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Shrinking the Violets: Phylogenetic Relationships of Infrageneric Groups in *Viola* (Violaceae) Based on Internal Transcribed Spacer DNA Sequences

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ABSTRACT. A phylogenetic study of the genus *Viola* used internal transcribed spacer (ITS) DNA sequences for 44 taxa representing many infrageneric groups in *Viola* plus outgroups *Hybanthus concolor* and *Noisettia orchidiflora*. Parsimony and maximum likelihood approaches place Latin American sections basal in *Viola*, supporting an Andean origin for the genus. Groups of sect. *Chamaemelanium*, mostly stemmed and yellow-flowered with $x = 6$ chromosomes, intermingle with groups of sect. *Nomimium* that are stemless and white- or blue-flowered with $x = 12$ or an aneuploid number. Neither section is monophyletic, and the assemblage forms a weak clade or grade, depending on the analysis. The remaining sect. *Nomimium* groups with primarily blue flowers and $x = 10$ and aneuploid or polyploid numbers form a clade including Hawaiian sect. *Nosphinium*, with pansies of sect. *Melanium* (typically stemmed with multicolored flowers and $x = 5$ to 17) at the base. Phylogenetic relationships from ITS data herald the need for drastic remodeling of *Viola*. Proposed changes include splitting sect. *Chamaemelanium* and some of sect. *Nomimium* into several sections, transferring the remainder of *Nomimium* to the segregate sect. *Viola*, and merging Hawaiian sect. *Nosphinium* with the amphi-Beringian *V. langsdorffii* complex under the *Langsdorffianae* in sect. *Viola*.

The Violaceae include about 25 genera (Melchior 1925; Hekking 1988) and approximately 900 species worldwide. Most genera are monotypic or contain a small number of species and are restricted to the New World or Old World tropics. By far the largest genus is *Viola*, with 525–600 species (Clausen 1964; Ballard 1996) and an extensive north-temperate distribution that belies the otherwise tropical affinities of the family. This genus is distributed throughout most frost-free regions of the world, ranging widely across temperate habitats of the Northern Hemisphere and into higher elevations of mountain systems near the equator and in the Southern Hemisphere. Primary centers of morphological and taxonomic diversity reside in the Alps and Mediterranean region, the Himalayas and mountainous eastern Asia, and the South American Andes and Patagonia. Secondary centers are the Pacific Coastal region of the United States, the Appalachian temperate forests and Atlantic Coastal Plain of the eastern United States, and the mountains of central and northern Mexico.

German monographer Wilhelm Becker was the first taxonomist in this century to study *Viola* on a worldwide scale, leaving behind a prodigious legacy of new species, regional treatments and taxonomic synopses (in particular Becker 1916, 1917a, 1917b, 1918, 1922, 1923a, 1923b, 1923c, 1923d, 1924, 1925a). His (1925b) conspectus of the genus provided the first comprehensive infrageneric reappraisal of *Viola* worldwide, recognizing 14 sections and approximately twice as many infrasectional groups (Table 1). Clausen (1927, 1929, 1931, 1964) later proposed extensive taxonomic revisions to Becker's classification, many of which have been adopted by subsequent specialists while others have remained in dispute. Bamford and Gershoy (1930), Gershoy (1934), and Yuzepchuk and Klokov (1974), as well as numerous other regional specialists, made additional modifications, primarily nomenclatural in nature. A graphical summary of Becker's classification and subsequent major modifications is presented in the "wire diagram" of Fig. 1.

Clausen segregated primarily stemless groups with a base number of $x = 12$ or presumed aneuploid derivatives into sect. *Plagiostigma*, leaving a much reduced sect. *Nomimium* (= Clausen's invalid sect. *Rostellatae*) consisting of groups with $x = 10$. He elevated the predominately stemmed and yellow-flowered groups of sect. *Chamaemelanium* from series to subsections and splintered sect. *Nuttallianae* into a number of subsections. While some

TABLE 1. Voucher information for Violaceae sequenced for ITS spacers, with group membership and group worldwide distribution for *Viola* taxa. Infrageneric classification of *Viola* taxa and their arrangement from putatively most primitive to most recent follows Becker (1925). Extractions from herbarium material are indicated by an asterisk (*).

INFRAGENERIC GROUP/ GEOGRAPHIC DISTRIBUTION	SPECIES/VOUCHER/(ITS1 & ITS2 GENBANK ACCESSION #)
Outgroup Taxa	
Hybanthus Jacq.	*Hybanthus concolor* (T. F. Forster) Spreng.
Worldwide, mostly tropical	USA, Michigan, 18 May 1992, *Ballard 92–013* (WIS) [AF097218, AF097264]
Noisettia Ging.	*Noisettia orchidiflora* (Rudge) Ging.*
n. South America	FRENCH GUIANA, Saül, 8 May 1986, *Mori & Pennington 17950* (MO) [AF097219, AF097265]
Genus *Viola* Tourn, ex L.	
Sect. *Chilenium* W. Becker	*V. reichei* Skottsberg*
sw. South America	CHILE, Prov. Ñuble, 11 Dec 1987, *Rechinger & Rechinger 64271* (M) [AF097223, AF097269]
Sect. *Andinium* W. Becker	
Annuae Reiche	*V. micranthella* Wedd.*
w. South America	PERU, Prov. Huaylas, 4 May 1987, *Mostacero, Leiva, Mejia, Peláez, Medina & Zelada 1959* (F) [AF097222, AF097268]
Sect. *Rubellium* W. Becker	*V. capillaris* Pers.*
Chile	CHILE, Prov. Concepción, 22 Oct. 1990, *Lammers, Baeza, Peñailillo & Mazzeo 7522* (F) [AF097220, AF097266]
Sect. *Nosphinium* W. Becker	*V. chamissoniana* Ging. subsp. *tracheliifolia* (Ging.) W. L. Wagner, Herbst & Sohmer
Hawaiian Islands	USA, Hawaii, 16 Jun 1993, *Nepokroeff 774* (WIS) [AF097261, AF097307]
	V. helenae C. Forbes & Lydgate
	USA, Hawaii, 30 Jun 1993, *Perlman s.n.* (NTBG) [AF097260, AF097306]
	V. kauaensis A. Gray
	USA, Hawaii, [no date] *Nepokroeff 786b* (WIS) [AF097262, AF097308]
	V. maviensis H. Mann
	USA, Hawaii, 15 Jul 1995, *Nepokroeff 913 & Lau* (WIS) [AF097263, AF097309]
Sect. *Leptidium* Ging.	*V. scandens* Willd. Ex Roem. & Schult.
Mesoamerica, Antilles, South America	COSTA RICA, 10°08'N, 84°09'30"W, 5 Jan 1994, *Ballard 94–001, Wetter & Baker* (WIS) [AF097221, AF097267]
Sect. *Melanium* Ging.	
Crenatifoliae W. Becker	*V. arvensis* Murray
Europe, w. Asia	USA, Maryland, 1993, *Hahn s.n.* (WIS) [AF097242, AF097288]
	V. calcarata L.*
	ITALY, Ligurian Alps, 5 Jun 1993, *Conti s.n.* (WIS) [AF097243, AF097289]
Sect. *Chamaemelanium* Ging.	*V. barroetana* Hemsley
Barroetanae W. Becker	
Mexico	MEXICO, Edo. Durango, 1 July 1993, *Ballard s.n.* (WIS) [AF097224, AF097270]
Nuttallianae W. Becker	*V. praemorsa* Kellogg*
w. North America, Baja California	USA, Nevada, 23 May 1987, *Tiehm 11076* (WIS) [AF097228, AF097274]
	V. purpurea Kellogg
	USA, Utah, 8 May 1995, *Ballard s.n.* (WIS) [AF097229, AF097275]
	V. vallicola A. Nelson
	USA, Utah, 7 May 1995, *Ballard s.n.* (WIS) [AF097230, AF097276]
Chrysanthae W. Becker	*V. beckwithii* T. & G.
w. North America	USA, Utah, 7 May 1995, *Ballard s.n.* (WIS) [AF097227, AF097273]
	V. sheltonii Torr.*
	USA, California, 2 Jul 1994, *Bartholomew 6787* (WIS) [AF097226, AF097272]
Erectae W. Becker	
Nudicaules W. Becker	*V. pubescens* Aiton
e. Asia, North America	USA, Michigan, 18 May 1992, *Ballard 92–011* (WIS) [AF097225, AF097271]
Canadenses W. Becker	*V. canadensis* L. ("NC")*
North America, n. Mexico	USA, North Carolina, 18 Apr 1967, *Greenlee 240* (WIS) [AF097231, AF097277]
	V. canadensis L. ("NM")
	USA, New Mexico, 2 May 1995, *Ballard & Meloche s.n.* (WIS) [AF097232, AF097278]
	V. cuneata S. Watson*
	USA, Oregon, 18 May 1942, *Constance & Rollins 2975* (US) [AF097234, AF097280]
Flagelliformes W. Becker	*V. flagelliformis* Hemsley*
Mexico	MEXICO, Edo. San Luis Potosi, 2 Dec 1983, *Fernández & Acosta 2041* (NY) [AF097233, AF097279]

TABLE 1. Continued.

