2$, 10 μL of 2 mmol/L dNTPs (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA), 0.005% bovine serum albumin (BSA), 0.4 μL of 5U/μL AmpliTaq DNA polymerase (Applied Biosystems, Branchburg, New Jersey, USA), 1 μL of each 100 mmol/L primer (MWG-Biotech, Ebersberg, Germany) and 2 μL of genomic DNA (approximately 200 ng). The PCR included an initial denaturation step of 5 min at 94°C, followed by 35 cycles with 1 min denaturation at 94°C, 1 min annealing at 50°C, 2 min extension at 72°C, and a final extension of 5 min at 72°C. Following Taberlet et al. (1991), primer pairs a/b were used to amplify the *trnT-trnL* intergenic spacer and primer pairs c/f were used to amplify the *trnL* intron and intergenic spacer between *trnL* and *trnF*. The *atpB-rbcL* spacer was amplified using forward primer *atpB*-107 (5′-GGAAARGCTACATCT-AGTAC-3′) and reverse primer *rbcL*-326 (5′-CATRTTAGTAACAGAACCT-TATT-3′). For the *rpl16* intron, amplification was performed using primers F71 and R1661 (Jordan et al., 1996); the *trnS-trnG* spacer was amplified using primers *trnS* (GCU) and *trnG* (UCC) from Hamilton (1999). A portion of the glutamine synthetase (*ncpGS*) was amplified with primers Gscp687f and Gscp994r (Emshwiller and Doyle, 1999). The PCR products were run in 1% agarose gels stained with ethidium bromide and purified from the gel using Prep-A-Gene. DNA sequencing was performed using BigDye Sequence kits (Applied Biosystems, Foster City, California, USA) and then run on an ABI 377 DNA sequencer according to the manufacturer's protocols (Applied Biosystems). Both strands were sequenced using the amplification primers; for the *atpB-rbcL* spacer the internal primers *atpB*-49 (5′-TTTCAAGCGTGGAA-ACCCCA-3′) and *rbcL*-188 (5′-TACAGTTGTCCATGTACCAG-3′) were used as well, and for the *rpl16* intron we used the additional primer R1516 designed by Kelchner and Clark (1997).

Direct sequencing of *ncpGS* produced heterogeneous electropherograms in 28 accessions. Therefore, purified PCR products were cloned by ligation into pGEM-T Vector Systems (Promega, Madison, Wisconsin, USA); XL2-Blue MRF ultra-competent cells were transformed according to the manufacturer's protocols (Stratagene, La Jolla, California, USA). Resulting colonies were screened for plasmids with inserts by PCR using pGEM/M13 primers, and five amplified inserts were then sequenced using primer Gscp687f as described above.

Sequences have been deposited in EMBL/GenBank (*trnS-trnG* accessions AJ438352–AJ438434; *trnT-trnL* accessions AJ439249–AJ439331; *trnL-trnF* accessions AJ439745–AJ439829; *atpB-rbcL* accessions AJ439900–AJ439984; *rpl16* intron accessions AJ487702–AJ487786; *ncpGS* accessions AJ459606–AJ459691).

Phylogenetic analyses—All sequences were aligned by eye; the aligned matrix is available from M. Perret and V. Savolainen. Three regions in the *trnS-trnG* (positions 69–116, 416–560, and 702–880 in the alignment of *trnS-trnG*), two regions in the *trnT-trnL* (positions 370–395 and 608–630) and two regions in the *rpl16* intron (positions 151–160 and 811–825) were unsatisfactorily aligned due to poly(AT) and poly(T) regions; therefore they were excluded from the analysis. Insertions/deletions (indels) were coded as binary characters using question marks for nested indels (Barriel, 1994).

Phylogenetic analyses were performed using PAUP* 4.0b8 (Swofford, 1999). Most-parsimonious trees were found with heuristic searches including 1000 replicates of random taxon additions and tree bisection-reconnection (TBR) branch swapping with only 10 trees held at each step; the trees found in these replicates were then used as starting trees in another search without a tree limit until 10 000 most parsimonious trees were found. Internal support was evaluated with the bootstrap (BS; Felsenstein, 1985): 1000 replicates were performed using TBR swapping, SIMPLE addition of taxa, and a tree limit of 500. The searches described above were performed separately for each DNA region, all plastid regions combined, and plastid DNA regions plus *ncpGS* combined.

Tests of incongruence—To compare the pattern of phylogenetic signal present in the different DNA regions, we used the ''incongruence length difference test'' (ILD test, Farris et al., 1995) implemented in PAUP* 4.0b8 as the ''partition homogeneity test'' (Swofford, 1999). The collection of most parsimonious trees obtained from analyses of each partition separately were also compared and conflicting nodes supported by a minimum of 70% bootstrap (BS) support were identified (i.e., hard incongruences; Johnson and Soltis, 1998). When hard incongruences were found between plastid-based and *ncpGS*-based topologies, we evaluated their significance using the ''Shimodaira-Hasegawa test'' (SH test; Shimodaira and Hasegawa, 1999; Goldman et al., 2000) implemented in PAUP* 4.0b8 (Swofford, 1999). These conflicting nodes were used as constraints with the rival data and a new heuristic search was performed as described above. Then, two subsets of 100 trees obtained with and without constraint were compared simultaneously with the RELL option of the SH test and 1000 bootstrap replicates as implemented in PAUP* 4.0b8 (Swofford, 1999). Because maximum likelihood calculations are necessary to achieve an SH test, we used the HKY85 model of evolution (Hasegawa et al., 1985) with a gamma distribution to accomodate for rate heterogeneity among sites (Yang, 1996); all parameters (i.e., base frequencies, transition/transversion ratios, alpha shape, proportion of variable sites) were estimated for plastid and *ncpGS* data sets from DNA sequences and a 50% majority-rule bootstrap consensus tree.

Character mapping—Floral and vegetative types were mapped onto the trees resulting from parsimony analyses of combined plastid DNA regions, *ncpGS* alone, and all data sets combined. Recontruction of ancestral states were performed after pruning 18 accessions to include only one representative per species and *Smithiantha* as outgroup (77 accessions in total). Using MacClade 3.1 (Maddison and Maddison, 1992), unambiguous changes were reconstructed with maximum parsimony and both accelerated (ACCTRAN)

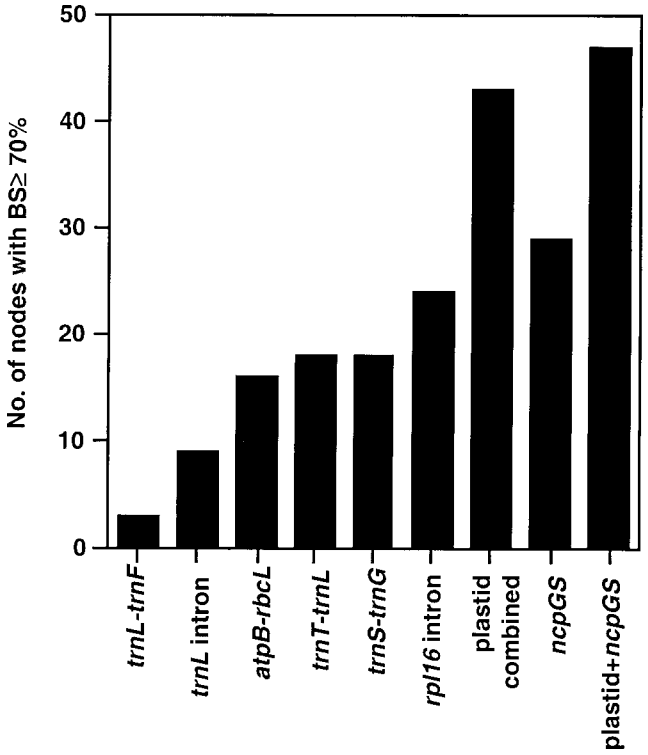


Fig. 1. Number of nodes with bootstrap support ≥70% in partitioned and combined analyses of plastid non-coding DNAs and *ncpGS*.

and delayed (DELTRAN) character state optimizations by considering all alternative topologies to accomodate for polytomies.

Four broad classes of pollination syndromes have been recognized in Sinningieae based on corolla shape, size, color, fragrance, nectar volume and sugar compositions. These are hummingbird (51 species), bee (20 species), bat (four species), and moth (one species, *S. tubiflora*).

The tuber of *Sinningia* has a hypocotyledonary origin and grows by incorporating the basal shoot internodia over the years (Weber, 1936 under *Corytholoma*). We recognized here three different habits: (1) tuberous plants with annual growth and with basal internodes giving rise to new axillary shoots every year (53 *Sinningia* species); (2) tuberous evergreen plants in which the tuber is small and growth restarts from the upper nodes of the perennial stem (six *Sinningia* species); (3) non-tuberous plants with perennial stem, which is suffrutescent to woody in *Paliavana* spp., *Vanhouttea* spp., *S. lindleyi*, *S. schiffneri*, and *S.* sp. nov. 4 (Wiehler, 1983; Chautems and Weber, 1999).

Odor collection and analysis—The moth-pollinated flowers of *S. tubiflora* produce a sweet fragrance to attract its pollinators. To identify the volatile compounds, a flower of *S. tubiflora* growing in a glasshouse at the Royal Botanic Gardens, Kew (accession number 1985-5527) was partially enclosed in a transparent forensic sample bag, and air from near the flower was drawn through an odor trap (100 mm × 3 mm diameter; SGE, Victoria, Australia) packed with 100 mg of Tenax TA (35–60 mesh, Jones Chromatography, Hengoed, Mid-Glamorgan, UK) at 25 mL/min for 3 h (21:00–23:00) by means of a portable pump. The trap was analyzed by thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS: TD injector, SGE; GC, Perkin Elmer 8500, Shelton, Connecticut, USA; MS, Finnigan Ion Trap Detector 800 Series, San Jose, California, USA). The odor trap was desorbed at 250°C for 3 min in a flow of helium and chromatography was performed on a 25 m × 0.22 mm (internal diameter) × 0.25 μm BPX5 capillary GC column (SGE) using an oven temperature program of 40–260°C at 4°C/min with a helium carrier gas pressure of 68947.6 Pa. During desorption of the trap, the front end of the GC column was looped through a polystyrene cup filled with liquid nitrogen so as to focus the volatile compounds. Electron ionization mass spectra of the column eluate were recorded every second using a scan range of *m/z* 38–400 (mass/charge).