INFRAGENERIC GROUP/ GEOGRAPHIC DISTRIBUTION	SPECIES/VOUCHER/(ITS1 & ITS2 GENBANK ACCESSION #)
Sect. *Nomimium* Ging.	
Uncinatae Kupffer	
Flagellatae Kittel	*V. odorata* L. ("GDN")
Europe, w. Asia	USA, Wisconsin, Apr 1993, *Ballard s.n.* (WIS) [AF097249, AF097295]
	V. odorata L. ("GY")
	GERMANY, Mainz region, 20 May 1995, *Ballard s.n.* (WIS) [AF097250, AF097296]
Rostratae Kupffer	
Rosulantes Borbas	*V. reichenbachiana* Boreau
Eurasia, North America	ITALY, Maritime Alps, 16 Jun 1993, *Conti s.n.* (WIS) [AF097248, AF097294]
	V. striata Aiton
	USA, Michigan, 18 May 1992, *Ballard 92–010* (WIS) [AF097247, AF097293]
Arosulatae Borbas	*V. elatior* Fries
Eurasia	GERMANY, Mainz region, 20 May 1995, *Ballard s.n.* (WIS) [AF097246, AF097292]
Stolonosae Kupffer	*V. blanda* Willd. var. *palustriformis* A. Gray*
Eurasia, North America to n. South America	USA, Minnesota, Jun 1993, *Ballard s.n.* (WIS) [AF097238, AF097284]
	V. domingensis Urban*
	DOMINICAN REPUBLIC, La Nevera, 3–5 Apr 1971, *Liogier 17983* (F) [AF097237, AF097283]
	V. jalapaensis W. Becker
	MEXICO, Edo. Veracruz, 21 Jun 1993, *Ballard s.n.* (WIS) [AF097235, AF097281]
	V. macloskeyi Lloyd ssp. *pallens* (Ging.) M. S. Baker
	USA, Michigan, 17 May 1992, *Ballard 92–005* (WIS) [AF097236, AF097282]
Adnatae W. Becker	*V. pinnata* L.
Eurasia, North America	USA, Ex horto Robert Faden of Smithsonian Institution, 1995, *Ballard s.n.* (WIS) [AF097240, AF097286]
Langsdorffianae W. Becker	*V. langsdorffii* Ging.*
e. Asia, w. North America	USA, Alaska, 20 July 1975, *Craighead s.n.* (WIS) [AF097259, AF097305]
Diffusae W. Becker	*V. nagasawai* Makino & Hayata*
s. Asia, Malaysia	Taiwan, Taipei Co., 12 Mar 1994, *Wang & Lin 9056* (WIS) [AF097239, AF097285]
Boreali-Americanae W. Becker	*V. affinis* Leconte
North America, n. Mexico	USA, Michigan, 17 May 1992, *Ballard 92–006* (WIS) [AF097251, AF097297]
	V. clauseniana M. S. Baker
	USA, Utah, 20–30 Apr 1994, *Welsh & Sidles s.n.* (WIS) [AF097254, AF097300]
	V. cucullata Aiton
	USA, Pennsylvania, May 1992, *Wiegman 92HB008* (Wiegman) [AF097252, AF097298]
Pedatae Pollard	*V. pedata* L.
e. North America	USA, Michigan, 17 May 1992, *Ballard 92–007a* (WIS) [AF097253, AF097299]
Orbiculares Pollard	*V. rotundifolia* Michx.
North America	USA, Pennsylvania, May 1992, *Wiegman 92HB004* (Wiegman) [AF097241, AF097287]
Mexicanae W. Becker	*V. sp. nov.* A*
Mexico, Central America	GUATEMALA, Departamento de Huehuetenango, 30 Jul 1965, Roe, *Roe & Mori 657* (WIS) [AF097256, AF097302]
	V. hemsleyana Calderón
	MEXICO, Edo. Veracruz, 21 Jun 1993, *Ballard s.n.* (WIS) [AF097258, AF097304]
	V. hookeriana HBK.
	MEXICO, Edo. Durango, 30 Jun 1993, *Ballard s.n.* (WIS) [AF097257, AF097303]
	V. nannei Polak.
	COSTA RICA, 9°57'N, 83°51'W, 10 Jan 1994, *Ballard 94–025 & Baker* (WIS) [AF097255, AF097301]
Umbraticolae W. Becker	*V. sp. nov.* B*
Mexico, sw. United States	MEXICO, Edo. Chihuahua, 15 Apr 1984, *Spellenberg & Soreng 7709* (US) [AF097245, AF097291]
	V. umbraticola HBK.
	MEXICO, Edo. Durango, 30 Jun 1993, *Ballard s.n.* (WIS) [AF097244, AF097290]

specialists have maintained Becker's sect. *Dischidium* and retained subsect. *Orbiculares* in sect. *Nomimium*, Clausen merged sect. *Dischidium* and the *Orbiculares* and subordinated this combined taxon under sect. *Chamaemelanium* under the invalidly published subsect. *Biflorae*. Clausen and other specialists have held widely divergent opinions on the circumscription and rank of Becker's

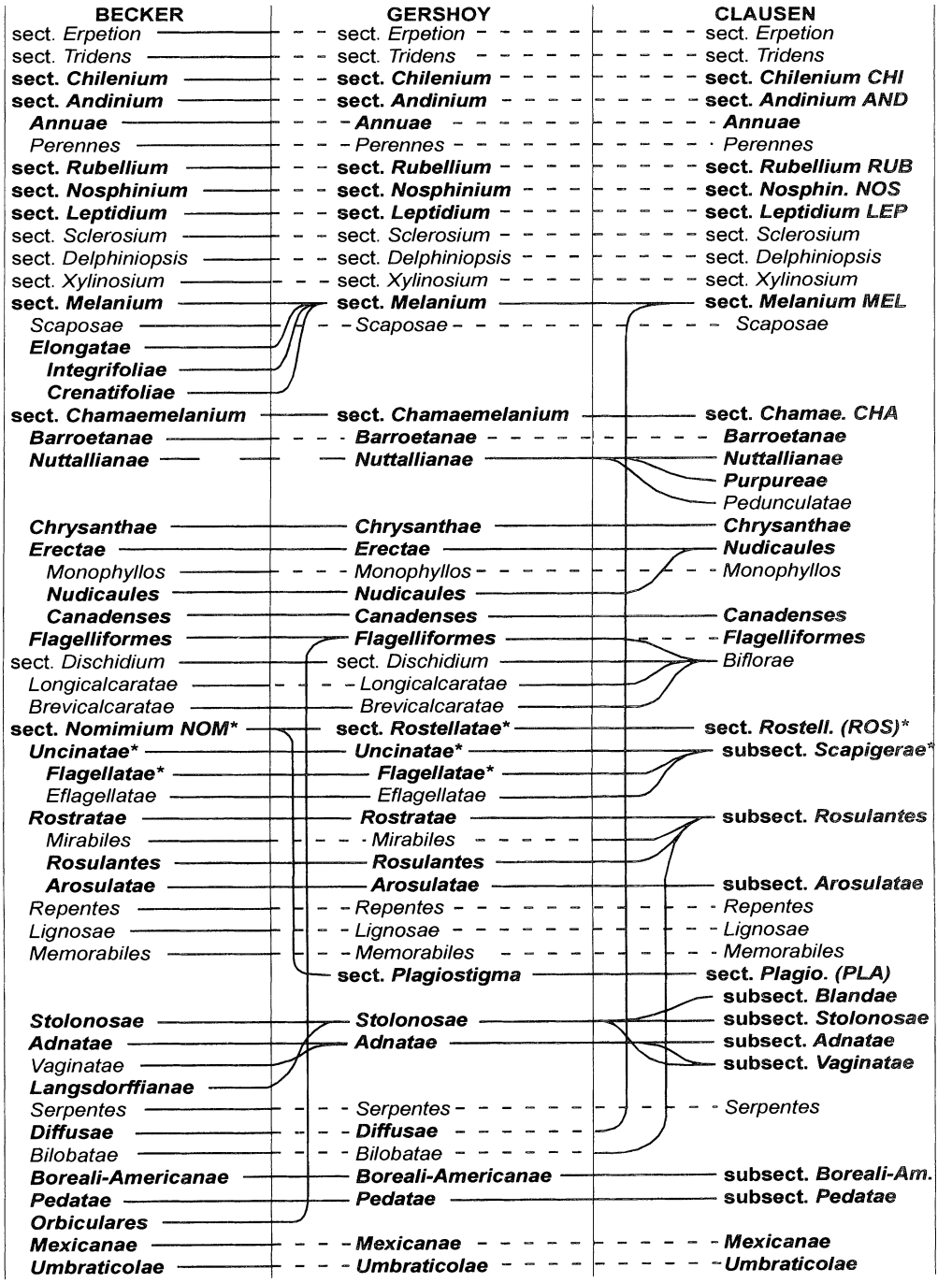

FIG. 1. "Wire" diagram illustrating major taxonomic differences between three previously published infrageneric classifications of *Viola* (note that infrasectional groups recognized by Becker received no explicit assignment of rank). A dashed line flanking a group in the second or third column indicates that the specialist did not deal explicitly with that group (hence no recommended change). Merging lines denote lumping of two or more infrageneric groups into one by a specialist, while splitting lines denote segregation into two or more groups. Groups represented in the present study are in boldface type.

Adnatae, *Diffusae*, *Langsdorffianae*, *Stolonosae* and *Vaginatae* groups.

Although some studies since Becker's alpha-taxonomic investigations have clarified the delimitation and taxonomic composition of certain groups (e.g., Nieuwland and Kaczmarek 1914; Brainerd 1921; Clausen 1927, 1929, 1964; Gershoy 1928, 1934; Holm 1932; and Valentine 1962), most have failed to provide much beyond a generalized quasi-phenetic sense of relationships in the genus. Nearly all have relied on general trends in a few morphological features and have utilized small numbers of chromosome counts representing a few groups, typically western European and North American ones. Reevaluation of previously recognized infrageneric groups in *Viola* demands a new and independent set of data, ideally one amenable to rigorous cladistic analysis and permitting testing of explicit hypotheses of relationship.

In many recent studies, the Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA has proven sufficiently variable and phylogenetically informative to give information on relationships among species and among closely related genera (see reviews by Baldwin et al. 1995; Sytsma and Hahn 1996). Sequence data from the ITS region have been used in several instances to clarify potential morphological parallelisms or the placement of aberrant taxa implicated in groups having undergone adaptive radiation (Nepokroeff and Sytsma 1998, and Nepokroeff et al., in press; and papers in Givnish and Sytsma 1997). In many cases phylogenetic relationships derived from ITS sequence data have shown strong agreement with relationships indicated by different kinds of traditional systematic data. In certain instances, however, ITS data have yielded novel and unexpected relationships never before suggested, especially in groups potentially subjected to adaptive radiation or convergent evolution (Baldwin et al. 1995).

A phylogenetic investigation of the genus *Viola* was undertaken using ITS sequence data to generate phylogenetic hypotheses of relationship. Taxa for the ingroup represented most previously recognized infrageneric groups in the genus. Phylogenetic analyses using different algorithms with different underlying assumptions were utilized. The primary objectives of the study were to produce a well supported infrageneric phylogeny, to reevaluate the composition and relationships of discernible groups in *Viola*, and to propose tentative realignments in the genus based on these phylogenetic relationships. Additional aims were to evaluate the taxonomic utility of the ITS region in *Viola*, resolve the placement of controversial groups (e.g., the stemless yellow-flowered *Orbiculares*) and aberrant ones (e.g., woody-stemmed, purportedly "ancient" Hawaiian sect. *Nosphinium*), and to test the monophyly of morphologically heterogeneous groups (e.g., Hawaiian sect. *Nosphinium* and the *Mexicanae*).

Materials and Methods

Taxon Sampling and DNA Extraction. DNA extractions of the 44 samples were prepared from fresh, frozen and silica gel-dried leaf tissue of single individuals (Table 1). Sixteen extractions came from herbarium specimens ranging in collection date from 1942 to 1995. Approximately two-thirds of the extractions (especially those from fresh and frozen leaf tissue) were made using a modified 6% CTAB method (Doyle and Doyle 1987; Smith et al. 1991); the remainder were made with an SDS "mini-extraction" protocol (Edwards et al. 1991) followed by the chloroform-isoamyl alcohol extraction, alcohol precipitations and acetate salt rinses of the CTAB method scaled down in volume for 1.5 ml plastic tubes. Although amplification generally provided greater quantities of product from recently collected material, herbarium tissue extractions yielded sufficient amplified product for sequencing almost as readily as fresh or silica-gel dried tissue in many groups, especially those of sect. *Chamaemelanium* and the South American sections.