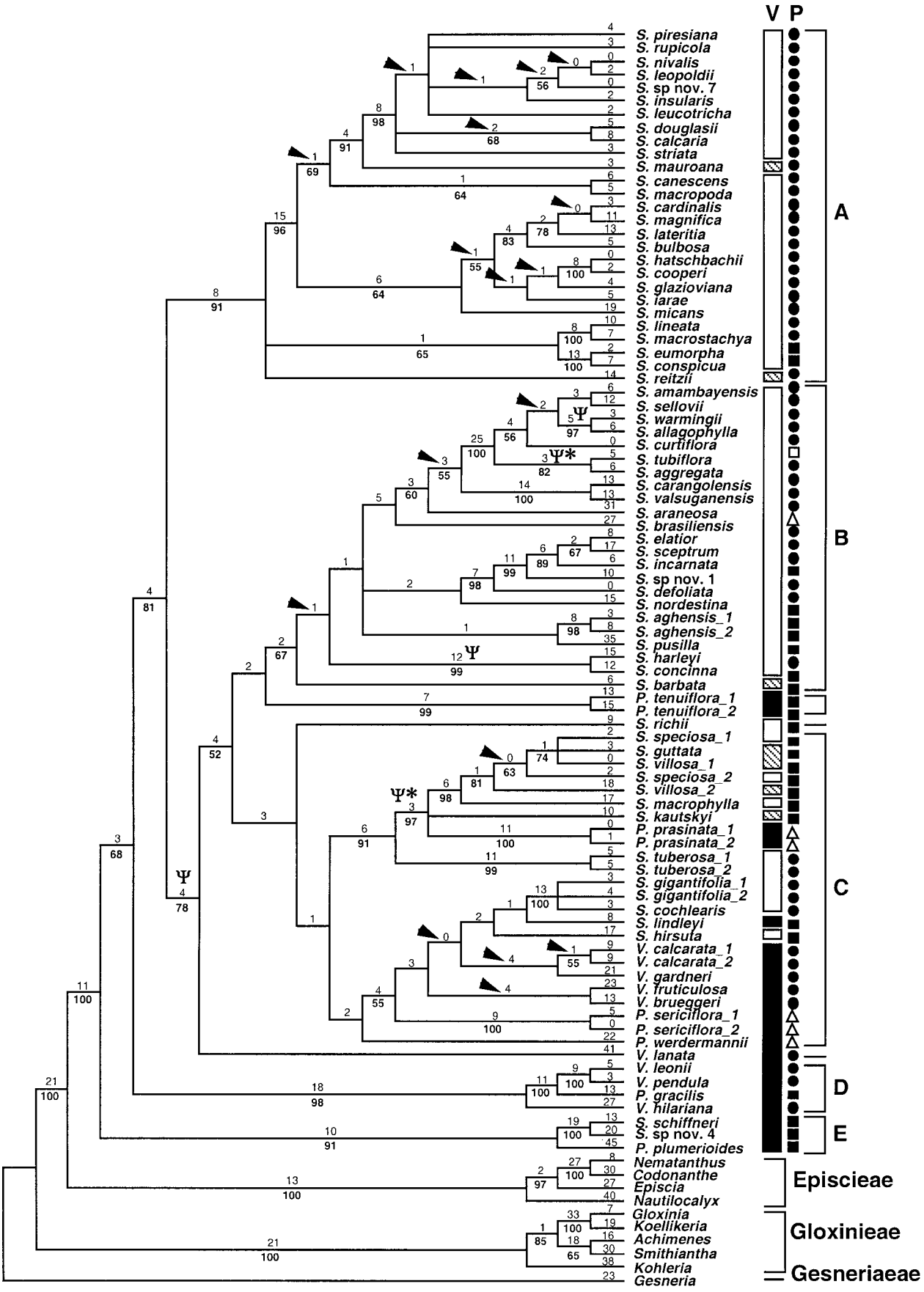
RESULTS

Plastid DNA—Approximately 4.1 kilobases were sequenced per taxon. Considering only ingroup taxa, sequence variation between species ranged from 0.79% (*trnL* intron) up to 1.81% (*trnS-trnG*) and 2.06% (*trnT-trnL*) with intermediate percentages in other spacers (*atpB-rbcL*: 0.93%, *trnL-trnF*: 1.13%) and in the *rpl16* intron (1.31%; Table 1). These pairwise divergences are well correlated with the proportion of potentially parsimony informative characters except for the *rpl16* intron, which had a higher proportion of informative characters compared to the *trnS-trnG* spacer but a lower level of sequence divergence (Table 1). The guanine and cytosine (G + C) content ranged from 26.3% in the *atpB-rbcL* spacer to 35.6% in the *trnL* intron (Table 1). Sequence length variation was particularly high in *trnT-trnL*, *trnL-trnF*, and *trnS-trnG* regions (115–304 base pairs [bp]). Length variation was caused by numerous small indels (1–20 bp) and a few larger deletions in *S. brasiliensis* (139 bp in the *trnT-trnL* spacer), *G. humilis* (60 bp in the *trnL* intron and 231 bp in the *trnL-trnF* spacer), and in *P. gracilis*, *P. plumerioides*, *S.* sp. nov. 4, *S. schiffneri*, *V. leonii*, *V. brueggeri*, and *V. pendula*, which share with the outgroup a 145–157-bp deletion in the *trnS-trnG* spacer. Overall, the alignment of all plastid DNA regions contained 5032 characters with 289 positions and 57 indels potentially parsimony informative within Sinningieae.

ncpGS sequences—The segment of the *ncpGS* gene amplified included five exons and four introns numbered 7–10 according to Tischer et al. (1986) and Emshwiller and Doyle (1999). Total length of the introns varied from 399 to 427 bp (intron 7, 95 bp; intron 8; 131–153 bp; intron 9; 78–81 bp;

TABLE 1. Characteristics of plastid non-coding DNA and *ncpGS*. Values in brackets are for the ingroup only (i.e., Sinningieae).

Region	Length range (bp)	Alignment length (sites)	Mean GC content (%)	Mean pairwise divergence (%)	Transitions/ transversion	Potentially informative characters		
						Number of substitutions	Number of indels	Total (%)
rpl16 intron	886–914	1151	32.6	1.639 (1.31)	1.12 (1.16)	92 (66)	14 (13)	9.2 (6.9)
trnL intron	443–485	526	35.6	1.01 (0.79)	1.31 (1.27)	32 (20)	5 (4)	7.0 (4.6)
trnL-trnF spacer	203–376	417	34.2	1.44 (1.13)	0.83 (0.86)	26 (16)	6 (5)	7.7 (5.0)
trnT-trnL spacer	597–712	867	26.3	2.26 (2.06)	1.08 (1.17)	98 (83)	21 (17)	13.7 (11.5)
trnS-trnG spacer	609–913	1000	33.5	2.48 (1.81)	1.27 (1.29)	103 (59)	13 (9)	11.6 (6.8)
atpB-rbcL spacer	960–984	1071	32.3	1.01 (0.92)	1.27 (1.12)	54 (45)	9 (9)	5.9 (5.0)
ncpGS exons	324	324	46.2	1.88 (1.52)	1.73 (1.82)	26 (18)	0	8.0 (5.6)
ncpGS introns	399–427	456	35.9	5.53 (4.40)	1.60 (1.74)	135 (102)	11 (7)	32.0 (23.9)


V P
S. piresiana
S. rupicola
S. nivalis
S. leopoldii
S. sp nov. 7
S. insularis
S. leucotricha
S. douglasii
S. calcaria
S. striata
S. mauroana
S. canescens
S. macropoda
S. cardinalis
S. magnifica
S. lateritia
S. bulbosa
S. hatschbachii
S. cooperi
S. glazioviana
S. larae
S. micans
S. lineata
S. macrostachya
S. eumorpha
S. conspicua
S. reitzii
S. amambayensis
S. sellovii
S. warmingii
S. allagophylla
S. curtiflora
S. tubiflora
S. aggregata
S. carangolensis
S. valsuganensis
S. araneosa
S. brasiliensis
S. elatior
S. sceptrum
S. incarnata
S. sp nov. 1
S. defoliata
S. nordestina
S. aghensis_1
S. aghensis_2
S. pusilla
S. harleyi
S. concinna
S. barbata
P. tenuiflora_1
P. tenuiflora_2
S. richii
S. speciosa_1
S. guttata
S. villosa_1
S. speciosa_2
S. villosa_2
S. macrophylla
S. kautskyi
P. prasinata_1
P. prasinata_2
S. tuberosa_1
S. tuberosa_2
S. gigantifolia_1
S. gigantifolia_2
S. cochlearis
S. lindleyi
S. hirsuta
V. calcarata_1
V. calcarata_2
V. gardneri
V. truticulosa
V. brueggeri
P. sericiflora_1
P. sericiflora_2
P. werdermannii
V. lanata
V. leonii
V. pendula
P. gracilis
V. hilariana
S. schiffneri
S. sp nov. 4
P. plumerioides
Nematanthus
Codonanthe
Episcia
Nautilocalyx
Gloxinia
Koellikeria
Achimenes
Smithiantha
Kohleria
Gesneria
A
B
C
D
E
Episcieae
Gloxinieae
Gesneriaeae

intron 10, 90–95 bp). In the ingroup, mean pairwise divergence for the *ncpGS* introns was 4.4% (maximum of 10%) with 23.9% of potentially informative characters, which is about 2–5 times higher than in the non-coding plastid DNAs (Table 1). When outgroup taxa were included, mean pairwise divergence for the introns increased to 5.5% (maximum of 13%) with about a third of the characters being potentially informative. Out of the 28 accessions with heterogeneous electrophenograms, 10 of these had a unique sequence for each of the five clones sequenced. Heterogeneity was therefore probably due to different sequences present in a minority of the PCR products. Within the 18 remaining plants, more than one sequence of *ncpGS* was found and eight of them display even more than two sequences (up to five). The sequences found within individuals differ by 1–2 bp except for *S. richii* (3 bp), *S. villosa* (5 bp), and *V. gardneri* (11 bp). Phylogenetic analysis including all clones showed all species to be monophyletic except for *V. gardneri*, where the most divergent clone clustered with *V. leonii*/*V. pendula* (data not shown). For subsequent analyses only one clone per individual was used, except for *V. gardneri* for which we discarded the most divergent clone.

Plastid-based phylogenetic analyses—Each data set (i.e., *atpB-rbcL, trnT-trnL, trnL-trnF* and *trnS-trnG* spacers, *trnL* and *rpl16* introns) was first analyzed separately, resulting in relatively poorly resolved/supported trees (Fig. 1). The highest number of nodes supported by at least 70% BS was retrieved with *rpl16* intron, whereas the lowest was provided with *trnL-trnF* spacer (Fig. 1). The ILD test between all pairs of individual plastid data sets revealed significant incongruences ($P < 0.01$) except between *trnL* and *rpl16* introns ($P = 0.86$). However, as we have not found any hard incongruence (BS $> 70\%$) between these topologies, we performed a combined analysis of all plastid regions (hereafter ''plastid data set''; Fig. 2). In comparison with partitioned analyses, simultaneous analysis of plastid sequences increased from 1.8 to 14 times the number of branches supported by at least 70% BS (Fig. 1). Sinningieae were monophyletic (BS 100%). Within Sinningieae, three major clades are observed and denoted clade A (BS 91%), B (BS 67%), and C (BS $< 50\%$). Clade C includes several *Paliavana* species and the majority of the species of *Vanhouttea*, which are embedded in *Sinningia*. Not assigned to a clade are *P. tenuiflora*, *S. richii*, and *V. lanata*, sisters to clades B, C, and B/C respectively. The remaining species fall into two small clades denoted clade D (*P. gracilis*, *V. hilariana*, *V. leonii*, and *V. pendula*; BS 98%) and clade E (*P. plumerioides*, *S. schiffneri*, and *S.* sp. nov. 4; BS 91%). When two individuals per species were used, they came out as sisters with the exception of the individuals of *S. speciosa* and *S. villosa*, which were embedded in a same clade together with *S. guttata* (Fig. 2). Infraspecific divergence ranged between 0.03 and 0.43%, which is below interspecific divergence within Sinningieae (0.79–2.06%; Table 1).

Nuclear ncpGS-based phylogenetic analysis—Phylogenetic analysis of *ncpGS* alone resulted in less resolved trees than combined plastid analysis but a greater number of nodes supported by BS $\geq 70\%$ than any single plastid regions (Fig. 1). The *ncpGS* tree depicted in Fig. 3 also supports the monophyly of Sinningieae (BS 100%). Three major clades emerge (A, B, and F), and *P. plumerioides* is sister to the rest of the species (BS $< 50\%$). Species included in clades C, D, and E based on plastid DNAs are lumped together with *P. tenuiflora* and *V. lanata* in a clade denoted F (BS $< 50\%$). Clade A (BS 91%) includes the same species as in the plastid-based analysis, but clade B (BS 77%) differs by the inclusion of *S. richii*, which is sister to clade C based on plastid DNAs (BS $< 50\%$; Fig. 2). In addition, relationships among clades are different between the nuclear and plastid-based analyses, with clade B sister to A based on *ncpGS* (BS 83%; Fig. 3), whereas it is sister to C based on plastid DNAs (BS 78%; Fig. 2). When we used two individuals per species, they came in sister positions (Fig. 3). Intraspecific pairwise divergences in introns was 0–1.69%, which is 2.5 times lower than mean interspecific divergence within Sinningieae (4.4%; Table 1).

Plastid vs. nuclear DNAs—Results from ILD tests indicate incongruence between *ncpGS* and each individual plastid region data sets ($P < 0.01$). When comparing *ncpGS* and plastid-based topologies in 70% bootstrap trees, 11 hard incongruences are identified (nodes indicated with a Ψ symbol on Figs. 2 and 3). In the plastid-based trees, *S. warmingii* is sister to *S. allagophylla*; *S. tubiflora* is sister to *S. aggregata*; *S. concinna* is sister to *S. harleyi*; *P. prasinata* clusters with *S. guttata*, *S. kautskyi*, *S. macrophylla*, *S. speciosa*, and *S. villosa*; and clades B and C together with *V. lanata* are monophyletic (Fig. 2). However, in the *ncpGS*-based tree, *S. warmingii* is sister to *S. tubiflora*; *S. concinna* is included in a clade with *S. aghensis*/*S. pusilla*; *P. prasinata* is sister to *P. tenuiflora*; and clade B is sister to clade A (Fig. 3). In most cases, constraining these conflicting nodes onto the rival data set retrieved trees with maximum likelihood scores that were significantly worse than those derived from an unconstrained search, according to the SH test (significant conflicted nodes at $P < 0.05$ were denoted by an asterisk in Figs. 2 and 3). The null hypothesis was, however, not rejected in four cases when *ncpGS* data was constrained by the sister relationship of *S. warmingii*/*S. allagophylla* and B/C clades and when plastid data was constrained by the sister relationship of *P. prasinata*/*P. tenuiflora* and A/B clades.