Sequences for the ITS region were obtained for 42 species of *Viola* representing the eight most widespread and taxonomically diverse of 14 sections recognized by Becker (1925b), plus an additional population each for *V. canadensis* and *V. odorata* to assess levels of intraspecific variation in ITS (Table 1). Two to four species spanning the range of morphological and cytogenetic diversity within each polytypic group were sequenced where material was available. Most species of Hawaiian sect. *Nosphinium* and all species in the *Mexicanae* group were sequenced to test the monophyly of these two morphologically heterogeneous groups. These taxa were included in preliminary analyses to confirm monophyly; for the full range of analyses and support measures, four exemplars of each group were retained in the final analyses to reduce computation time and make figures clearer.

Selection of the outgroups *Hybanthus concolor* (T. F. Forster) Sprengel and *Noisettia orchidiflora* (Rudge) Ging. (nearest to *Viola*) was based on pre-

liminary results from a study of *rbcL* sequence data for the Violaceae in progress (Hodges et al. 1995).

Amplification and Sequencing of ITS. The polymerase chain reaction (PCR, Mullis et al. 1986) was used to amplify ITS for sequencing using primers ITS leu1 (Bruce Baldwin, pers. comm.) and ITS4 (White et al. 1990). Reaction constituents included those of Baum et al. (1994) scaled down to 50 µl reactions, with the addition of 10 µl $MgCl_2$ (20 mM), 1 µl bovine serum albumin (4 µg/µl) and 0.75–2.5 µl undiluted DNA template. Thermal cycler conditions followed Baum et al. (1994). Successful reactions were cleaned with a QIAquick spin-column PCR purification kit (QIAgen) and quantified with a fluorimeter. Samples were cycle-sequenced with dye-terminator chemistry (Applied Biosystems) using primer ITS 5 (White et al. 1990) for ITS 1 and primer ITS 3B (Baum et al. 1994) for ITS2. Reverse primers ITS 2 and ITS 4 (White et al. 1990) were used initially to obtain the complementary strand for several taxa; however, the reverse complements were identical to the forward primer sequences in all cases, and sequencing of the complementary strands was discontinued. After ethanol-sodium acetate precipitation, products were analyzed on an ABI 377 automated sequencer at the Department of Horticulture, University of Wisconsin. Sequences have been submitted to GenBank, and accession numbers are provided in Table 1. The aligned data set and trees illustrated in Figs. 2–5 have been submitted to TreeBASE.

Phylogenetic Analysis. Sequencer trace files of ITS sequences were edited with Sequencher 3.0 software and aligned initially by visual means with PAUP* version 4.050d (kindly made available by David Swofford). The boundaries of the ITS1 and ITS2 spacers were established by comparison with published sequences of *Daucus* L. and *Epilobium* L., and the 5.8S region and coding regions for 18S and 26S were deleted due to the dearth of informative characters and absence of complete 5.8S sequence for some taxa. The provisionally aligned spacer sequences, with less than 0.5% missing data across all taxa, were submitted to CLUSTAL V (Higgins and Sharp 1989), with a range of incremental gap penalties from 2 to 18 specified in separate alignment submissions. The five different CLUSTAL analyses yielded essentially identical alignments. The shortest alignment was subjected to a preliminary maximum parsimony analysis to obtain the number of most-parsimonious trees, number of steps (with informative characters only), and consistency index (CI) and retention index (RI) values. Minor adjustments were made to the alignment, punctuated by further parsimony analysis and production of internal homoplasy statistics, following the strategy of Bogler and Simpson (1996). Adjustments were curtailed when tree length did not diminish and homoplasy indices did not increase with further changes.

Phylogenetic analysis of the accepted aligned ITS data matrix included (1) maximum parsimony under the Fitch (1971) criterion; (2) weighted parsimony using transversion/transition ratios calculated from the two spacers using the method of Baum et al. (1994) and applied to their respective nucleotide positions in user-defined stepmatrices, and (3) maximum likelihood (Felsenstein 1981). The first three algorithms were employed in PAUP* and the fourth, in fastDNAml version 1.0.6 (Olsen et al. 1994). Following some of the recommendations and practices of Baum et al. (1994), three separate parsimony analyses were conducted to explore the phylogenetic significance of different indel treatments: (1) gaps treated as missing data; (2) gaps treated as missing data and synapomorphic indels coded as additional binary or multistate characters at the end of the matrix, each weighted equal to a nucleotide position, and (3) same as #2 but with gap code characters scaled to a value of one.

All parsimony analyses employed random-addition replicates (1,000 for unweighted and weighted analyses, 10 for determination of decay values) using all four combinations of NNI-and

TBR-swapping with MULPARS off and on (Olmstead et al. 1993), to search for multiple islands of equally most-parsimonious trees (Maddison 1991; Page 1993). Internal support for the resulting strict consensus tree from unweighted parsimony was evaluated from the consistency index or CI (Kluge and Farris 1969) and the retention index or RI (Archie 1989; Farris 1989). To help circumvent biases in inferring support for particular nodes using bootstrap resampling alone (Felsenstein 1985; Sanderson 1989), Bremer decay values were generated for each resolved node in the strict consensus (Bremer 1988). Decay analysis used the converse constraint method (Baum et al. 1994). Support for internal branches in the ITS phylogeny was also evaluated using the "parsimony jackknife" procedure of Farris et al. (1996) in PAUP*, emulating JAC resampling with 36.8% nominal deletion and "collapse=amb" under the condense trees option. Both bootstrap and jack-knife analyses used 100 replicates, each beginning with random taxon-addition and using NNI-swapping with MULPARS on. To

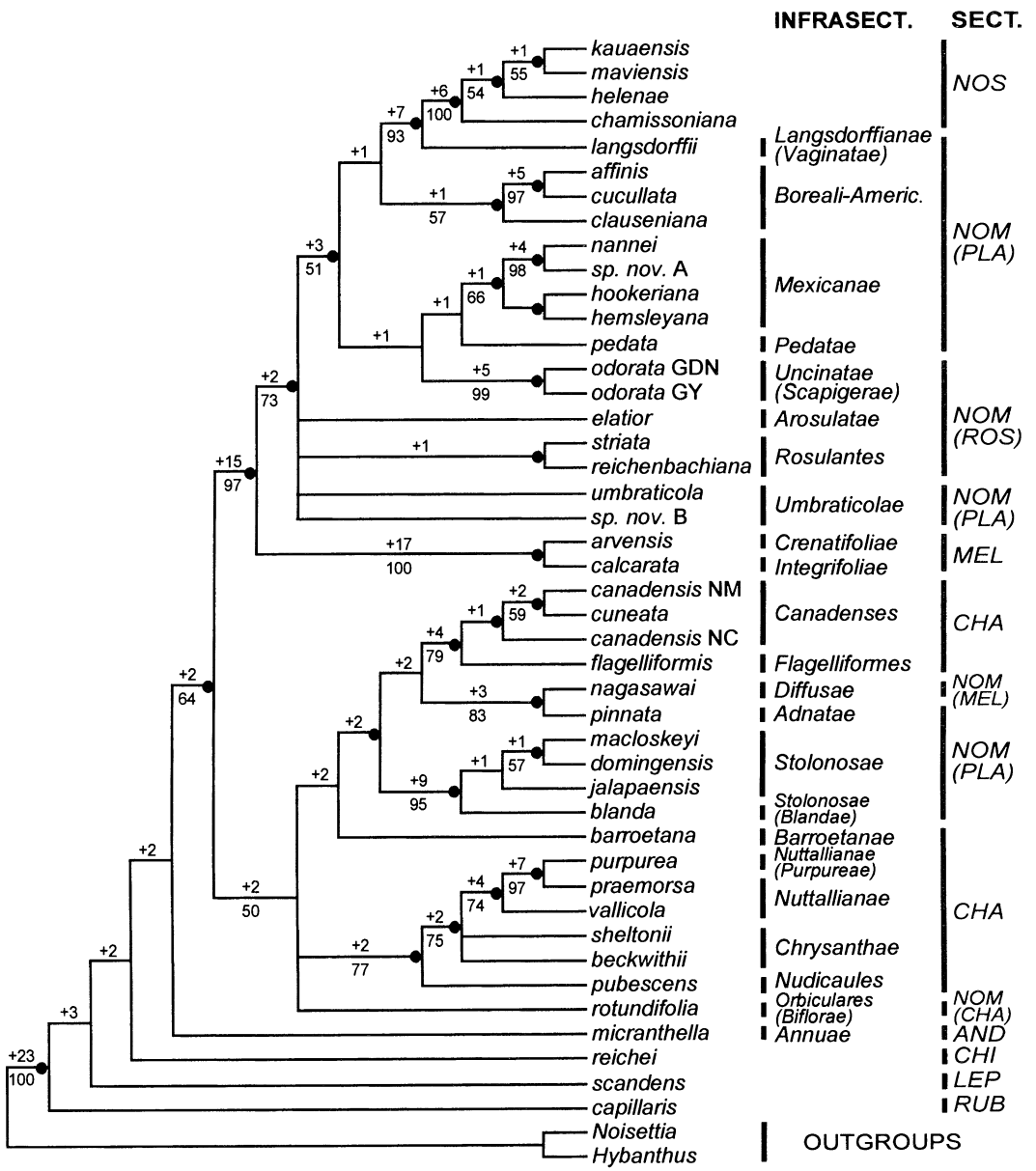

FIG. 2. Strict consensus tree of the eight most parsimonious trees from unweighted parsimony analysis of *Viola* and outgroup ITS sequences (CI = 0.500 excluding uninformative characters, RI = 0.716). Numbers above branches represent decay values, those below are the percentage of bootstrap replicates (out of 100 replicates) supporting each branch; a filled circle indicates at least 50% jackknife support. Column of names to right of species epithets are infrasectional groups recognized by Becker (or Clausen, in parentheses). Acronyms at far right are first three letters of sections listed in Table 1.

determine which treatment of indel regions provided the best representation of phylogenetic relationships from maximum parsimony analysis, the number of trees, number of steps, CI, RI, and average bootstrap, jackknife and decay values across resolved nodes were compared among the three data sets (gaps as missing data only, gaps as missing data with additional unscaled binary and multi-state characters, and gap codes scaled to a value of one).