Total evidence—We also combined these two data sets in a ''total evidence'' approach (Kluge, 1989; Fig. 4). Combining

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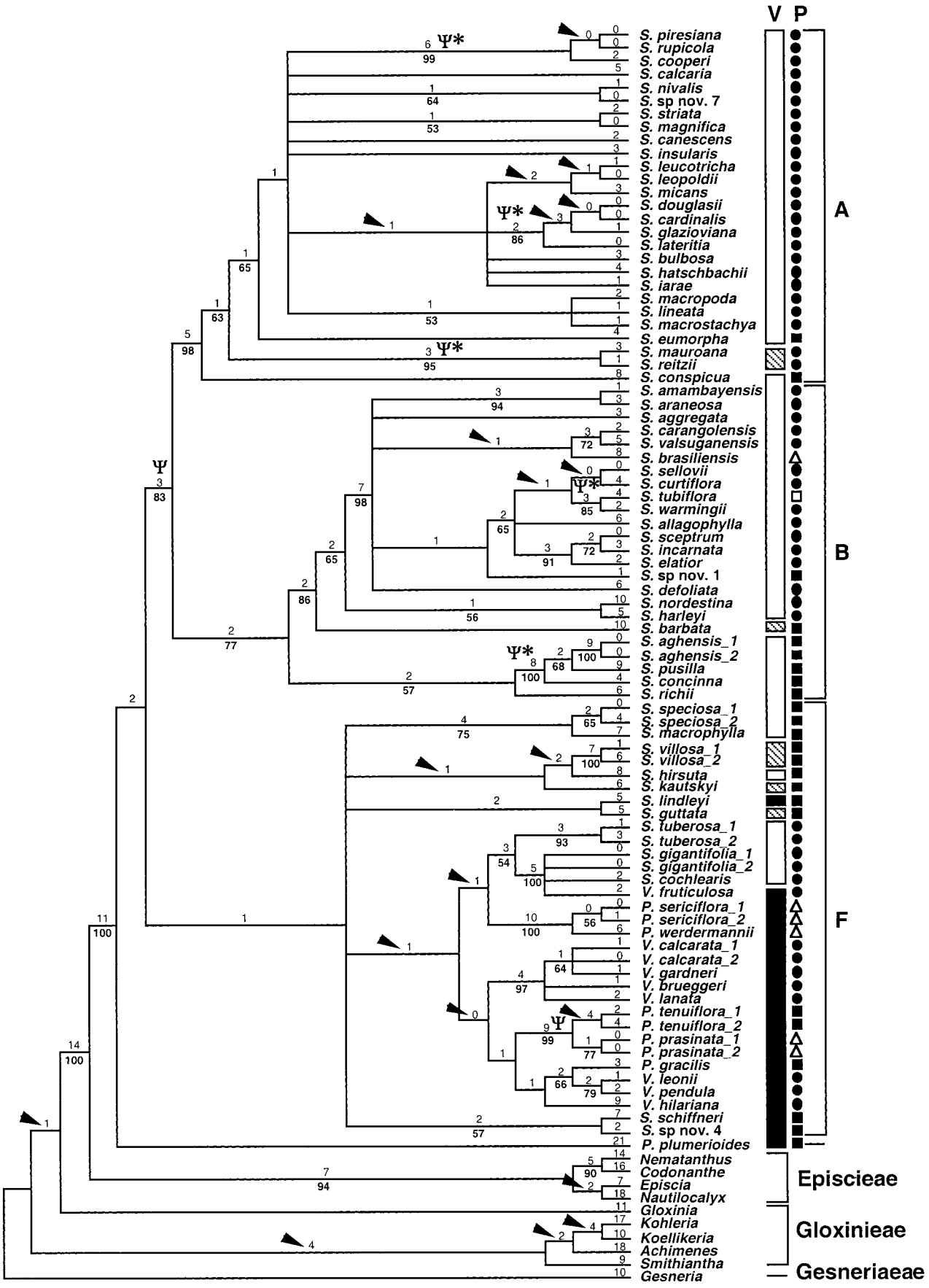
Fig. 2. One of the shortest trees resulting from a maximum parsimony analysis of a combined data set with *trnT-trnL, trnL-trnF, trnS-trnG, atpB-rbcL* spacers and *trnL* and *rpl16* introns from the plastid genome sequenced for 95 accessions including 85 Sinningieae and 10 outgroups within tribes Episcieae, Gloxinieae, and Gesnerieae. Tree length = 1585 steps, CI (consistency index, excluding uninformative characters) = 0.52, RI (retention index) = 0.80. Numbers above the branches are branch lengths; those below are bootstrap values ≥50%. Arrows indicate branches that collapse in a strict consensus tree of 10 000 most parsimonious trees. Ψ symbols indicate nodes in conflict with *ncpGS* topology at a confidence limit of 70% bootstrap value. Asterisks indicate nodes that conflict significantly with the *ncpGS* data set according to the SH test ($P < 0.05$). V, vegetative habit defined in three classes: tuberous with annual stem (white); tuberous with perennial stem (dashed lines); non-tuberous and perennial (black). P, pollination syndromes divided in four classes: hummingbird flowers (●); bee flowers (■); bat flowers (△); moth flower (□).


V P
S. piresiana
S. rupicola
S. cooperi
S. calcaria
S. nivalis
S. sp nov. 7
S. striata
S. magnifica
S. canescens
S. insularis
S. leucotricha
S. leopoldii
S. micans
S. douglasii
S. cardinalis
S. glazioviana
S. lateritia
S. bulbosa
S. hatschbachii
S. iarae
S. macropoda
S. lineata
S. macrostachya
S. eumorpha
S. mauroana
S. reitzii
S. conspicua
S. amambayensis
S. araneosa
S. aggregata
S. carangolensis
S. valsuganensis
S. brasiliensis
S. sellovii
S. curtiflora
S. tubiflora
S. warmingii
S. allagophylla
S. sceptrum
S. incarnata
S. elatior
S. sp nov. 1
S. defoliata
S. nordestina
S. harleyi
S. barbata
S. aghensis_1
S. aghensis_2
S. pusilla
S. concinna
S. richii
S. speciosa_1
S. speciosa_2
S. macrophylla
S. villosa_1
S. villosa_2
S. hirsuta
S. kautskyi
S. lindleyi
S. guttata
S. tuberosa_1
S. tuberosa_2
S. gigantifolia_1
S. gigantifolia_2
S. cochlearis
V. fruticulosa
P. sericiflora_1
P. sericiflora_2
P. werdermannii
V. calcarata_1
V. calcarata_2
V. gardneri
V. brueggeri
V. lanata
P. tenuiflora_1
P. tenuiflora_2
P. prasinata_1
P. prasinata_2
P. gracilis
V. leonii
V. pendula
V. hilariana
S. schiffneri
S. sp nov. 4
P. plumerioides
Nematanthus
Codonanthe
Episcia
Nautilocalyx
Gloxinia
Kohleria
Koellikeria
Achimenes
Smithiantha
Gesneria
A
B
F
Episcieae
Gloxinieae
Gesneriaeae

data sets did not decrease global bootstrap support as the number of nodes with at least 70% BS was slightly higher than in the plastid-based trees (47 vs. 43; Fig. 1). Resulting topologies are similar to those obtained from the plastid data set and include five major clades (A to E). Due to the higher number of informative characters in the plastid data set, conflicting relationships with *ncpGS* are mainly resolved towards the plastid-based topologies with the exception of a few relationships that follow the *ncpGS*-based topologies (i.e., position of *S. richii* within clade B [BS 61%], sister positions of *P. tenuiflora*/*P. prasinata* [BS 57%] and *P. sericiflora*/*P. werdermanii* [BS 98%], position of *V. fruticulosa* clustered with *S. gigantifolia* and *S. cochlearis* [BS 87%], and *V. lanata* clustered with other *Vanhouttea* [BS 68%]). Within the subclade that includes *S. aggregata*, *S. allagophylla*, *S. amambayensis*, *S. curtiflora*, *S. sellovii*, *S. tubiflora*, and *S. warmingii* (BS 100%), only two nodes were present in the strict consensus of the total evidence analysis (Fig. 4), whereas four are found in the plastid-based strict consensus (Fig. 2); this decrease of resolution is certainly the consequence of conflicts between plastid and *ncpGS* data sets in the relationships of these species.

Mapping pollination syndromes—Mapping pollination syndromes on all combined and partitioned trees indicated an equivocal ancestral state in Sinningieae between the hummingbird and the bee syndromes. Bee and hummingbird flowers represent 93% of the Sinningieae species and occur together in all the clades except clade E; most of the changes recorded are therefore between these two syndromes (Fig. 5). Unambiguous changes from hummingbird to bee syndromes were recorded on terminal branches leading to *S.* sp. nov. 1 (clade B) and to *P. gracilis* (clade D). Location of the remaining changes between these two syndromes varied depending on the arrangements of the basal node in the different analyses. Bat syndrome evolved independently in *S. brasiliensis* (clade B) and in *P. prasinata*, *P. sericiflora*, and *P. werdermanii* (clades C or F; Figs. 2, 3, and 4). The bat syndrome evolved unambiguously from hummingbird syndrome in *S. brasiliensis*, but the bee syndrome was reconstructed as a possible ancestral state for bat-pollinated *Paliavana* based on the plastid and plastid + *ncpGS* analyses (Fig. 5). The only moth flower, *S. tubiflora*, originated from hummingbird syndrome in clade B (Fig. 5); it differs from its closest extant relatives by having a longer corolla (approximately 7 cm), being white instead of red, and producing a sweet fragrance.

Odor analysis of Sinningia tubiflora—The TD-GC-MS analysis of the odor of *S. tubifera* trapped on Tenax revealed one major component (Fig. 6). This was identified as linalool by comparison of its retention index (RI = 1102) and mass spectrum with a standard. The peak of linalool in the total ion chromatogram accounted for over 60% of the total area of all the peaks detected. The remaining peak area was due to numerous components (none having a peak area greater than 5% of the total) eluting between RI 1200–1400 and showing mass spectra typical of alkanes, except one at RI = 1420 that showed a mass spectrum typical of many sesquiterpenes.

Mapping vegetative habit—Clade A and B only include tuberous taxa whereas non-tuberous plants are restricted to the clades C, D, and E. The ancestral habit in Sinningieae was found to be a perennial without a tuber based on combined and plastid analyses, whereas it was equivocal based on the tree derived only from *ncpGS*. Acquisition of a tuber in *Sinningia* is likely to have occurred independently several times (2–6 times; Fig. 7). The acquisition of a tuber occurred mostly in plants with annual shoots, whereas the shift from non-tuberous to tuberous plants with perennial stems was less frequent and not firmly supported when based on the plastid analysis (Fig. 7). Subsequent changes from annual stems to perennial stems have independently occurred one to four times, depending on the different reconstructions and optimizations (Fig. 7). This shift occurred unambiguously along branches leading to *S. reitzii*, *S. mauroana* (clade A), and *S. barbata* (clade B), supporting the notion that perennial stems in tuberous plants evolve after tuber acquisition and thus constituting a reversion.