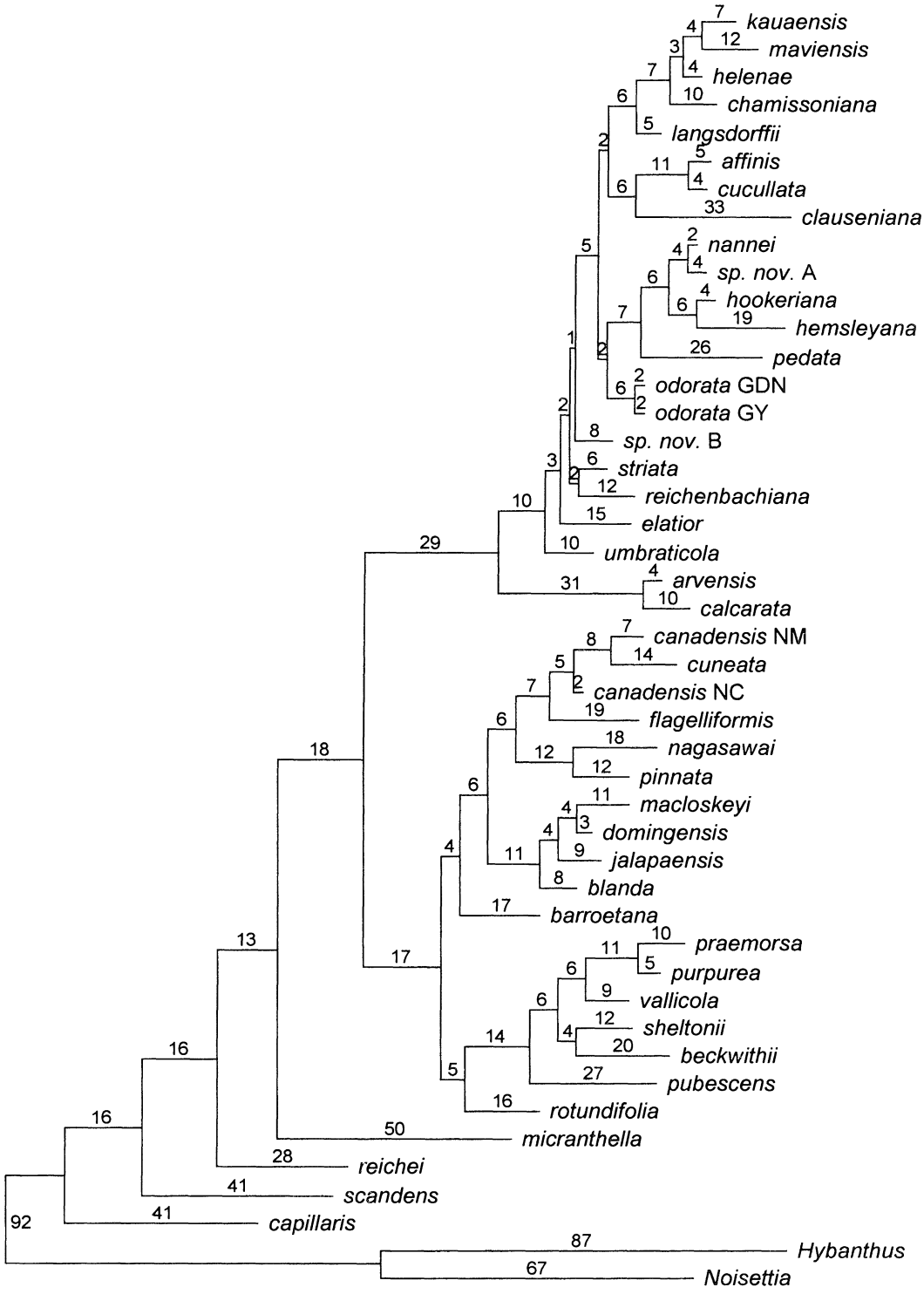

FIG. 3. Phylogram for one of the eight most parsimonious trees from unweighted parsimony analysis of *Viola* and outgroup ITS sequences. Numbers above branches represent nucleotide substitutions.

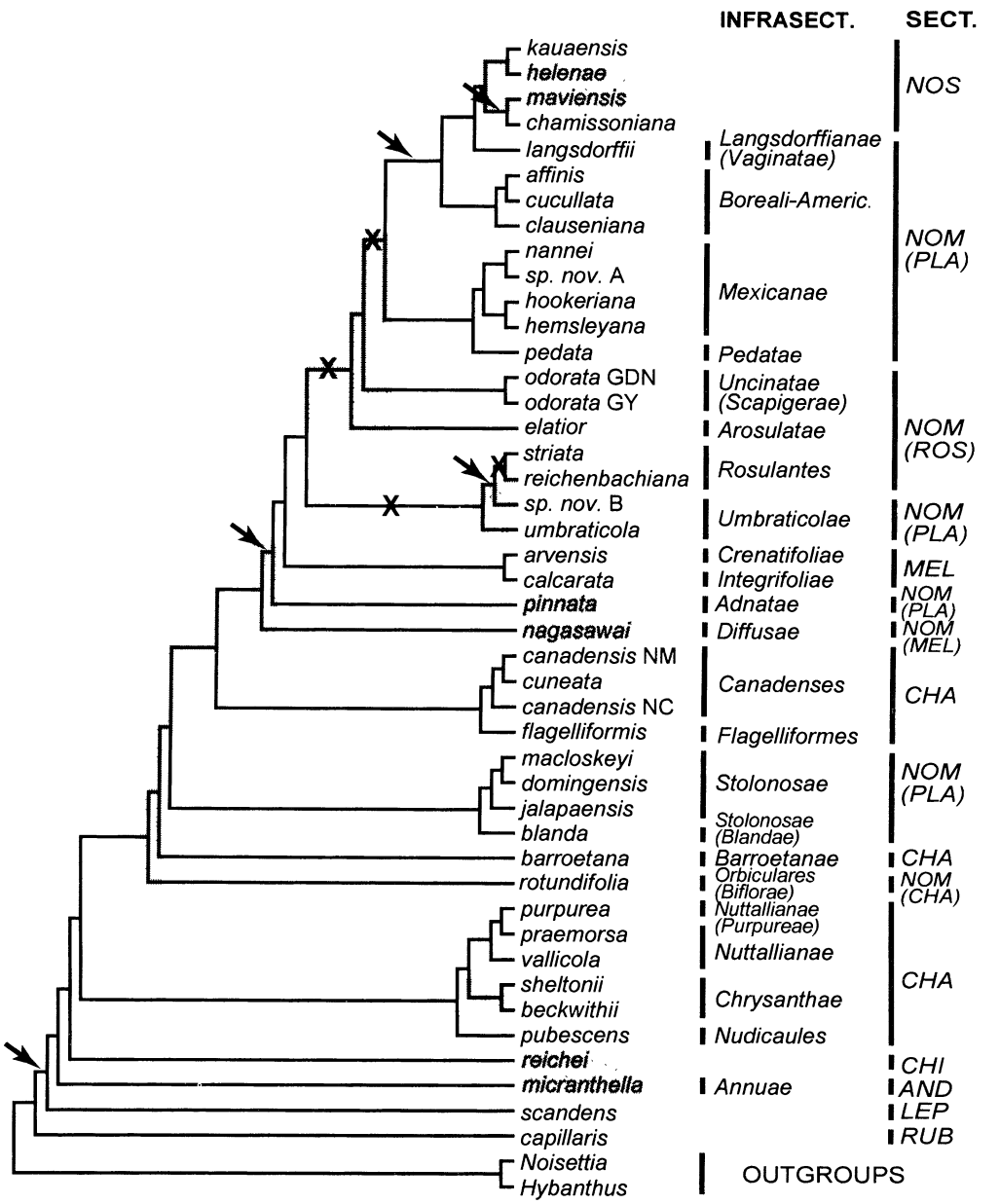

FIG. 4. Maximum likelihood tree based on nucleotide variation in *Viola* and outgroup ITS sequences, with phylogenetic positions differing from those in the maximum parsimony tree denoted in gray. Arrows denote statistically significant support at p = 0.05 and X's denote no support; all other branches are highly statistically significant at p = 0.1. Column of names to right of species epithets are infrasectional groups recognized by Becker (or Clausen, in parentheses). Acronyms at far right are first three letters of sections listed in Table 1.

Maximum likelihood used the substitution model of Felsenstein that is the default in PHYLIP (Felsenstein 1993). This analysis used empirically determined nucleotide substitution rates and a 2:1 transition/transversion ratio.

Interspecific and intergroup sequence divergence values, uncorrected for multiple substitutions, were obtained from PAUP*. All analyses were performed on a 7100/80av Power Macintosh personal computer.