DISCUSSION

Molecular evolution of non-coding plastid DNAs and ncpGS gene—Sequences of the six different non-coding plastid DNA regions exhibit a lower (3.5 times on average) proportion of potentially informative characters than the introns of *ncpGS* as expected; this is due to the lower substitution rates in plastid than nuclear DNA (Wolfe et al., 1987; Randall et al., 1998). Among the plastid regions we sequenced, the *trnT-trnL* and *trnS-trnG* spacers and the *rpl16* intron provided more variable sites than the commonly used *atpB-rbcL*, *trnL-trnF* spacers, and the *trnL* intron; this result corroborates results of Small et al. (1998) for *trnT-trnL* spacer and the *rpl16* intron. As observed by Gielly and Taberlet (1994), the evolutionary rate of the *trnL* intron is only slightly lower than the adjacent *trnL-trnF* spacer despite conserved stem regions and secondary structures of this group I intron (Kuhsel et al., 1990). Similarly, the number of variable positions in the *rpl16* intron range within values obtained for intergenic spacers, despite the fact that this group II intron was suspected to be strongly constrained by structural features (Clegg et al., 1994). Among intergenic spacers, *atpB-rbcL* spacer exhibits the lowest pairwise divergence. Unlike other spacer between *trn* genes, *atpB-rbcL* spacer contains conserved elements involved in gene regulation like *atpB* and *rbcL* promoters and leader sequences of both genes; these could account for the low evolution rates comparable to the *rbcL* exon, as observed in Rubieae (Manen et al., 1994b; Manen and Natali, 1995).

Plastid non-coding regions were rich in strings of mononucleotides repeats and small tandem repeat units, which are known to be subject to slipped-strand mispairing, a major mechanism in length mutations (Levinson and Gutman, 1987).

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Fig. 3. One of the shortest trees resulting from a maximum parsimony analysis of 95 *ncpGS* sequences; tree length = 595 steps; CI = 0.57 (excluding uninformative characters); RI = 0.84. Numbers above the branches indicate number of steps, numbers below indicate bootstrap values ≥50%. Arrows indicate branches that collapse in a strict consensus tree of 10 000 most parsimonious trees. Ψ symbols indicate nodes in conflict with the plastid combined regions topology at a confidence limit of 70% bootstrap value. Asterisks indicate nodes that conflict significantly with the plastid combined data set according to the SH test ($P < 0.05$).

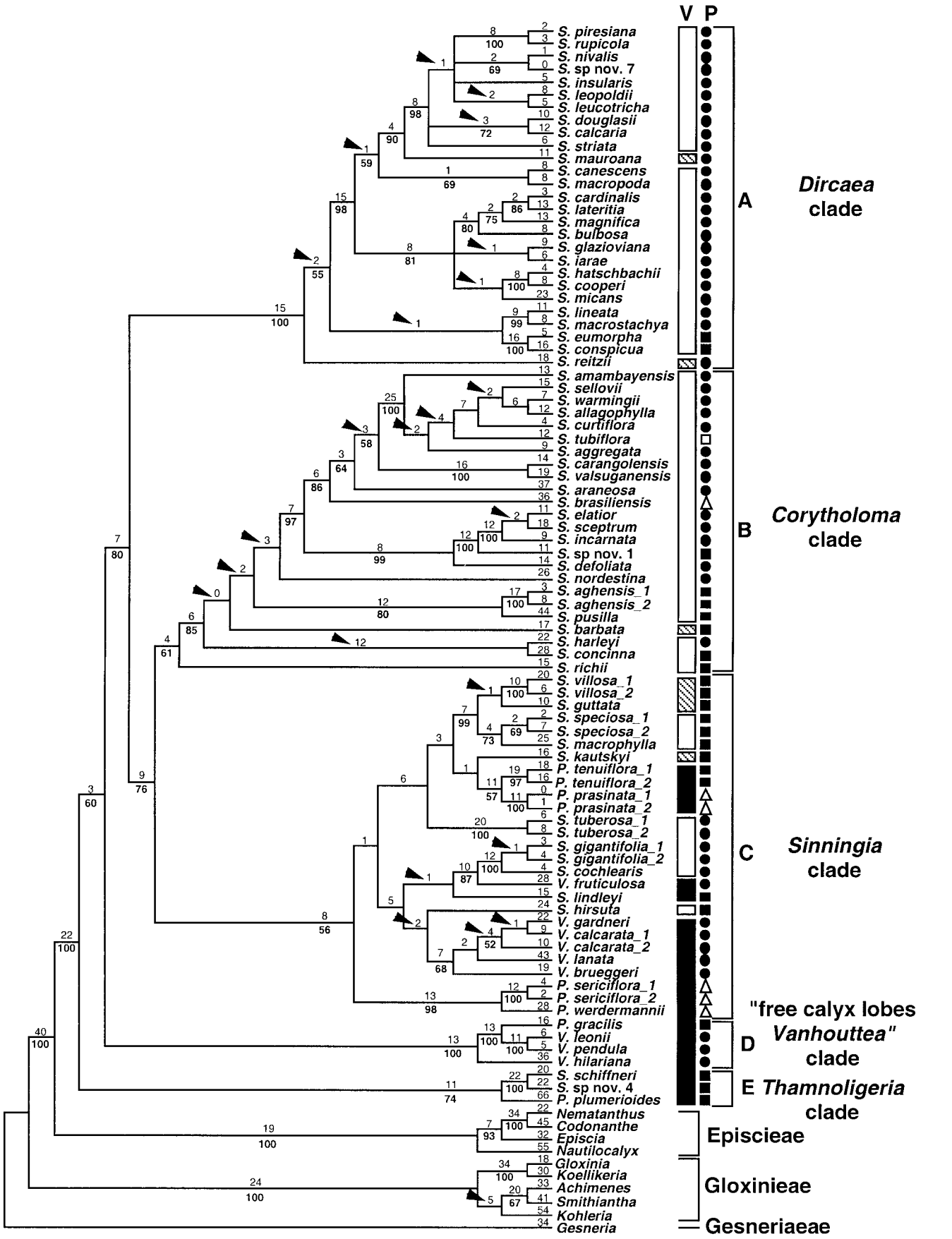

Fig. 4. One of the shortest trees resulting from a maximum parsimony analysis of all combined data; tree length = 2275 steps; CI = 0.50 (excluding uninformative characters); RI = 0.79. Numbers above the branches indicate number of steps; numbers below indicate bootstrap values ≥50%. Arrows indicate branches that collapse in a strict consensus tree of 10 000 most parsimonious trees.

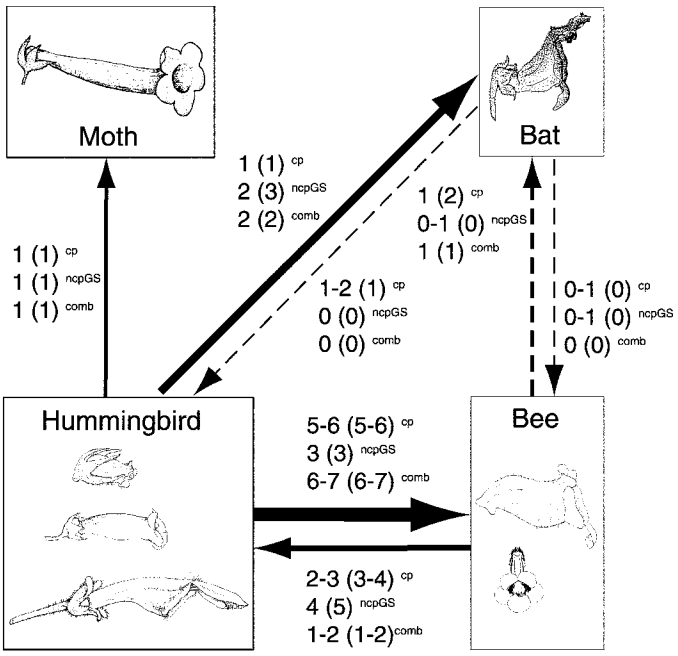

Fig. 5. Evolution of pollination syndromes in Sinningieae. Arrows indicate the direction of changes. Maximum and minimum numbers of changes were calculated based on different resolving options of the trees in Figs. 2, 3, and 4 for plastid (cp), *ncpGS*, and combined (comb) analysis assuming ACCTRAN and DELTRAN optimizations (former values in brackets). The width of the arrows is proportional to the frequency of changes. Arrow is dashed when a change is not supported by one of the alternative trees.

Sequence repeats and extensive length variation were particularly high in the spacers *trnT-trnL* and *trnS-trnG*, requiring the introduction of a large number of gaps in their alignments, which potentially limited their use to closely related species between which indel types can be recognized and used for alignment (Kelchner, 2000). Although indels were recorded as being as frequent as nucleotide substitution between closely related species (Zurawski and Clegg, 1987; Gielly and Taberlet, 1994), our results indicate that potentially informative indel characters represent only one-sixth (in *rpl16* intron) to one-third (in *trnL-trnF* spacer) of the total number of informative characters (Table 1).

The four introns of *ncpGS* sequenced in Sinningieae amounted to a length slightly longer than in *Oxalis* (360–394 bp). They occupied the same locations as in *Oxalis* and *Medicago*, indicating that the intron positions among rosids and asterids are conserved (Tischer et al., 1986; Emshwiller and Doyle, 1999). Restricted length variation in *ncpGS* and a maximum pairwise divergence of 13% between Sinningieae and outgroups allow an unambiguous alignment, and we assume that the phylogenetic utility of this gene might be not only at the generic or tribal level but also at higher taxonomic levels. The mean pairwise divergence found in the *ncpGS* intron among Sinningieae species (averaging 4.4%) is slightly higher than among closely related *Oxalis* species according to the results of Emshwiller and Doyle (1999) and comparable at the same taxonomic level with intron divergence detected in other low copy nuclear genes like *Adh*, *pistillata*, or *cycloidea* (Randall et al., 1998; Bailey and Doyle, 1999; Citerne et al., 2000).

The presence of more than one *ncpGS* sequence within individual plants revealed by cloning indicates apparent heterozygosity for this locus. The level of *ncpGS* variation does not however exceed intra-individual polymorphism detected within paralogous *Adh* genes (Sang et al., 1997b) or between multiple copies of the internal transcribed spacers of nuclear ribosomal DNA (nrITS; Denduangboripant and Cronk, 2000; Kita and Ito, 2000). Presence of multiple *ncpGS* sequences within individuals was also found in *Oxalis*, in which variants were interpreted as either normal allelic polymorphism, homeologous loci, or artifacts due to recombination during PCR amplification (Emshwiller and Doyle, 1999). Contrary to *Oxalis*, a maximum of two variants is expected in *Sinningia*, in which cytological data report only diploids with $2n = 26$ (Clayberg, 1967). Further investigation are thus required to determine the origin of more than two sequences in eight individuals by examining alternatives such as the presence of

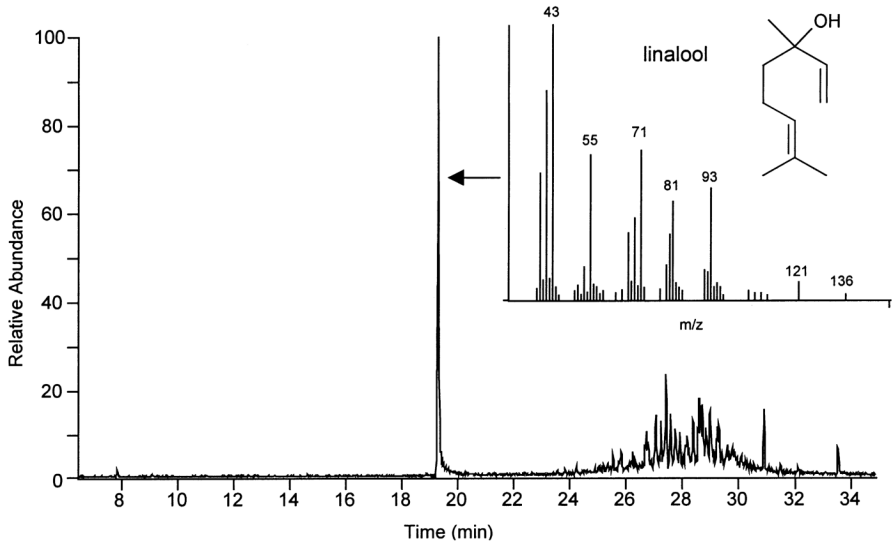

Fig. 6. Total ion chromatogram from the odor analysis of *Sinningia tubifera* by headspace trapping with Tenax followed by thermal desorption-gas chromatography-mass spectrometry. Inset is a graph of an ion trap electron ionization (EI) mass spectrum of the major peak, identified as linalool.

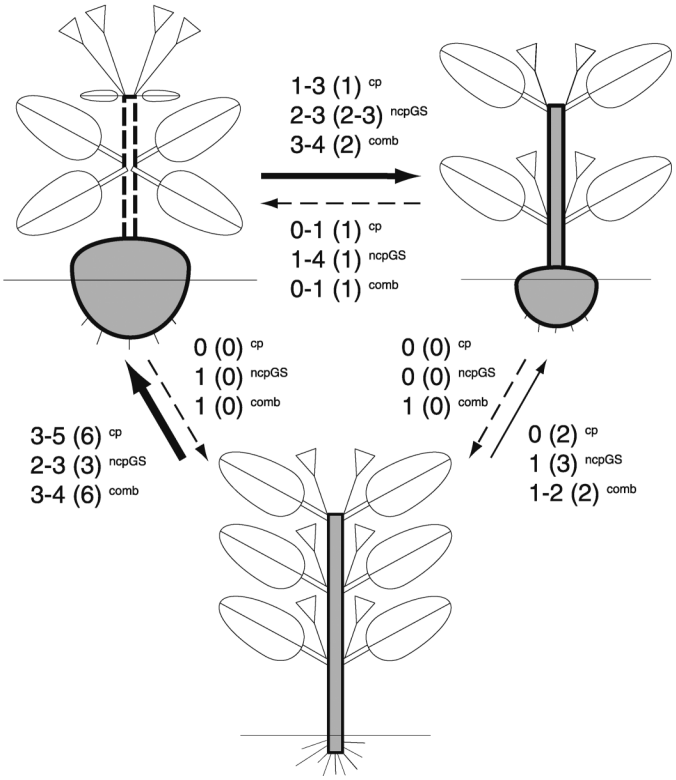

Fig. 7. Evolutionary transitions between three forms of vegetative habit in Sinningieae (tuberous with annual shoot [top left], tuberous with perennial stem [top right], non-tuberous and perennial [bottom]). Perennial structures (i.e., tuber and stem) are in grey and annual shoot is drawn in dashed line. Arrows indicate the direction of changes. Maximum and minimum numbers of changes were calculated based on different resolving options of the trees in Figs. 2, 3, and 4 for plastid (cp), *ncpGS*, and combined (comb) analyses assuming ACCTRAN and DELTRAN optimization (former values in brackets). The width of the arrows is proportional to number of changes. Arrow is dashed when a change was not supported by one of the alternative trees.

undetected polyploids, weakly divergent paralogous copies, or putative PCR artifacts.

Incongruence between data sets—Separate analyses of each of the six plastid DNA regions were shown to be significantly incongruent according to the ILD test (except between *trnT-trnL* and *rpl16*). However, the topologies obtained were congruent at a confidence limit of 70% BS, exemplifying the difference between characters and topological incongruences (Allard and Carpenter, 1996). Because no hard incongruence was found, the inclusion of all linked plastid DNA regions into a single analysis should maximize the explanatory power regardless of the level of character incongruences between data sets detected with the ILD (Nixon and Carpenter, 1996; Yoder et al., 2001). Sequence variation and resolution of *ncpGS* are higher than for any plastid non-coding regions, and several topological incongruences were found between the plastid and *ncpGS*-based phylogenetic trees, as inferred from bootstrap analyses and SH test (Figs. 2 and 3). Some of the taxa included in the conflicting clades probably have separate evolutionary histories for plastid and *ncpGS* gene, and thus introgression, lineage sorting, or gene duplication/deletion processes should be considered (Doyle, 1992; Maddison, 1997; Wendel and Doyle, 1998). The probability that independent duplication of a single locus has occurred among closely related species is low (Maddison, 1997; Sang and Zhang, 1999). However, the presence of multiple sequences detected within individuals of Sinningieae (e.g., *V. gardneri*) does not exclude completely that paralogous copies are responsible for incongruences between *ncpGS* and plastid-based analyses. On the other hand, at a low taxonomic level, many discrepancies between plastid and nuclear phylogenetic analyses were attributed to presumed introgression or lineage sorting events in a wide range of plant groups like *Ceanothus*, *Gossypium*, *Helianthus*, *Heuchera*, *Paeonia*, and Triticeae (Rieseberg, 1991; Wendel et al., 1991; Soltis and Kuzoff, 1995; Mason-Gamer and Kellogg, 1996; Sang et al., 1997a; Seelanan et al., 1997; Hardig et al., 2000). In Sinningieae, our results show significant topological incongruences as identified by the SH test at nodes including terminal branches, and few individual species can be identified as the cause of the conflict. At this within-clade level, experimental hybridization studies and cytological analyses demonstrated a weak post-zygotic reproductive barrier between *Sinningia* species (Clayberg, 1968, 1970, 1996; Wiehler and Chautems, 1995), and it is therefore possible that introgression explains the incongruences observed between the plastid and nuclear-based phylogenetic trees.

Plastid introgression was found to occur more frequently than nuclear gene flow as reviewed by Rieseberg and Soltis (1991). This process was mainly inferred from discordant distributions between organellar and nuclear characters among interfertile plant species (Whittemore and Schaal, 1991; Rieseberg et al., 1991; Soltis and Kuzoff, 1995; Wolfe and Elisens, 1995). In Sinningieae, hypothesis of plastid capture could be evoked for morphologically related species (i.e., *S. reitzii*/*S. mauroana*, *S. tubiflora*/*S. warmingii*, *P. prasinata*/*P. tenuiflora*, *S. pusilla*/*S. concinna*) that have a sister relationship with the *ncpGS* tree but significantly divergent positions in the plastid tree (Fig. 2 vs. Fig. 3). In several other cases, the plastid phylogenetic analysis agrees with previous ideas about taxonomic relationships better than does the *ncpGS* tree. For example, the fusion of two dorsal extended corolla lobes might be seen as a ''good'' synapomorphy for the clade including *S. bulbosa*, *S. cardinalis*, *S. magnifica*, *S. lateritia*, *S. glaziovana*, *S. hatschbachii*, *S. cooperi*, *S. iarae*, and *S. micans* based on plastid DNAs (BS 64%; Fig. 2). Based on *ncpGS*, these latter species are oddly embedded in three different clades with species displaying tubular corolla with lobes of equal size (i.e., *S. glaziovana*/*S. cardinalis*/*S. lateritia* with *S. douglasii*, BS 86%, and *S. cooperi* with *S. piresiana*/*S. rupicola*, BS 99%, Fig. 3). Such incongruences could indicate nuclear introgression events, although the possibility exists that we did not amplify homologues *ncpGS* in all cases.

Systematics of Sinningieae—The combined analysis of plastid DNAs and *ncpGS* (Fig. 4) provides insights in the systematics of Sinningieae, but only relationships that are not in significant conflict between plastid and *ncpGS* single analyses are discussed in this section. All analyses confirm that Sinningieae constitute a monophyletic group in agreement with Smith et al. (1997) and Zimmer et al. (2002), distinct from Gloxinieae within which they were included by Wiehler (1983). *Paliavana*, *Vanhouttea*, and *Sinningia* are primarily Brazilian genera currently distinguished by their habit and floral morphology: *Paliavana* and *Vanhouttea* are shrubby species with respectively campanulate to bell-shaped flowers or red tubular flowers, whereas *Sinningia* is characterized by the

presence of a tuber irrespective to corolla shape. This generic circumscription is however not congruent with our phylogenetic analyses: both *Paliavana* and *Vanhouttea* are found to be polyphyletic and nested within *Sinningia*. A new circumscription of these genera is thus required. Bearing in mind the need for nomenclatural stability, the inclusion of all taxa in a single redefined genus appears to be the best solution (McNeill, 2000).

Monophyly of clade A is strongly supported in both separate and combined analyses (BS 91%, 98%, 100% in Figs. 2, 3, and 4, respectively). Most species in this clade are characterized by a verticillate habit corresponding to a pseudo-whorl of leaves at the extremity of an elongated basal internode (Chautems and Weber, 1999). Exceptions are *S. magnifica* and *S. cooperi*, which have internodes of equal size and *S. eumorpha* and *S. conspicua*, which have a rosulate habit. Morpholgical synapomorphies for this clade are a calyx base shortly adherent to the ovary, calyx lobes generally short and narrow, two separate enlarged dorsal nectary glands, and five nectary chambers equal in size. Corollas are red and tubular in all species except in the sister species *S. conspicua* and *S. eumorpha*, which have respectively yellow or white to light lavender campanulate flowers. Among red tubular species, fusion and expansion of the two dorsal corolla lobes (''hooded'' corolla) is a synapomorphy supporting the monophyly of *S. bulbosa*, *S. cardinalis*, *S. cooperii*, *S. glazioviana*, *S. hatschbachii*, *S. iarae*, *S. magnifica*, *S. micans*, and *S. lateritia* (BS 81%; Fig. 4), whereas the remaining species have five lobes of equal size. Based mostly on corolla shape, Fritsch (1894) classified species within clade A in two different genera: campanulate species were placed in *Sinningia*, whereas red and tubular species were included either in *Corytholoma* section *Dircaea* (hooded corolla) or in section *Thamnocaula* (corolla lobes equal in size). However, based on tests of crossability, Clayberg (1968, 1996) first suggested a close affinity of these species because he was able to produce fully fertile hybrids from all crosses between *S. eumorpha*, *S. cardinalis*, *S. magnifica*, *S. cooperi*, *S. macrorhiza* (= *bulbosa*), *S. douglasii*, *S. macropoda*, *S. lineata*, *S. leucotricha*, and *S. reitzi*. Congruence of our results with Clayberg supports that clade A constitutes a taxonomical entity for which the name ''Dircaea'' is appropriate for nomenclatural reasons.

Clade B (BS 61%) includes species with various floral morphologies and vegetative habit. Basal species are particularly diverse and have habits ranging from verticillate (*S. aghensis*), rosulate-caulescent (*S. barbata*) to minute rosulate (*S. pusilla*), and flowers with tiny funnel-shaped (*S. pusilla*, *S. concinna*), bell-shaped (e.g., *S. aghensis*), or tubular (e.g., *S. harleyi*) corolla. The 16 species included in a well-supported subclade (BS 97%, from *S. amambayensis* to *S. defoliata* in Fig. 4) have a more uniform habit, i.e., annual herbaceous stems with equal size internodes bearing caulescent to spicate florescences (Chautems and Weber, 1999). A noticeable exception to this above-described morphology is *S. defoliata*, which displays a stemless, unifoliate habit. Within this subclade corolla size and color are highly labile, ranging from red or orange tubular corolla ($\leq$1 cm long in *S. allagophyla* and *S. curtiflora*) through long white tubular corolla ($\geq$7 cm in *S. tubiflora*) to a greenish gullet-shaped corolla in *S. brasiliensis*. Despite this diversity, crosses between several species in clade B produced fertile hybrids (Wiehler and Chautems, 1995; Clayberg, 1996). Furthemore, we identified some morphological synapomorphies for this clade such as calyx lobes that are foliaceous and non-adherent to the ovary, two enlarged and fused dorsal nectary glands (excepted in *S. brasiliensis* having a ring composed of five fused glands), and two dorsal nectary chambers more developed than the three remaining ones. Early on, Decaisne (1848) used this same set of characters to circumscribe the genus *Corytholoma* Decaisne. Fritsch (1894) then expanded this definition of *Corytholoma* and divided it in five sections of which three are part of clade B; ''*Corytholoma*'' is therefore an appropriate name for this group.

Clade C (BS 56%) includes *Paliavana* and *Vanhoutta* species embedded within *Sinningia* species. Habit is suffrutesent to shrubby in both *Paliavana* and *Vanhouttea* (up to 3 m tall), whereas the *Sinningia* species included in this clade are herbaceous caulescent, rosette-like, or unifoliate (Chautems and Weber, 1999). *Sinningia lindleyi*, *S. villosa*, and *S. kautskyi* have a reduced tuber producing a perennial stem. Flowers are also highly diversified, ranging from purple and greenish bell-shaped to red and tubular. However, all species in clade C have five nectary glands of equal size surrounding the ovary base, except in *S. gigantifolia* and *S. cochlearis*, which display two enlarged and fused nectary glands in dorsal position. Several subclades within clade C are well supported, although their relationships are poorly resolved. The bee-pollinated species *S. guttata*, *S. macrophylla*, *S. speciosa*, and *S. villosa* are clearly monophyletic (BS 99%). All species of *Vanhouttea* except one are clustered in a subclade (BS 68%) that includes the type species of the genus, *V. calcarata*. Not included in this latter subclade is *V. fruticulosa*, which is clustered with *S. gigantifolia* and *S. cochlearis* (BS 87%). *Paliavana* appears paraphyletic with *P. sericiflora*/*P. werdermannii* sister to clade C and *P. tenuiflora*/*P. prasinata* embedded within *Sinningia*, but the position of *P. prasinata* is discordant between plastid and *ncpGS* analyses. The positions of the remaining species of *Sinningia* within clade C are poorly resolved. Some of them (e.g., *S. lindleyi* and *S. guttata*) were among the first species recognized in this genus and are morphologically close to the type species *S. helleri*; we thus propose to include all species of clade C under the name ''*Sinningia*.''