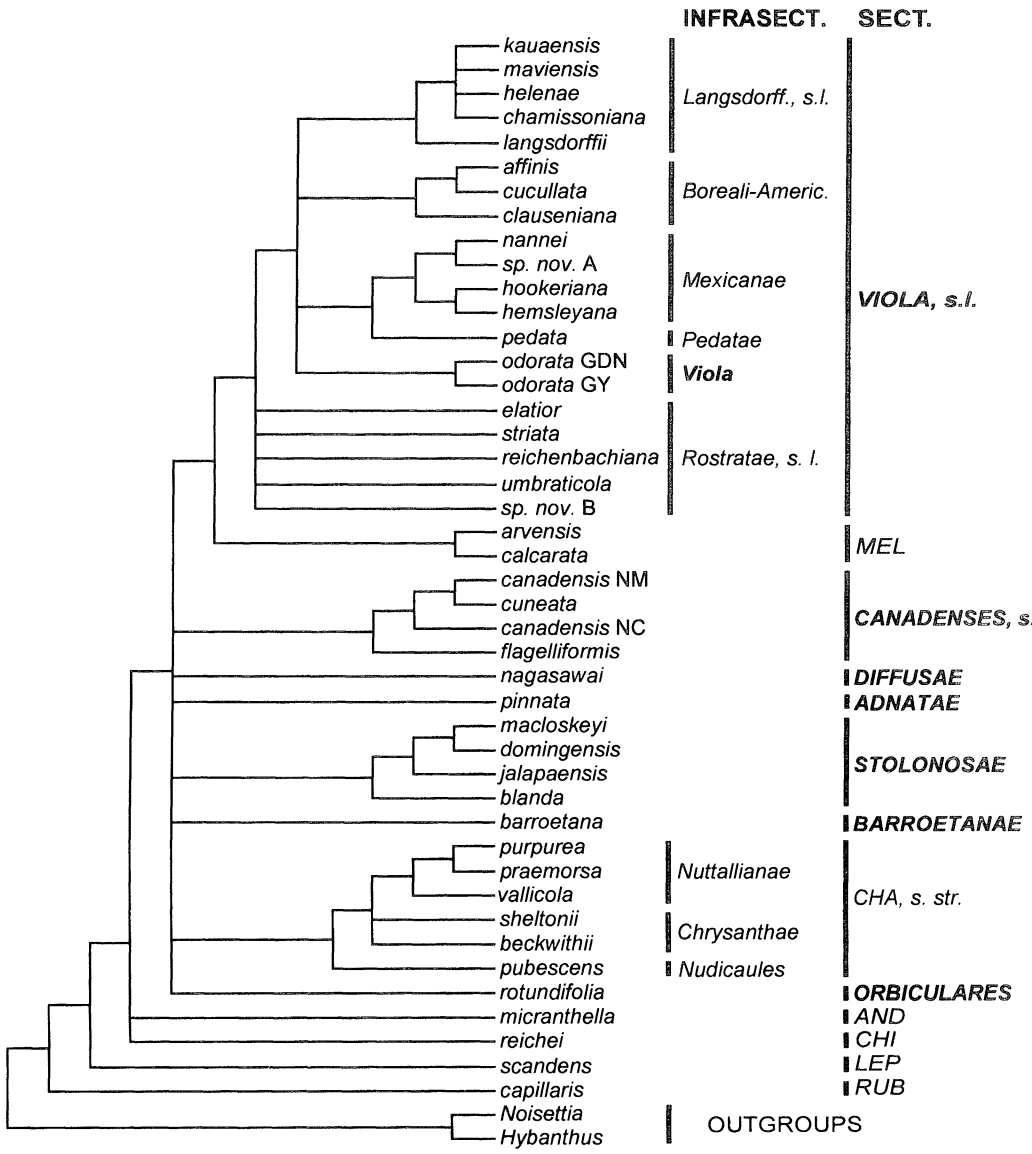

FIG. 5. Strict consensus of the maximum parsimony strict consensus cladogram in Fig. 2 (retaining branches with +2 or more decay and 50% bootstrap or jackknife support) and the maximum likelihood cladogram in Fig. 4 (with p = 0.5 or p = 0.1 statistical significance). Proposed phylogenetic groups at the infrasectional and sectional levels are named provisionally using the earliest available name. Provisional names used for groups proposed for recognition at sectional rank are in bold; the name *Viola* (in bold) is accepted at both levels following nomenclatural rules for groups including the type species of the genus).

RESULTS

Sequence Characteristics. Sequence characteristics of the ITS spacers in *Viola* and outgroups are summarized in Table 2. The ITS1 spacer is somewhat longer than the ITS2 spacer, and includes more indels and generally more variable characters with a higher proportion of these being phylogenetically informative. The ITS1 spacer also has a slightly lower transition/transversion ratio and, thus, a relatively higher proportion of transversions, than the ITS2 spacer. Across *Viola* the ITS region, particularly ITS1, may be approaching satu-

TABLE 2. Sequence characteristics of the ITS region for Viola taxa and outgroups.

	ITS1	ITS2	BOTH
Unaligned length			
Within *Viola*	215–268	193–209	413–472
Outgroups only	203–272	202–209	405–481
Aligned length	299	221	520
All indels	32	20	52
Informative indels	26	14	40
GC content (%)	64.3	63.4	63.9
Transition/transversion ratio	1.20	1.27	1.23
Variable nucleotide positions	224	175	399
Informative (% of total) nucleotides			
Within *Viola*	131 (43.8)	71 (32.1)	202 (38.8)
Viola + outgroups	146 (48.8)	87 (39.4)	233 (44.8)
Sequence divergence (%)			
Within *Viola*	1.2–29.7	0.0–28.7	1.1–28.8
Viola + outgroups	1.2–45.0	0.05–42.5	1.1–40.4

ration in the rate of nucleotide substitution at certain sites because of the high sequence divergence and numerous positions with four-nucleotide "hits." Both spacers have very similar GC content. The unaligned and aligned lengths of the ITS1 and ITS2 spacers, transition/transversion ratios, and GC content fall within the ranges reported for other angiosperm groups (Baldwin et al. 1995).

The upper limit of combined ITS spacer divergence within *Viola*, 28.8% between *V. capillaris* and *V. clauseniana*, is greater than values reported for most angiosperm genera thus far studied. The genera *Viola*, *Arceuthobium* Bieb. (Nickrent et al. 1994) and the aggregate genus *Senecio* L. (Bain and Jansen 1995) in particular show greater maximal intrageneric ITS sequence divergence than in most genera, e.g., twice as high as in *Epilobium* with 12.9% (Baum et al. 1994). Maximum species divergence equals or surpasses that found among genera of the Rosaceae (Campbell et al. 1995), Caryophyllaceae (Oxelman and Lidén 1995), Polemoniaceae (Porter 1993), Fabaceae (Sanderson and Wojciechowski 1996; Wojciechowski et al. 1993), Saxifragaceae (Soltis and Kuzoff 1995), Winteraceae (Suh et al. 1993), Asteraceae (Baldwin 1992, 1993; Kim and Jansen 1994; Susanna et al. 1995) and Gentianaceae (Yuan and Küpfer 1995).

Sequence differences are substantial even among closely related species and show infraspecific variation in *V. canadensis*, *V. odorata*, *V. chamissoniana* subsp. *tracheliifolia*, *V. kauaensis* and *V. maviensis*. Data are also robust enough to resolve relationships among groups throughout the genus *Viola*. Conversely, sequence differences are so great between the outgroups, *Noisettia* and *Hybanthus*, and the ingroup (*Viola*) that the former are alignable visually only with difficulty. An ITS sequence for *Hybanthus mexicanus*, shown by *rbcL* data to actually belong to a different clade of genera from that containing *H. concolor* (Hodges et al. 1995), cannot be aligned with the outgroup or ingroup sequences used here. The lowest sequence divergence between an outgroup taxon and *Viola* taxon is 31.1%, between *Noisettia orchidiflora* and *V. capillaris*. Thus, ITS sequence data appear to be informative and useful predominately below the generic level in the Violaceae. The divergence in ITS sequences among outgroup and ingroup genera also suggest that genera in the Violaceae are phylogenetically very divergent and quite isolated from each other. Genera in the family are the focus of ongoing phylogenetic studies by the first author and collaborators using more slowly evolving gene regions.

Phylogenetic Analyses. The strict consensus trees in the three maximum parsimony analyses (treating indels as missing data and without gap codes, then with binary and multistate gap codes unscaled, and finally with gap codes scaled to a value of one) are all moderately to highly resolved. The data matrix that includes unscaled binary and multi-state gap codes as additional characters yields fewer, better resolved trees with higher CI and RI values and generally higher clade support statistics compared with other treatments of indels. This phylogeny is used for discussion as the best portrayal of phylogenetic relationships in *Viola* from unweighted maximum parsimony analysis (Fig. 2). Our experimental treatments of gaps support the empirical conclusions of Baum et al. (1994) and others arguing for inclusion of gap codes as additional characters to help capture the maximum amount of phylogenetic signal from ITS data for maximum parsimony analysis. The analysis using empirically determined transversion weightings, excluding gap codes, produces fewer most-parsi-

monious trees than the unweighted parsimony analysis but yields comparable resolution. The trees are in nearly all respects a subset of those generated by the maximum parsimony analysis discussed at length above, and are not elaborated on here.

The strict consensus tree from maximum parsimony (Fig. 2) places Chilean sect. *Rubellium* at the very base of the genus, with Mesoamerican-South American sect. *Leptidium* and South American sect. *Chilenium* and sect. *Andinium* above it, the last being sister to the rest of *Viola*. Northern Hemisphere groups representing the remainder of the genus form two clades, one with sect. *Chamaemelanium* groups paraphyletic and basal to certain sect. *Nomimium* groups, and the other with the pansies of sect. *Melanium* sister to the rest of sect. *Nomimium* and Hawaiian sect. *Nosphinium*.

The paraphyletic predominately yellow-flowered sect. *Chamaemelanium* disintegrates into a grade of four distinct and moderately to very well supported groups in trees two steps longer than the strict consensus. The sect. *Chamaemelanium* groups consist of the stoloniferous *Orbiculares*, the stemmed *Nudicaules* plus *Nuttallianae*, the rosulate (=rosette-forming) *Barroetanae* and the stemmed *Flagelliformes* plus *Canadenses*. Embedded within the loose sect. *Chamaemelanium* assemblage are two moderately well supported, stemless white-flowered subclades of sect. *Nomimium*, the *Stolonosae*, and the *Adnatae* plus *Diffusae*.

The remaining Northern Hemisphere groups are extremely well supported as a clade, with the pansies of sect. *Melanium* sister to a moderately well supported clade composed of the remaining groups of sect. *Nomimium* and the mostly woody Hawaiian sect. *Nosphinium*. Moderately to highly supported subclades terminal to the *Rostratae* plus *Umbraticolae* are represented by the stemless *Nomimium* s. str., *Pedatae*, *Mexicanae*, and *Boreali-Americanae*, and the stemmed *Langsdorffianae* plus sect. *Nosphinium*.

The *Langsdorffianae*-sect. *Nosphinium* subclade is one of the most strongly supported. The very close sister relationship between the Hawaiian species of *Viola*, whose monophyly is extremely strongly supported, and amphi-Beringian *V. langsdorffii* s.l., is entirely unexpected on the basis of morphological and other traditional systematic data. The morphologically heterogenous *Mexicanae* are monophyletic with moderate support (preliminary analyses with all taxa, not shown here, gave higher support for this particular subclade).

A representative phylogram of the unweighted maximum parsimony analysis indicates the number of nucleotide substitutions along internal and terminal branches (Fig. 3). Both species and subclades nearer the base generally have higher nucleotide substitution and indel rates relative to derived taxa in more terminal positions.

The results of maximum likelihood analysis (Fig. 4) agree with the maximum parsimony consensus tree in most respects, showing strong statistical support in clades well supported by the maximum parsimony analysis and weaker or no suppport for relationships not well supported by maximum parsimony. Key differences include the switch of positions between *V. micranthella* and *V. reichei* near the base, suggesting an equivocal relationship of these basal and very divergent groups, fragmenting of sect. *Chamaemelanium* and sect. *Nomimium* into an intermingling grade of subclades leading to the sect. *Melanium*-sect. *Nomimium*-sect. *Nosphinium* clade, and splitting apart of the *Adnatae* and *Diffusae*. Group circumscription and support of subclades in the sect. *Melanium*-sect. *Nomimium*-sect. *Nosphinium* clade, and of the relationships among them, are essentially identical to those portrayed by the maximum parsimony analysis.