Monophyly of clades D and E are well supported in the plastid-based analyses and in the total evidence analyses (BS $>$ 74%); however in the *ncpGS* analyses, species of clade D and E are lumped together within with clade C under clade F (Fig. 3), but with a BS of $<$50%. Taxa of both clades D and E share characters with the majority of the members of clade C (clade *Sinningia*), such as absence of a tuber, suffrutescent/shrubby perennial stem, and five equally sized nectary glands. Clade D includes four shrubby species of which one matches the definition of *Paliavana* because of its blue campanulate flowers and three were initially considered as *Vanhouttea* because of their red tubular corolla (Chautems, 2002). The segregated position of the latter three taxa is surprising as they do not differ from the other *Vanhouttea* species of clade C, with the exception of calyx lobes that are free in the bud instead of being fused. Based on this character we named this lineage ''free calyx lobes *Vanhouttea*.'' Clade E includes *S. schiffneri*, *P. plumerioides*, and one unpublished new species; their corollas are white and trumpet-shaped in *S. schiffneri* and *S.* sp. nov. 4, whereas they are yellowish and gullet-like in *P. plumerioides*. Fritsch (1908) early recognized *S. schiffneri* as being the only member of the *S.* section *Thamnoligeria*, and we adopt this name for this clade.

Based on the molecular data, five lineages are now recognized, whereas the lability of flower morphology and habit had

made their identification previously difficult. However, nectary glands and adherence of calyx to the ovary characters, already used by Decaisne (1848) in an attempt to classify Brazilian Sinningieae, turn out to be most useful features for diagnosing the principal lineages within Sinningieae.

Pollination syndromes—Optimization of pollination syndromes in phylogenetic analyses indicates that multiple and rapid changes have occurred. Similar results were found in the Old World Gesneriaceae, *Streptocarpus* and *Saintpaulia* (Harrison et al., 1999), and Plantaginaceae (Reeves and Olmstead, 1998). Genetic studies in *Mimulus* and *Antirrhinum* have furthermore demonstrated that floral color, shapes, symmetry, and nectar production were controlled only by a few loci or were epigenetic (Bradshaw et al., 1995; Luo et al., 1996; Cubas et al., 1999).

Multiple origins of hummingbird and bee syndromes in Sinningieae and their co-occurrence in every major clades apart from E indicate numerous changes in pollination syndromes; however, because in all reconstructions both hummingbird and bee syndromes can be optimized at the root node, the ancestral state was not determined unambiguously. In more recent cladogenesis, unambiguous changes from hummingbird to bee syndromes were reconstructed in contrast to the general view that hummingbird pollination is derived from bee pollination (e.g., Grant, 1994).

Shifts to the hummingbird from the bee syndrome involved the acquisition of floral features, such as the red color and the straight tubular corolla, that are considered to play a role in deterring insect visitors that may rob nectar (Heinrich and Raven, 1972; Raven, 1972; Schemske and Bradshaw, 1999; Chittka et al., 2001). Field observations confirm hummingbird specificity for a few red tubular *Sinningia* and *Vanhouttea* species (Snow and Teixeira, 1982; Sazima et al., 1996; Chautems et al., 2000; Buzato et al., 2000; Vasconcelos and Lombardi, 2000). On the contrary, shifts to bee syndrome involve numerous colors (blue, purple, yellow, and white) independently of campanulate or funnel-shaped flowers.

Shifts to bat syndrome in *S. brasiliensis*, *P. prasinata*, *P. sericiflora*, and *P. werdermannii* occurred independently in clade B and C. The greenish, widely opened, gullet-like flower of *S. brasiliensis* is unambiguously derived from a red tubular flower, which characterizes its closely related species (*S. araneosa*, *S. carangolensis*, and *S. valsuganensis*; Fig. 4). Within clade C, the bat syndrome is restricted to the three *Paliavana* species displaying green to cream robust bell-shaped corollas. These latter species are embedded with bee- and hummingbird-pollinated species in clade C, and their ancestral state remains ambiguous between these two syndromes. Associated with these morphological features, bat flowers also produce fragrance reminiscent of burnt plastic. The chemical composition of this fragrance has not been identified in Sinningieae, but similar scents were recorded in several bat-pollinated flowers from different angiosperm families; these all consisted of sulfur-containing compounds (Knudsen and Tollsten, 1995). The production of nectar measured in *S. brasiliensis* and *P. prasinata* was 4–6 times higher than in other Sinningieae species pollinated by moths, bees, or hummingbirds (Perret et al., 2001). Nectar sugar concentration and composition were also different from hummingbird and bee flowers: total sugar concentration in bat flowers averaged 7.1 ± 3.4 % (mass/total mass) and is much lower than in hummingbird (23.9 ± 10.6%) and bee flowers (28.7 ± 10.6%), whereas sucrose proportion in bat flowers is on average 12 times lower than in the other syndromes (Perret et al., 2001). This sugar concentration in bat-pollinated species is below optimal nectar concentration established from models and feeding experiments with hummingbirds (Heyneman, 1983; Kingsolver and Daniel, 1983; Tamm and Gass, 1986) and below nectar concentration among bee-pollinated flowers in temperate and tropical regions (Pyke and Waser, 1981). Diluted nectar in bat flowers could consequently deter hummingbirds and bees more efficiently than the morphology of the corolla, paralleling the hypothesis of Bolten and Feinsinger (1978).

Moth pollination in *S. tubiflora* is likely due to features such as the long and slender flower and the white color, but as far as we know, no observations have been reported in the wild (Silberbauer-Gottsberger and Gottsberger, 1975; Endress, 1994). *Sinningia tubiflora* occurs at the southwestern limit of Sinningieae distribution in Paraguay and in northern Argentina. Because all species related to *S. tubiflora* display hummingbird flowers (Fig. 4), this moth flower is derived unambiguously from a red and tubular morphology. Close relationships between these two pollination syndromes were also recorded in the genera *Ipomopsis* and *Aquilegia* in North America, where sphingophilous species occurred at higher elevations and in generally dryer habitats than their ornithophilous relatives (Grant, 1992). The shift to moth pollination is also associated with a sweet fragrance, which was identified as linalool in this study (Fig. 6); this compound is found in moth-pollinated flowers worldwide (Knudsen and Tollsten, 1993; Raguso and Pichersky, 1999). The evolution of a species having a linalool-dominated odor from a non-scented species has also occurred in *Clarkia*, where it was found that de novo production of linalool in *C. breweri* was due to the up-regulation of linalool synthase (Dudareva et al. 1996; Raguso and Pichersky, 1999). The gene encoding linalool synthase has also arisen frequently during plant evolution, apparently by the duplication of terpene synthase genes followed by divergence and/or domain swapping (Cske et al., 1998).

Vegetative habit and geographical distribution—The ancestral habit in Sinningieae is likely to comprise suffrutescent stems and a lack of a tuber, as reconstructed in the two clades (D and E) that are successively sister to the rest (Fig. 4). The acquisition of tubers in *Sinningia* has occurred independently several times and is a feature that was considered a key character defining the genus, as the rest of Gloxinieae have scaly rhizomes (Wiehler, 1983). This storage organ, characterizing all members of clades A and B, is coincident with the ability of many species to colonize rupicolous habitats in open or forested areas. Cyclic annual growth and tuber dormancy are well adapted with the seasonal climate of southeastern Brazil (i.e., rainfall and warm temperatures in November to March, and dry and cool conditions in June to August). These conditions can be extreme when species grow in rocky habitats. Multiple reversions to perennial growth in association with the reduction of tuber size could then reflect a habitat shift to less seasonally extreme environments, relaxing constraints on the dormancy period.

The geographical distribution of clade A is restricted to the southeast and south regions of Brazil (Fig. 8). These taxa have marked affinities for well-drained substrates, where water is available from rain, fog, or dripping water. Such conditions occur mainly in rupiculous habitats, but two species are also found in epiphytic situations (*S. douglasii*, *S. cooperi*). Some

Fig. 8. Geographical distribution of the principal lineages resolved in the tribe Sinningieae according to the tree in Fig. 4 (combined analysis). Dashed line, range of the *Dircaea* clade; grey line, range of the *Corytholoma* clade; solid line, range of the *Sinningia* clade; dotted line, range of the two clades ''free calyx lobes *Vanhouttea*'' and *Thamnoligeria*.

taxa occupy impressive cliffs along the coastal range (Serra do Mar) including the well-known granite rock outcrops in Rio de Janeiro (Meirelles et al., 1999). The popular name ''queen of the abyss'' was therefore given to *S. canescens* and *S. leucotricha*. The largest tubers (over 30 cm for *S. lineata* and *S. macrostachya*) were recorded within this clade for the species occurring in southern Brazil, where seasonality is more pronounced than in southeastern Brazil. Conversely, reversion to a perennial suffrutescent stem with a reduced tuber was recorded in *S. reitzii* and *S. mauroana* living in rich pockets of humus in high rainfall sections of the Atlantic forest.

The species included in clade B occupy the widest distribution, ranging from northern Argentina to southern Mexico (Fig. 8). The majority of these species are restricted to the Atlantic ranges, but a few, such as *S. allagophylla*, *S. elatior*, *S. incarnata* and *S. warmingii*, have a more continental distribution. The ecological tolerance of these latter species for less strictly rupicolous habitats and their ability to colonize savanna vegetation has certainly facilitated their wider distribution (e.g., the terrestrial species *S. elatior* is found all over Brazil with a northern limit in Colombia). Reversion to a perennial stem and reduction of tuber recorded for *S. barbata* took place in the absence of marked seasonality characterizing the coastal forest of eastern Brazil.

Species of clades C, D, and E are restricted to the coastal region in Brazil north of the Tropic of Capricorn (Fig. 8). They grow on rocky substrates in various open, mostly montane habitats (*Vanhouttea* spp. and *Paliavana* spp.) or in the understory of rain forest at low altitudes (*S. schiffneri*, *S. lindleyi*). The co-occurrence of the three habits within clade C (tuberous with annual stem, tuberous with perennial stem, non-tuberous and perennial; Fig. 4) implies multiple shifts in life history between these closely related taxa. However, low bootstrap support for relationships within clade C prevent solid conclusions about location of these shifts except between the tuberous species *S. gigantifolia* and *S. cochlearis* and their non-tuberous sister species *V. fruticulosa* (BS 87%, Fig. 4).

Conclusions—Our phylogenetic analyses, including nearly all species of Sinningieae, clearly support the monophyly of this tribe as previously suggested (Smith et al., 1997; Zimmer et al., 2002). Within Sinningeae, *Paliavana* and *Vanhouttea* were found to be paraphyletic and nested in *Sinningia*. Based on this result, we first propose to recircumscribe *Sinningia* with the inclusion of *Paliavana* and *Vanhouttea*. Second, the five clades *Dircaea*, *Corytholoma*, *Sinningia*, ''free calyx lobes Vanhouttea,'' and *Thamnoligeria* might be considered at subgenus ranks in future formal proposals for nomenclatural changes. However, we do not exclude further modifications in the circumscription of the *Sinningia* lineage because the relationships among these highly diversified species are only poorly supported. Mapping pollination syndromes on cladograms indicates multiple shifts between bee and hummingbirds syndromes. The phylogenetic hypotheses support also two distinct origins of the bat syndrome, and a change from hummingbird to moth syndromes. These latter shifts in pollination syndromes involved not only rapid changes in flower color and morphology, but also the acquisitions of chemical features related with nectar sugar composition and floral fragrance. Perennial tubers were derived from perennial stems in non-tuberous plants. Multiple acquisitions of this storage organ were generally associated with a cyclic annual growth, but reversions to perennial stems in tuberous plants were also recorded. Finally, this study shows a higher substitution rate in the nuclear introns of *ncpGS* than in plastid non-coding regions and confirms the utility of this gene to resolve phylogenetic relationships at the species level.

LITERATURE CITED

ALLARD, M. W., AND J. M. CARPENTER. 1996. On weighting and congruence. *Cladistics* 12: 183–198.

ARMBRUSTER, W. S. 1993. Evolution of plant pollination systems: hypotheses and tests with the neotropical vine *Dalechampia*. *Evolution* 47: 1480–1505.

BAILEY, C. D., AND J. J. DOYLE. 1999. Potential phylogenetic utility of the low-copy nuclear gene *pistillata* in dicotyledonous plants: comparision to nrDNA ITS and *trnL* intron in *Sphaerocardamum* and other Brassicaceae. *Molecular Phylogenetics and Evolution* 13: 20–30.

BARRACLOUGH, T. G., J. E. HOGAN, AND A. P. VOGLER. 1999. Testing whether ecological factors promote cladogenesis in a group of tiger beetles (Coleoptera: Cicindelidae). *Proceedings of the Royal Society London B* 266: 1061–1067.

BARRACLOUGH, T. G., AND S. NEE. 2001. Phylogenetics and speciation. *Trends in Ecology and Evolution* 16: 391–399.

BARRIEL, V. 1994. Molecular phylogenies and how to code insertion/deletion events. *Compte Rendu de l'Académie des Science de la Vie/Life Science* 317: 693–701.

BOGGAN, J. K. 1991. A morphological study and cladistic analysis of *Sinningia* and associated genera with particular reference to *Lembocarpus*, *Lietzia*, *Paliavana* and *Vanhouttea* (Gesneriaceae: Gloxinieae). M.S. thesis, Cornell University, Ithaca, New York, USA.

BÖHLE, U.-R., H. H. HILGER, AND W. F. MARTIN. 1996. Island colonization and evolution of the insular woody habit in *Echium* L. (Boraginaceae). *Proceedings of the National Academy of Sciences, USA* 93: 11 740–11 745.

BOLTEN, A. B., AND P. FEINSINGER. 1978. Why do hummingbird flowers secrete dilute nectar? *Biotropica* 10: 307–308.

BRADSHAW, H. D., S. M. WILBERT, K. G. OTTO, AND D. W. SCHEMSKE. 1995. Genetic mapping of floral traits associated with reproduction isolation in monkeyflowers (*Mimulus*). *Nature* 376: 762–765.

BUZATO, S., M. SAZIMA, AND I. SAZIMA. 2000. Hummingbird-pollinated floras at tree Atlantic forest sites. *Biotropica* 32: 824–841.

CHAUTEMS, A. 1990. Taxonomic revision of *Sinningia* Nees: nomenclatural changes and new synonymies. *Candollea* 45: 381–388.

CHAUTEMS, A. 1991. Taxonomic revision of *Sinningia* Nees (Gesneriaceae) II: new species from Brazil. *Candollea* 46: 411–425.

CHAUTEMS, A. 1995. Taxonomic revision of *Sinningia* Nees (Gesneriaceae) III: new species from Brazil and new combinations. *Gesneriana* 1: 8–14.

CHAUTEMS, A. 2002. New Gesneriaceae from Minas Gerais, Brazil. *Candollea* 52: 261–279.

CHAUTEMS, A., G. S. BARACHO, AND J. A. SIQUEIRA FILHO. 2000. A new species of *Sinningia* (Gesneriaceae) from northeastern Brazil. *Brittonia* 52: 49–53.

CHAUTEMS, A., AND A. WEBER. 1999. Shoot and inflorescence architecture in the neotropical genus *Sinningia* (Gesneriaceae). *In* M. H. Kurmann and A. R. Hemsley [eds.], The evolution of plant architecture, 305–322. Royal Botanic Gardens, Kew, UK.

CHITTKA, L., J. SPAETHE, A. SCHMIDT, AND A. HICKELSBERGER. 2001. Adaptation, constraint, and chance in the evolution of flower color and pollinator color vision. *In* L. Chittka and J. D. Thomson [eds.], Cognitive ecology of pollination, animal behavior and floral evolution, 106–126. Cambridge University Press, Cambridge, UK.

CITERNE, H. L., M. MÖLLER, AND Q. C. B. CRONK. 2000. Diversity of *cycloidea*-like genes in Gesneriaceae in relation to floral symmetry. *Annals of Botany* 86: 167–176.

CLAYBERG, C. D. 1967. Chromosome numbers in *Sinningia* and *Rechteineria* (Gesneriaceae). *Baileya* 15: 33–35.

CLAYBERG, C. D. 1968. Biosystematic studies in *Sinningia* and *Rechsteineria* (Gesneriaceae). *American Journal of Botany* 55: 829–833.

CLAYBERG, C. D. 1970. Cytology of interspecific hybrids in *Sinningia* and *Rechsteineria* (Gesneriaceae). *Canadian Journal of Genetics and Cytology* 12: 759–768.

CLAYBERG, C. D. 1996. Interspecific hybridisation in *Sinningia* (Gesneriaceae). *Baileya* 23: 184–194.

CLEGG, M. T., B. S. GAUT, G. H. LEARN, JR., AND B. R. MORTON. 1994. Rates and patterns of chloroplast DNA evolution. *Proceedings of the National Academy of Sciences, USA* 91: 6795–6801.

CSKE, L., N. DUDAREVA, AND E. PICHERSKY. 1998. Structure and evolution of linalool synthase. *Molecular Biology and Evolution* 15: 1491–1498.

CUBAS, P., C. VINCENT, AND E. COEN. 1999. An epigenetic mutation responsible for natural variation in floral symmetry. *Nature* 401: 157–161.

DECAISNE, J. 1848. Gesneriaceae. *Revue Horticole* 20: 461–469.

DENDUANGBORIPANT, J., AND Q. C. B. CRONK. 2000. High intraindividual variation in internal transcribed spacer sequences in *Aeschynanthus* (Gesneriaceae): implication for phylogenetics. *Proceedings of the Royal Society London B* 267: 1407–1415.

DOYLE, J. J. 1992. Gene trees and species trees: molecular systematics as one-character taxonomy. *Systematic Botany* 17: 144–163.

DOYLE, J. J., AND J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

DUDAREVA, N., L. CSEKE, V. M. BLANC, AND E. PICHERSKY. 1996. Evolution of floral scent in *Clarkia*: novel patterns of S-linalool synthase gene expression in the *C. breweri* flower. *Plant Cell* 8: 1137–1148.

EMSHWILLER, E., AND J. J. DOYLE. 1999. Chloroplast-expressed glutamine synthetase (*ncpGS*): potential utility for phylogenetic studies with an example from *Oxalis* (Oxalidaceae). *Molecular Phylogenetics and Evolution* 12: 310–319.

ENDRESS, P. K. 1994. Diversity and evolutionary biology of tropical flowers. Cambridge University Press, Cambridge, UK.

FARRIS, J. S., M. KÄLLERSJÖ, A. G. KLUGE, AND C. BULT. 1995. Testing significance of incongruence. *Cladistics* 10: 315–319.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.

FRITSCH, K. 1894. Gesneriaceae. *In* A. Engler and K. Prantl [eds.], Die natürlichen Pflanzenfamilien, vol. 4, 145–185. Wilhelm Engelmann, Leipzig, Germany.

FRITSCH, K. 1908. Gesneriaceae. *In* Ergebnisse der botanischen Expedition der kaiserlichen Akademie der Wissenschaften nach Südbrasilien 1901. *Denkschriften der Kaiserlichen Akademie Wissenschaften, Wien. Mathematich-naturwissenschatliche Klasse* 79: 286–292.

GIELLY, L., AND P. TABERLET. 1994. The use of chloroplast DNA to resolve plant phylogenies: noncoding versus *rbcL* sequences. *Molecular Biology and Evolution* 11: 769–777.

GIVNISH, T. J. 1997. Adaptative radiation and molecular systematics: issues and approaches. *In* T. J. Givnish and K. J. Sytsma [eds.], Molecular evolution and adaptive radiation, 1–54. Cambridge University Press, Cambridge, USA.

GIVNISH, T. J., T. M. EVANS, M. L. ZJHRA, T. B. PATTERSON, P. E. BERRY, AND K. H. SYTSMA. 2000. Molecular evolution, adaptative radiation, and geographic diversification in the amphiatlantic family Rapateaceae: evidence from *ndhF* sequences and morphology. *Evolution* 54: 1915–1937.

GOLDMAN, N., J. P. ANDERSON, AND A. G. RODRIGO. 2000. Likelihood-based tests of topologies in phylogenetics. *Systematic Biology* 49: 652–670.

GRANT, V. 1992. Floral isolation between ornithophilous and sphingophilous species of *Ipomopsis* and *Aquilegia*. *Proceedings of the National Academy of Sciences, USA* 89: 11 828–11 831.

GRANT, V. 1994. Historical development of ornithophily in the western North America flora. *Proceedings of the National Academy of Sciences, USA* 91: 10 407–10 411.

HAMILTON, M. B. 1999. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology* 8: 521–523.

HANSTEIN, J. 1864. Gesneraceae. *In* C. F. P. Martius [ed.], Flora Brasiliensis, vol. 8, 341–428. F. Fleischer, Leipzig, Germany.

HARDIG, T. M., P. S. SOLTIS, AND D. E. SOLTIS. 2000. Diversification of the north American shrub genus *Ceanothus* (Rhamnaceae): conflicting phylogenies from nuclear ribosomal DNA and chloroplast DNA. *American Journal of Botany* 87: 108–123.

HARRISON, C. J., M. MOELLER, AND Q. C. B. CRONK. 1999. Evolution and development of floral diversity in *Streptocarpus* and *Saintpaulia*. *Annals of Botany* 84: 49–60.

HARVEY, P. H., AND A. PURVIS. 1991. Comparative methods for explaining adaptations. *Nature* 351: 619–624.

HASEGAWA, M., H. KISHINO, AND T. YANO. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 21: 160–174.

HEINRICH, B., AND P. H. RAVEN. 1972. Energetics and pollination ecology. *Science* 176: 597–176.

HEYNEMAN, A. J. 1983. Optimal sugar concentration of floral nectars: dependence on sugar intake efficiency and foraging costs. *Oecologia* 60: 198–213.

JOHNSON, L. A., AND D. E. SOLTIS. 1998. Assessing congruence: empirical examples from molecular data. *In* D. E. Soltis, P. S. Soltis, and J. J. Doyle [eds.], Molecular systematic of plants II: DNA sequencing, 265–296. Kluwer, Norwell, Massachusetts, USA.

JORDAN, W., M. W. COURTNEY, AND J. E. NEIGEL. 1996. Low levels of intraspecific genetic variation at a rapidly evolving chloroplast DNA locus in North America duckweeds (Lemnaceae). *American Journal of Botany* 83: 430–439.

KELCHNER, S. A. 2000. The evolution of non-coding chloroplast DNA and its application in plant systematics. *Annals of the Missouri Botanical Garden* 87: 482–498.

KELCHNER, S. A., AND L. G. CLARK. 1997. Molecular evolution and phylogenetic utility of the chloroplast *rpl16* intron in Chusquea and Bambusoideae (Poaceae). *Molecular Phylogenetics and Evolution* 8: 385–397.

KINGSOLVER, J. G., AND T. L. DANIEL. 1983. Mechanical determinants of nectar feeding strategy in hummingbirds: energetics, tongue morphology, and licking behavior. *Oecologia* 60: 214–226.

KITA, Y., AND M. ITO. 2000. Nuclear ribosomal ITS sequences and phylogeny in East Asian *Aconitum* subgenus *Aconitum* (Ranunculaceae), with special reference to extensive polymorphism in individual plants. *Plant Systematic and Evolution* 225: 1–13.

KLUGE, A. G. 1989. A concern for evidence and a phylogenetic hypothesis of relationships among *Epicrates* (Boidae, Serpentes). *Systematic Zoology* 38: 7–25.

KNUDSEN, J. T., AND L. TOLLSTEN. 1993. Trends in floral scent chemistry in pollination syndromes: floral scent composition in moth-pollinated taxa. *Botanical Journal of the Linnean Society* 113: 263–284.

KNUDSEN, J. T., AND L. TOLLSTEN. 1995. Floral scent in bat-pollinated plants: a case of convergent evolution. *Botanical Journal of the Linnean Society* 119: 45–57.

KUHSEL, M. G., R. STRICKLAND, AND J. D. PALMER. 1990. An ancient group I intron shared by Eubacteria and chloroplasts. *Science* 250: 1570–1573.