Discussion

In spite of very different assumptions and algorithms used by the parsimony and maximum likelihood analyses, all produce very similar topologies, with relationships among terminal taxa almost identical in each case. This indicates that the ITS data contain a very strong phylogenetic signal that was successfully captured by the various analyses. In general, phylogenetic relationships derived from ITS sequence data corroborate the major infrageneric—particularly strongly the infrasectional—groupings and alliances postulated by previous specialists on the basis of macromorphology, style micromorphology, and chromosome numbers. The ITS data also greatly clarify the relationships of lower-level infrageneric groups, which are obscure based on morphology and cytology. They illuminate the affinities of problematical species, e.g., *V. rotundifolia* representing the yellow-flowered *Orbiculares* group with its prostrate-stemmed habit and *Plagiostigma*-like style. They also reveal monophyly in the heterogeneous *Mexicanae* group and Hawaiian sect. *Nosphinium*, where morphology suggests polyphyly.

All analyses place the South American sections at the base of the genus *Viola*, with sect. *Rubellium* most basal. Parsimony (Fig. 2) and maximum likelihood

(Fig. 4) analyses arrange groups of sect. *Chamaemelanium* and certain groups of sect. *Nomimium* (Clausen's *Plagiostigma* in part, namely *Adnatae*, *Diffusae* and *Stolonosae* groups) differently. Maximum parsimony places these in a weakly monophyletic clade of strongly supported groups that is sister to a clade containing the rest of the genus, whereas maximum likelihood splinters them into a grade of groups with poorly resolved basal relationships. In all analyses, at any rate, the sect. *Nomimium* groups intermingle with those comprising sect. *Chamaemelanium*. All analyses also place the rest of the groups in Becker's sect. *Nomimium* (Clausen's sect. *Rostellatae* plus what is left of his sect. *Plagiostigma*) together with Hawaiian sect. *Nosphinium* into a large and exceedingly well supported clade, with the pansies of sect. *Melanium* at the base. Segregation of these other sect. *Nomimium* groups together with Hawaiian *Nosphinium* in a segregate section, *Viola* (named so because it includes the type species of the genus), is another new hypothesis of relationships generated by the ITS data.

Further details are mentioned below, under three major subheadings representing (1) the basal Latin American sections; (2) groups in the sect. *Chamaemelanium*-sect. *Nomimium* (in part) aggregate as a weak clade or grade; and (3) groups in the sect.-*Melanium*-sect. *Nomimium* (in part)-sect. *Nosphinium* clade.

The Basal Latin American Sections. The molecular data support the hypothesis that the genus probably arose in the Andes and that South American groups are the most primitive in *Viola* (Clausen 1929; Valentine 1962). Relationships among these divergent basal groups are not well supported at present, although it appears that sect. *Rubellium* may be the most primitive group in the genus and sect. *Leptidium*, morphologically similar to it, may be the next most primitive. Much additional sampling within each of these groups must be done before any degree of resolution may be achieved.

The* Chamaemelanium-Nomimium *Aggregate. Clausen (1929) surmised that the partly or entirely yellow-flowered species comprising *Chamaemelanium* are primitive among north-temperate members of the genus, and most of the molecular phylogenies are consistent with this idea. Derivation of some groups of sect. *Nomimium* (segregated as sect. *Plagiostigma* by Clausen) from within sect. *Chamaemelanium* was also suggested by Clausen (1929, 1964) on the basis of tetraploid (n= 12) or putatively derived aneuploid chromosome numbers in the former, but his hypothesis has not been taken up by subsequent specialists. The ITS data support Clausen's suggestion, placing certain sect. *Nomimium* groups among groups of sect. *Chamaemelanium* (in a weak clade or a grade, depending on the analysis) such that neither section is monophyletic.

The placement of the *Orbiculares* has been problematic in large part due to disagreement among specialists regarding the relative importance of its prostrate-stemmed or stoloniferous growth habit and *Plagiostigma*-like style morphology. Clausen (1964) transferred it to sect. *Chamaemelanium* as an aberrant complex, whereas most specialists (e.g., Gershoy 1934; Gil-ad 1995) have kept it in Becker's sect. *Nomimium*. Various analyses of the ITS sequence data place the *Orbiculares* at or near the base of the sect. *Chamaemelanium*-sect. *Nomimium* aggregate of groups but firmly exclude it from individual *Nomimium* groups themselves. Whether the *Orbiculares* group should be merged with members of the *V. biflora* complex, as part of Clausen's *Biflorae* group, must await ITS sequences of the circumpolar *V. biflora* complex and additional sequences of other *Orbiculares*.

The ITS sequence data further clarify relationships within and among lower-level infrageneric groups in Becker's *Nuttallianae*. Included are the *V. purpurea* and *V. nuttallii* complexes, which Clausen (1964) recognized as commensurate in rank with the *Chrysanthae* group. The molecular phylogenies indicate a more complex set of relationships. The two members of the *V. nuttallii* complex, diploid *V. vallicola* and polyploid *V. praemorsa*, form a paraphyletic group relative to diploid *V. purpurea*. This supports retention of the two complexes within a broader *Nuttallianae* group as done by Becker and indicated by morphological data and natural hybridization. The ITS data provide moderate support for a monophyletic *Chrysanthae-Nuttallianae* subclade. Together with natural hybridization among the complexes and the existence of allopolyploids connecting different complexes, these molecular phylogenetic relationships justify merger of the *Nuttallianae* with Clausen's *Purpureae* and probably his *Pedunculatae* into a single arid-land western North American *Nuttallianae* group equal in rank to the closely related *Chrysanthae*. Further study of these groups, including members of the *Nudicaules* as sister taxa, are needed.

The monotypic *Barroetanae* group, represented by *V. barroetana*, is excluded by the ITS data from the *Nuttallianae* despite very close morphological similarity to the latter (excepting its stemless habit),

suggesting that the *Barroetanae* should be retained as a distinct and phylogenetically isolated monotypic group.

The *Adnatae* and *Diffusae* groups have a relatively close but somewhat uncertain phylogenetic relationship to the *Stolonosae* and demand further investigation with additional representatives of all three groups as well as other Australasian groups (e.g., *Bilobatae*) not sampled here. The *V. blanda* complex was segregated by Gershoy (1934) as subsection *Blandae*. However, frequent hybridization with species in the *Stolonosae* s. str. and its placement in close association with other *Stolonosae* representatives in the molecular analyses argue for retaining the *V. blanda* complex in a more broadly circumscribed *Stolonosae* sensu Becker.

Two accessions of *V. canadensis* from the Midwest and the Southeast, representing the *Canadenses*, are commonly paraphyletic with respect to narrowly endemic species in the complex. *Viola flagelliformis* represents one species of two complexes originally assigned to the *Flagelliformes* by Becker based on a misinterpretation that its flowers were yellow (*V. flagelliformis* and *V. galeanaensis* actually have pale blue to whitish corollas and yellow throats). The molecular data support the transfer of *V. flagelliformis* to the *Canadenses*; the status of its morphologically very similar relative in northern Mexico, *V. galeanaensis*, must await studies now in progress. All analyses portray this expanded *Canadenses* group as a well supported monophyletic unit that deserves recognition on par with other distinct groups in the *Chamaemelanium-Nomimium* aggregate. The *Canadenses* are the focus of ongoing systematic and evolutionary studies (Ballard et al. 1997).

***The* Melanium-Nomimium-Nosphinium *Clade*.** Section *Melanium* has long been viewed as having a close relationship to sect. *Chamaemelanium*. Its status as a transitional group to sect. *Nomimium* has never been suggested, but the ITS results support such a position for this fascinating and cytogenetically diverse section. Clausen's (1931) removal of the *Diffusae* from sect. *Nomimium* to sect. *Melanium* on the basis of its base chromosome number of $x = 13$ and a supposedly annual habit is not supported by the molecular data. Rather, the molecular data place it sister to, or closely related to, the *Adnatae*. In the context of the ITS phylogeny, the *Diffusae* may be interpreted as a rare example of aneuploid increase (from $x = 12$) rather than of aneuploid drop as inferred for other aneuploid taxa in the genus.

Clausen's (1964) splitting of Becker's sect. *Nomimium* into sect. *Rostellatae* with $x = 10$ on the one hand, and sect. *Plagiostigma* with $x = 12$ and derivatives on the other, is supported by the ITS data insofar as segregation of the euploid groups is concerned. However, the molecular data suggest that Clausen did not go far enough: several more groups in his sect. *Plagiostigma* with $x = 20$ or 30 and probable aneuploid derivatives, are actually phylogenetically related to groups in his *Rostellatae* and are well removed from other *Nomimium* groups that nestle in the sect. *Chamaemelanium*-sect. *Nomimium* aggregate.

Considerable sequence divergence and poor basal resolution among subclades within the *Nomimium-Nosphinium* clade, but modest support for the clade itself, may reflect an abrupt radiation leading to these comparatively recent sublineages. Equivocal relationships within the *Rostratae* group, and for that matter among representatives of the *Stolonosae* and between the *Adnatae* and *Diffusae* groups, however, is likely due to sparse taxon sampling and "exemplar effects" (see general discussion of these two potential problems in Sytsma and Baum 1996).

Members of the *Rostratae* are consistently placed basal to other sect. *Nomimium* groups falling out in this clade, and with sect. *Nosphinium* in a highly derived position. Equivocal relationships and intermingling of Becker's *Arosulatae* and *Rosulantes* subgroups in the *Rostratae* together with the *Umbraticolae* group support morphological suggestions of a very close and even reticulate relationship among these complexes. The molecular data also support the recognition of another new species, *V.* sp. nov. B, that (as yet unpublished) monographic studies have distinguished from *V. umbraticola* in the latter group. The monophyly of Becker's *Rostratae* subgroups and *Umbraticolae* cannot adequately be judged, perhaps owing partly to the poor taxon sampling of the very diverse and speciose *Rostratae*. However, the current ITS data would seem to argue for retaining the *Arosulatae*, *Rosulantes* and *Umbraticolae* in a single and variable infrasectional group, the *Rostratae*, without subdivisions.

The relationships among the subclades terminal to the *Rostratae*, namely *Viola*, *Boreali-Americanae*, *Mexicanae*, *Pedatae* and *Nosphinium*-plus-*Langsdorffianae*, are equivocal, as are relationships within the *Rostratae* and the *Stolonosae*, although most groups are modestly to very strongly supported as monophyletic. The molecular data indicate a more distant relationship between the *Pedatae* and *Boreali-Americanae* than thought by most recent specialists (McKinney 1992; Russell 1965). A cladistic analysis of

macromorphological features and micromorphological patterns of seed coats in the *Boreali-Americanae* and *Pedatae* also points to a more distant relationship (Gil-ad 1995). Like the situation with the *Pedatae*, the molecular data suggest a weaker relationship between *V. clauseniana* and the other *Boreali-Americanae* than previously appreciated; this is further bolstered by the divergent chromosome numbers of n = 22 and n = 27, respectively (Clausen 1964). Analyses diverge in indicating a weak (parsimony, Fig. 2) or well supported (maximum likelihood, Fig. 4) sister relationship between the *Pedatae* and the *Mexicanae*, a relationship never before suggested. In considering the *Mexicanae* themselves, sequence divergence is considerable among the species. Sequence differences between a morphologically and ecologically distinct northern Guatemalan and southern Mexican alpine plant and *V. nannei* also support morphological evidence for the recognition of a geographically restricted new violet species in Mesoamerica, here indicated as *V.* sp. nov. A.

The highly derived phylogenetic position of the Hawaiian sect. *Nosphinium* and the very close sister relationship with the amphi-Beringian *V. langsdorffii* complex comprising the *Langsdorffianae* group could not have been guessed from available morphological or cytogenetic evidence alone, nor has such a placement ever been proposed. The very close morphological similarity between many members of sect. *Nosphinium* and neotropical sections *Leptidium* and *Rubellium* include branching, woody stems, lanceolate or narrowly ovate leaves, short corolla spur, and simple style. Hawaiian species of *Nosphinium* produce inflorescences, and at least those species in sect. *Leptidium* produce a second floral bud in addition to the first that fully develops as the chasmogamous flower. The ITS data suggest that these shared traits are apparently a remarkable instance of morphological convergence with montane Latin American violets in a group of plants that have diversified in high-elevation mountain sites under tropical oceanic conditions.

***Provisional Phylogenetic Groups in* Viola.** Available ITS data permit the recognition of several distinct phylogenetic groups at various levels in the genus *Viola*, especially where a consensus of the primary analytical results—maximum parsimony and maximum likelihood—is used as a conservative portrayal of relationships (Fig. 5). In the consensus tree of phylogenies, with all poorly supported branches collapsed in each phylogeny prior to taking the consensus, distinct groups become apparent that may be recognized formally, at the sectional or infrasectional (primarily subsectional) levels. Groups identified as such here have been given names currently in use where ranks have not changed. Groups that should ostensibly be elevated in rank because of phylogenetic isolation and modest to strong support as monophyletic taxa have been given provisional names representing the earliest available name (regardless of orthography). The groups identified here are intended only as informally designated taxa with provisional names; formally recognized phylogenetic groups will require much nomenclatural research and, of course, explicit circumscription beyond molecular phylogenetic characters. Much additional study will also be important to understand the composition, circumscription and relationships of diverse groups that are sparsely sampled in the present study. Nevertheless, the interpreted phylogenetic taxa, as infrageneric groups, will serve as a useful springboard for future studies of species-level relationships within groups in *Viola*.

Phylogenetic Predictions for Other Groups. Chromosome numbers show a clear and interpretable pattern of cytogenetic change (Ballard 1996) when superimposed on the ITS phylogeny (Fig. 6). All near-basal groups retain chromosome numbers based on x = 6, a number that is widespread in the Violaceae and may be primitive in the genus (although several genera have numbers based on x = 4). The *Adnatae*, *Diffusae* and *Stolonosae* groups have a base number of x = 12 or an aneuploid number derived from it, by comparison with members of sect. *Chamaemelanium* with x = 6, and in this context these groups represent eu-or aneutetraploid sublineages derived from various sublineages of the sect. *Chamaemelanium* assemblage. Section *Melanium* includes x = 6 to 17 with polyploid series based on many of these base numbers. As the cytogenetically most diverse group in *Viola*, it also includes x = 6 (in sect. *Chamaemelanium*) and x = 10 lineages as well as two annual (and presumably highly derived) species with n = 5. In many respects the section is morphologically transitional between groups with x = 6 and polyploids or derivatives on the one hand and groups with x = 10 and polyploids or derivatives on the other, making its phylogenetic position based on ITS sequence data very intriguing.

Chromosome numbers can be used in the context of the ITS phylogeny (Fig. 6), to make predictions of phylogenetic placement for morphologically and cytogenetically distinct groups in *Viola* that have not yet been sequenced. Section *Tridens* will likely

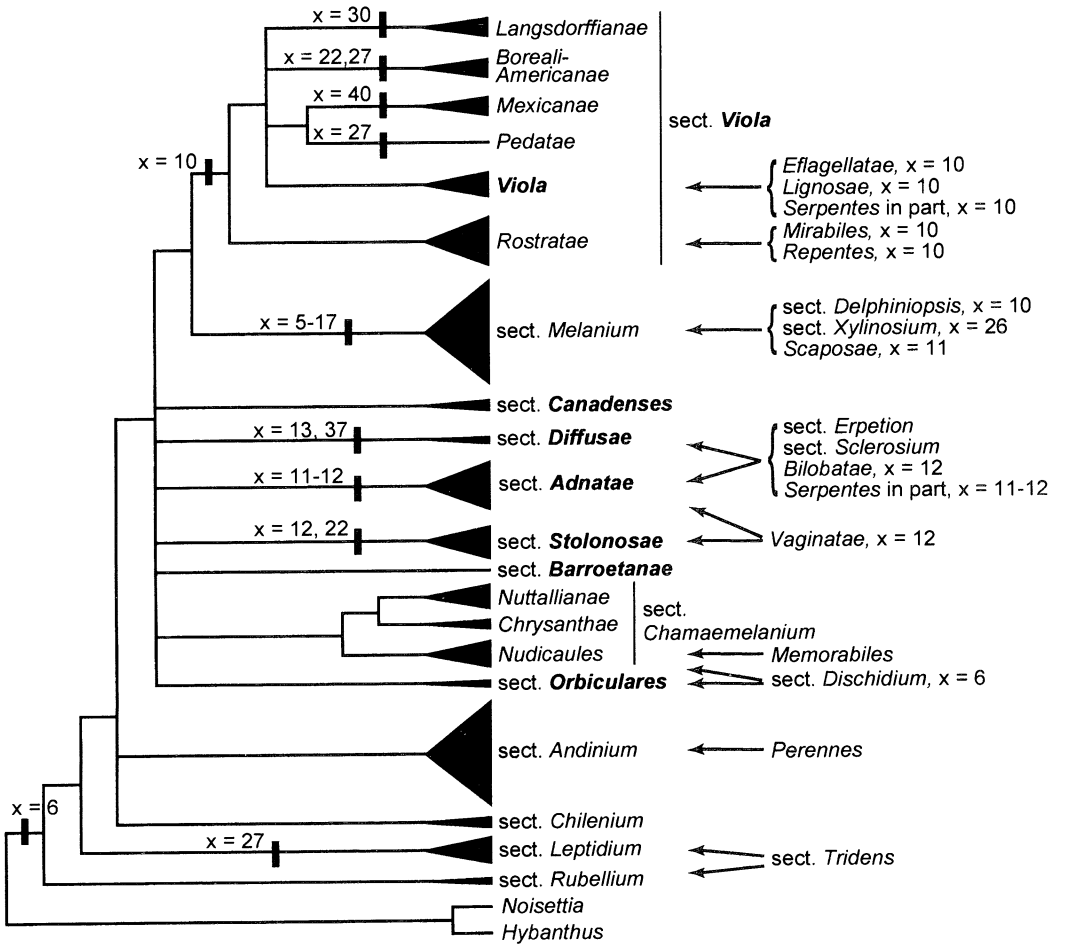

FIG. 6. Correspondence of *Viola* ITS phylogeny with base chromosome numbers, and predictions of placement for all remaining *Viola* groups not sequenced (see Fig. 1). Abbreviated graphical representation of the parsimony tree and groups delineated in Fig. 4, where the width of the triangle's base for each group is roughly proportional to the number of species (e.g., *Andinium* and *Melanium* contain ca. 100 species; *Stolonosae*, ca. 20 species; *Rubellium*, 3–4 species); names follow those accepted for Fig. 5. Base chromosome numbers (Ballard et al. 1996) are mapped onto the parsimony tree; verifiable chromosome numbers are lacking for Latin American sections except for *Leptidium*, but the chromosome number of outgroup *Hybanthus concolor* (as a polyploid) is consistent with $x = 6$ being primitive in *Viola*. Groups not sequenced lie on the right, with arrows showing predictions of placement based on morphological and cytogenetic traits shared with groups examined in this study.

join the other South American groups at the base of the genus, and may well be a part of sect. *Rubellium* or sect. *Leptidium* considering morphological features alone.

Clausen's *Biflorae* group of sect. *Chamaemelanium*, also segregated by many as sect. *Dischidium*, will probably fall within the larger assemblage of groups previously comprising sect. *Chamaemelanium*, because all yellow-flowered groups based on $x = 6$ thus far have been placed there by the ITS data. In addition, most groups of *Chamaemelanium* form well supported and morphologically well defined subclades that would not be expected to include the *V. biflora* complex. The *V. biflora* complex could join the phylogenetically divergent *Orbiculares* group (represented by *V. rotundifolia*) as suggested by Clausen or could end up isolated in the aggregate of *Chamaemelanium* and some *Nomimium* groups, as both *V. barroetana* and *V. rotundifolia* are presently isolated here.

All taxa sequenced with a base number of $x = 12$ or putatively derived from it fall into the *Adnatae*,

Diffusae or *Stolonosae* groups previously assigned by Clausen to sect. *Plagiostigma*, but which are found to be loosely associated and derived from within a grade (or weak clade) of *Chamaemelanium* groups. The following groups will probably fall among the *Adnatae-Diffusae* assemblage, based on similar morphological features and $x = 12$ or polyploid base numbers: the western Asian and African sect. *Sclerosium*, the southeastern Asian and Indo-Malaysian *Bilobatae* group, the enigmatic Indo-Malaysian complex including *V. kjellbergii*, *V. papuana* and *V. caleyana*, and sect. *Erpetion*. It is possible that the caulescent sect. *Sclerosium* and *Bilobatae* group above will be basal to the remaining, acaulescent groups in this assemblage. The loose association of the *Adnatae*, *Diffusae* and *Stolonosae* may be due to the low level of taxon sampling and absence of several groups which may be basal or "transitional." Further studies are needed to evaluate the relationships and circumscription of all these $x = 12$ groups.

The woody stems of most species of Hawaiian *Viola* are clearly a derived state and not evidence of a primitive phylogenetic position. By analogy, the supposedly primitive status of the woody Old World sect. *Delphiniopsis* and sect. *Xylinosium* of the Mediterranean region is also called into question, as it is based primarily on the presence of secondary growth. In *Delphiniopsis* primitiveness is also contradicted by its specialized hawk-moth pollination syndrome, but which has been reported in long-spurred pansy groups such as the *V. calcarata* complex. Suspiciously parallel morphological traits (e.g., deeply lobed stipules and details of floral architecture) and identical base chromosome numbers suggest a derivation of *Delphiniopsis* from within the *V. calcarata* complex, and also derivation of *Xylinosium* from within the *V. tricolor* complex, in sect. *Melanium*.