LARSON, A., AND J. B. LOSOS. 1996. Phylogenetic systematics of adaptation. *In* M. R. Rose and G. V. Lauder [eds.], Adaptation, 187–220. Academic Press, San Diego, California, USA.

LEVINSON, G., AND G. A. GUTMAN. 1987. Slipped-strand mispairing: a major

mechanism for DNA sequence evolution. *Molecular Biology and Evolution* 4: 203–221.

LUO, D., R. CARPENTER, C. VINCENT, L. COPSEY, AND E. COEN. 1996. Origin of floral asymmetry in *Antirrhinum*. *Nature* 383: 794–799.

MACDADE, L. A., AND M. L. MOODY. 1999. Phylogenetic relationships among Acanthaceae: evidence from noncoding *trnL-trnF* chloroplast DNA sequences. *American Journal of Botany* 86: 70–80.

MADDISON, W. P. 1997. Gene trees in species trees. *Sytematic Biology* 46: 523–536.

MADDISON, W. P., AND D. R. MADDISON. 1992. MacClade, version 3.01. Sinauer, Sunderland, Massachusetts, USA.

MANEN, J.-F., AND A. NATALI. 1995. Comparison of the evolution of ribulose-1, 5-bisphospate carboxylase (*rbcL*) and the *atpB-rbcL* spacer sequences in a recent plant-group, the tribe Rubieae (Rubiaceae). *Journal of Molecular Evolution* 41: 920–927.

MANEN, J.-F., A. NATALI, AND F. EHRENDORFER. 1994a. Phylogeny of Rubiaceae: Rubieae inferred from the sequence of cpDNA intergene region. *Plant Systematic and Evolution* 190: 195–211.

MANEN, J.-F., V. SAVOLAINEN, AND P. SIMON. 1994b. The *atpB* and *rbcL* promoters in plastid DNAs of a wide dicot range. *Journal of Molecular Evolution* 38: 577–582.

MASON-GAMER, R. J., AND E. A. KELLOGG. 1996. Testing for phylogenetic conflict among molecular data sets in the tribe Triticeae (Gramineae). *Systematic Biology* 45: 524–545.

MCNEILL, J. 2000. Naming the groups: developing a stable and efficient nomenclature. *Taxon* 49: 705–720.

MEIRELLES, S. T., V. R. PIVELLO, AND C. A. JOLY. 1999. The vegetation of granite rock outcrops in Rio de Janeiro, Brazil, and the need for its protection. *Environmental Conservation* 26: 10–20.

MÖLLER, M., AND Q. C. B. CRONK. 2001. Evolution of morphological novelty: a phylogenetic analysis of growth patterns in *Streptocarpus* (Gesneriaceae). *Evolution* 55: 918–929.

MOORE, H. E. 1973. Comments on cultivated Gesneriaceae. *Baileya* 19: 35–41.

NIXON, K. C., AND J. M. CARPENTER. 1996. On simultaneous analysis. *Cladistics* 12: 221–241.

PERRET, M., A. CHAUTEMS, R. SPICHIGER, M. PEIXOTO, AND V. SAVOLAINEN. 2001. Nectar sugar composition in relation to pollination syndrome in Sinningieae (Gesneriaceae). *Annals of Botany* 87: 267–273.

PROCTOR, M., P. YEO, AND A. LACK. 1996. The natural history of pollination. Timber Press, Portland, Oregon, USA.

PYKE, G. H., AND N. M. WASER. 1981. The production of diluted nectar by hummingbird and honeyeater flowers. *Biotropica* 13: 260–270.

RAGUSO, R. A., AND E. PICHERSKY. 1999. A day in the life of a linalool molecule: chemical communication in plant–pollinator system. Part 1: Linalool biosynthesis in flowering plants. *Plant Species Biology* 14: 95–120.

RANDALL, L. S., J. A. RYBURN, R. C. CRONN, T. SEELANAN, AND J. F. WENDEL. 1998. The tortoise and the hare: choosing between noncoding plastome and nuclear *Adh* sequences for phylogeny reconstruction in a recently diverged plant group. *American Journal of Botany* 85: 1301–1315.

RAVEN, P. H. 1972. Why are bird-visited flowers predominantly red? *Evolution* 26: 674.

REEVES, P. A., AND R. G. OLMSTED. 1998. Evolution of novel morphological and reproductive traits in a clade containing *Antirrhinum majus* (Scrophulariaceae). *American Journal of Botany* 85: 1047–1056.

RIESEBERG, L. H. 1991. Homoploid reticulate evolution in Helianthus (Asteraceae): evidence from ribosomal genes. *American Journal of Botany* 78: 1218–1237.

RIESEBERG, L. H., S. M. BECKSTROM-STERNBERG, A. LISTON, AND D. M. ARIAS. 1991. Phylogenetic and systematic inferences from chloroplast DNA and isozyme variation in *Helianthus* sect. *Helianthus* (Asteraceae). *Systematic Botany*: 50–76.

RIESEBERG, L. H., AND D. E. SOLTIS. 1991. Phylogenetic consequences of cytoplasmic gene flow in plants. *Evolutionary Trends in Plants* 5: 65–84.

SAFFORD, H. D., AND G. MARTINELLI. 2000. Southeast Brazil. *In* S. Porembski and W. Barthlott [eds.], Inselbergs: biotic diversity of isolated rock outcrops in tropical and temperate regions, 339–389. Springer-Verlag, Berlin, Germany.

SAMUEL, R., W. PINSKER, AND M. KIEHN. 1997. Phylogeny of some species of *Cyrtandra* (Gesneriaceae) inferred from *atpB/rbcL* cpDNA intergene region. *Botanica Acta* 110: 503–510.

SANG, T., D. J. CRAWFORD, AND T. F. STUESSY. 1997a. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). *American Journal of Botany* 84: 1120–1136.

SANG, T., M. J. DONOGHUE, AND D. ZHANG. 1997b. Evolution of alcohol dehydrogenase genes in Peonies (*Paeonia*): phylogenetic relationships of putative nonhybrid species. *Molecular Biology and Evolution* 14: 994–1007.

SANG, T., AND D. ZHANG. 1999. Reconstructing hybrid speciation using sequences of low copy nuclear genes: hybrid origin of five *Paeonia* species based on *Adh* gene phylogenies. *Systematic Botany* 24: 148–163.

SAVOLAINEN, V., P. CUENOUD, R. SPICHIGER, M. D. P. MARTINEZ, M. CREVECOEUR, AND J.-F. MANEN. 1995. The use of herbarium specimens in DNA phylogenetics: evaluation and improvement. *Plant Systematics and Evolution* 197: 87–98.

SAZIMA, I., S. BUZATO, AND M. SAZIMA. 1996. An assemblage of hummingbird-pollinated flowers in a montane forest in southeastern Brazil. *Botanica Acta* 109: 149–160.

SCHEMSKE, D. W., AND H. D. BRADSHAW. 1999. Pollinator preferences and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences, USA* 96: 11 910–11 915.

SEELANAN, T., A. SCHNABEL, AND J. F. WENDEL. 1997. Congruence and consensus in the cotton tribe (Malvaceae). *Systematic Botany* 22: 259–290.

SHIMODAIRA, H., AND M. HASEGAWA. 1999. Multiple comparisons of log-likelihoods with application to phylogenetic inference. *Molecular Biology and Evolution* 16: 1114–1116.

SILBERBAUER-GOTTSBERGER, I., AND G. GOTTSBERGER. 1975. Ueber sphingophile angiospermen brasiliens. *Plant Systematics and Evolution* 123: 157–184.

SILVERTOWN, J., AND M. DODD. 1997. Comparing plants and connecting traits. *In* J. Silvertown, M. Franco, and J. L. Harper [eds.], Plant life histories, 3–16. Cambridge University Press, Cambridge, UK.

SMALL, R. L., J. A. RYBURN, R. C. CRONN, T. SEELANAN, AND J. F. WENDEL. 1998. The tortoise and the hare: choosing between noncoding plastome and nuclear *Adh* sequences for phylogeny reconstruction in a recently diverged plant group. *American Journal of Botany* 85: 1301–1315.

SMITH, J. F., J. C. WOLFRAM, K. D. BROWN, C. L. CARROLL, AND D. S. DENTON. 1997. Tribal relationships in the Gesneriaceae: evidence from DNA sequences of the chloroplast gene *ndhF*. *Annals of the Missouri Botanical Garden* 84: 50–66.

SNOW, D. W., AND D. L. TEIXEIRA. 1982. Hummingbirds and their flowers in the coastal mountains of southeastern Brazil. *Journal für Ornithologie* 123: 446–450.

SOLTIS, D. E., AND R. K. KUZOFF. 1995. Discordance between nuclear and chloroplast phylogenies in the *Heuchera* group (Saxifragaceae). *Evolution* 49: 727–742.

SWOFFORD, D. L. 1999. PAUP*: phylogenetic analysis using parsimony (and other methods), version 4. Sinauer, Sunderland, Massachusetts, USA.

TABERLET, P., L. GIELLY, G. PAUTOU, AND J. BOUVET. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.

TAMM, S., AND C. L. GASS. 1986. Energy intake rates and nectar concentration preferences by hummingbirds. *Oecologia* 70: 20–23.

TISCHER, E., S. DASSARMA, AND H. M. GOODMAN. 1986. Nucleotide sequence of alfalfa glutamine synthetase gene. *Molecular General Genetics* 203: 221–229.

VASCONCELOS, M. F., AND J. A. LOMBARDI. 2000. Espécies vegetais visitadas por beija-flores durante meio do verão no Parque estadual da Pedra Azul, Esperito Santo. *Melopsittacus* 3: 36–41.

VOGEL, S. 1969. Chiropterophilie in der neotropischen flora (Neue mitteilungen II). *Flora* 158: 185–222.

WEBER, H. 1936. Vergleichend-morphologische Studien über die sprossbürtige Bewurzelung. *Nova Acta Leopoldina* 4: 227–298.

WENDEL, J. F., AND J. J. DOYLE. 1998. Phylogenetic incongruence: window into genome history and molecular evolution. *In* D. E. Soltis, P. S. Soltis, and J. J. Doyle [eds.], Molecular systematic of plants II: DNA sequencing, 265–296. Kluwer, Norwell, Massachusetts, USA.

WENDEL, J. F., J. M. D. STEWART, AND J. H. RETTIG. 1991. Molecular evidence for homoploid reticulate evolution among Australian species of *Gossypium*. *Evolution* 45: 684–711.

WIEHLER, H. 1983. A synopsis of the neotropical Gesneriaceae. *Selbyana* 6: 1–219.

WIEHLER, H. 1984. Miscellaneous new species in the Gesneriaceae. *Selbyana* 7: 328–347.

WIEHLER, H., AND A. CHAUTEMS. 1995. A reduction of *Lietzia* to *Sinningia*. *Gesneriana* 1: 5–7.

WHITTEMORE, A. T., AND B. A. SCHAAL. 1991. Interspecific gene flow in sympatric oaks. *Proceedings of the National Academy of Sciences, USA* 88: 2540–2544.

WOLFE, A. D., AND W. J. ELISENS. 1995. Evidence of chloroplast capture and pollen-mediated gene flow in *Penstemon* sect. *Peltanthera* (Scrophulariaceae). *Systematic Botany* 20: 395–412.

WOLFE, K. H., W.-H. LI, AND P. M. SHARP. 1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences, USA* 84: 9054–9058.

YANG, Z. 1996. Among site rate variation and its impact on phylogenetic analyses. *Trends in Ecology and Evolution* 11: 367–372.

YODER, A. D., J. A. IRWIN, AND B. A. PAYSEUR. 2001. Failure of the ILD to determine data combinability for slow lorise phylogeny. *Systematic Biology* 50: 408–424.

ZIMMER, E. A., E. H. ROALSON, L. E. SKOG, J. K. BOGGAN, AND A. IDNURM. 2002. Phylogenetic relationships in the Gesnerioideae (Gesneriaceae) based on nrDNA ITS and cpDNA *trnL-F* and *trnE-T* spacer region sequences. *American Journal of Botany* 89: 296–311.

ZURAWSKI, G., AND M. T. CLEGG. 1987. Evolution of higher-plant chloroplast DNA-encoded genes: implication for structure-function and phylogenetic studies. *Annual Review in Plant Physiology* 38: 391–418.