Other groups segregated from the *Rostratae* by Becker that are not distinct on the basis of morphology and natural hybridization, and that possess the same chromosome numbers as the *V. odorata* complex in the *Viola* group, will undoubtedly fall somewhere among the basal taxa in the sect. *Nomimium*-sect. *Nosphinium* clade. Non-stoloniferous species in Becker's *Eflagellatae* group will likely end up in the *Viola* group with the "*flagellate*" *V. odorata* complex. Additional taxon sampling for the *Rostratae* and *Viola* groups may provide further topological stability to this area of the phylogeny.

Limitations of Present Molecular Data, and Future Investigations. While taxon sampling and representation of groups was probably adequate to assess phylogenetic trends and deduce major clades and subclades, future studies must include certain highly anomalous or "pivotal" groups and, ideally, additional representatives of all morphologically and cytogenetically distinct groups. Representatives should be obtained for South American sect. *Tridens*, the circumboreal *Biflorae*, several Old World groups with $x = 12$ or derivatives, e.g., sect. *Sclerosium*, *Bilobatae* and African and Indo-Malaysian complexes, Mediterranean shrub species of sect. *Delphiniopsis* and sect. *Xylinosium*, and additional subgroups in the *Rostratae*.

The relationships presented are based on a nuclear ribosomal data set that shows generally close congruence with relationships indicated by chromosome numbers. Careful scrutiny of sequences in groups in which natural hybridization is frequent revealed no evidence of sequence additivity and no unusual amounts of polymorphism that would suggest interspecific hybridization. However, neither ancient hybridization nor lineage sorting can be ruled out (Sang et al. 1995; Wendel et al. 1995a, b). Another data set, for example consisting of chloroplast DNA markers, would be desirable to reevaluate relationships inferred from the ITS data. Nevertheless, the ITS data corroborate some previous hypotheses of relationship and reveal new insights into the phylogenetic diversification of *Viola*.

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Literature Cited

Archie, J. W. 1989. Homoplasy excess ratios: New indices for measuring levels of homoplasy in phylogenetic systematics and a critique of the consistency index. Systematic Zoology 38: 253–269.

Bain, J. F. and R. K. Jansen. 1995. A phylogenetic analysis of the aureoid *Senecio* (Asteraceae) complex based on ITS sequence data. Plant Systematics and Evolution 195: 209–219.

Baldwin, B. G. 1992. Phylogenetic utility of the Internal Transcribed Spacers of nuclear ribosomal DNA in plants: an example from the Compositae. Molecular Phylogenetics and Evolution 1: 3–16.

———. 1993. Molecular phylogenetics of *Calycadenia* (Compositae) based on ITS sequences of nuclear ribosomal DNA: chromosomal and morphological evolution reexamined. American Journal of Botany 80: 222–238.

———, M. J. Sanderson, J. M. Porter, M. F. Wojciechowski, C. S. Campbell, and M. J. Donoghue. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Annals of the Missouri Botanical Garden 82: 247–277.

Ballard, H. E., Jr. 1996. Phylogenetic relationships and infrageneric groups in *Viola* (Violaceae) based on morphology, chromosome numbers, natural hybridization and Internal Transcribed Spacer (ITS) sequences. Doctoral dissertation. Madison: University of Wisconsin.

———, S. Carlquist, and K. J. Sytsma. 1996. ITS sequence data illuminate the biogeographic origin and adaptive radiation of Hawaiian *Viola*. American Journal of Botany (supplement) 83: 139–140.

———, M. D. Devine, and K. J. Sytsma. 1997. Centrifugal allopatric speciation in the *Viola canadensis* complex (Violaceae) based on Internal Transcribed Spacer DNA sequences. American Journal of Botany (supplement) 84: 177.

Bamford, R. and A. Gershoy. 1930. Studies in North American violets. The cytology of some sterile F_1 violet hybrids. Vermont Agricultural Experiment Station Bulletin 325.

Baum, D. A., K. J. Sytsma, and P. C. Hoch. 1994. A phylogenetic analysis of *Epilobium* (Onagraceae) based on nuclear ribosomal DNA sequences. Systematic Botany 19: 363–388.

Becker, W. 1916. Violae Asiaticae et Australenses. I. Beihefte zum botanischen Centralblatt, Abt. II, 34: 208–266.

———. 1917a. Violae Asiaticae et Australenses. I. Bearbeitung der Gruppe "Sandvicenses" W. Bckr. ined. Beihefte zum botanischen Centralblatt 34, Abt. II: 209–216.

———. 1917b. Violae Asiaticae et Australenses. II. Beihefte zum botanischen Centralblatt, Abt. II, 34: 373–433.

———. 1918. Violae Asiaticae et Australenses. III. Beihefte zum botanischen Centralblatt, Abt. II, 36: 15–59.

———. 1922. Violae novae Americae meridionalis. Feddes Repertorium 18: 180–186.

———. 1923a. Violae Asiaticae et Australenses. IV. Beihefte zum botanischen Centralblatt, Abt. II, 40: 20–68.

———. 1923b. Violae Asiaticae et Australenses. V. Beihefte zum botanischen Centralblatt, Abt. II, 40: 69–171.

———. 1923c. Violae Mexicanae et Centrali-Americanae. I. Feddes Repertorium 19: 392–400.

———. 1923d. Violae Mexicanae et Centrali-Americanae. I. Feddes Repertorium 19: 392–400.

———. 1924. Violae Mexicanae et Centrali-Americanae, II. Feddes Repertorium 20: 1–12.

———. 1925a. Beiträge zur Kenntnis der südamerikanischen Violae. Feddes Repertorium 21: 349–361.

———. 1925b. *Viola*. Pp. 363–376 in *Die natürlichen Pflanzenfamilien, Volume 21*, eds. A. Engler and K. Prantl. Leipzig: Verlag von Wilhelm Engelmann.

Bogler, D. J. and B. B. Simpson. 1996. Phylogeny of Agavaceae based on ITS rDNA sequence variation. American Journal of Botany 83: 1225–1235.

Brainerd, E. 1921. Violets of North America. Vermont Agricultural Experiment Station Bulletin No. 224.

Bremer, K. 1988. The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. Evolution 42: 795–803.

Campbell, C. S., M. J. Donoghue, B. G. Baldwin, and M. F. Wojciechowski. 1995. Phylogenetic relationships in *Maloideae* (Rosaceae): evidence from sequences of the internal transcribed spacers of nuclear ribosomal DNA and its congruence with morphology. American Journal of Botany 82: 903–918.

Clausen, J. 1927. Chromosome number and the relationship of species in the genus *Viola*. Annals of Botany 41: 677–714.

———. 1929. Chromosome number and relationship of some North American species of *Viola*. Annals of Botany 43: 741–764.

———. 1931. Cyto-genetic and taxonomic investigations in *Melanium* violets. Hereditas 15: 219–304.

———. 1964. Cytotaxonomy and distributional ecology of western North American violets. Madroño 17: 173–204.

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

EDWARDS, K., C. JOHNSTONE, and C. THOMPSON. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Research 19: 1349.

FARRIS, J. S. 1989. The retention index and homoplasy excess. Systematic Zoology 38: 406–407.

———, V. A. ALBERT, M. KALLERSJO, D. LIPSCOMB, and A. G. KLUGE. 1996. Parsimony jackknifing outperforms neighbor-joining. Cladistics 12: 99–124.

FELSENSTEIN, J. 1981. Evolutionary trees from DNA sequences: A maximum likelihood approach. Journal of Molecular Evolution 17: 368–376.

———. 1985. Confidence limits of phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

———. 1993. *PHYLIP (Phylogeny Inference Package), version 3.5c*. Seattle: Univ. of Washington.

FITCH, W. M. 1971. Toward defining the course of evolution: minimum change for a specific tree topology. Systematic Zoology 20: 406–416.

GERSHOY, A. 1928. Studies in North American violets. I. General considerations. Vermont Agricultural Experiment Station Bulletin no. 279.

———. 1934. Studies in North American violets. III. Chromosome numbers and species characters. Vermont Agricultural Experiment Station Bulletin no. 367.

GIL-AD, N. 1995. Systematics and evolution of *Viola* L. subsection *Boreali-Americanae* (W. Becker) Brizicky. Doctoral dissertation. Ann Arbor: University of Michigan.

GIVNISH, T. J. and K. J. SYTSMA (eds.). 1997. *Molecular evolution and adaptive radiation*. Cambridge: Cambridge Univ. Press.

HEKKING, W. H. A. 1988. Violaceae Part I-*Rinorea* and *Rinoreocarpus*. Flora Neotropica Monograph 46.

HIGGINS, D. G. and P. M. SHARP. 1989. Fast and sensitive multiple sequence alignments on a microcomputer. Computer Applications in the Biosciences 5: 151–153.

HODGES, S. A., H. E. BALLARD, JR., M. L. ARNOLD, and M. W. CHASE. 1995. Generic relationships in the Violaceae: data from morphology, anatomy, chromosome numbers and *rbcL* sequences. American Journal of Botany (supplement) 82: 136.

HOLM, T. 1932. Comparative studies of North American violets. Beihefte zum Botanischen Centralblatt 50, Abt. II: 135–182.

KIM, K.-J. and R. K. JANSEN. 1994. Comparisons of phylogenetic hypotheses among different data sets in dwarf dandelions (*Krigia*, Asteraceae): additional information from internal transcribed spacer sequences of nuclear ribosomal DNA. Plant Systematics and Evolution 190: 157–185.

KLUGE, A. G. and J. S. FARRIS. 1969. Quantitative phyletics and the evolution of anurans. Systematic Zoology 18: 1–32.

MADDISON, D. R. 1991. The discovery and importance of multiple islands of most-parsimonious trees. Systematic Zoology 40: 315–328.

MCKINNEY, L. E. 1992. A taxonomic revision of the acaulescent blue violets (*Viola*) of North America. Sida, Botanical Miscellany no. 7.

MELCHIOR, H. 1925. Violaceae excepting *Viola*. Pp. 329–363 in *Die natürlichen Pflanzenfamilien, Volume 21*, eds. A. Engler and K. Prantl. Leipzig: Verlag von Wilhelm Engelmann.

MULLIS, K. F. FALOONA, S. SCHARF, R. SIAKI, G. HORN, and H. ERLICH. 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harbor Symposia of Quantitative Biology 51: 263–273.

NEPOKROEFF, M. and K. J. SYTSMA. 1998. Evolution in cloud forest members of *Psychotria* section *Notopleura*, Rubiaceae. In *Natural history of the Monteverde Cloud Forest Preserve*, eds. N. Nadkarni and N. Wheelwright. Oxford: Oxford Univ. Press.

———, B. BREMER, and K. J. SYTSMA. 1998. Reorganization of the genus *Psychotria* and tribe *Psychotrieae* (Rubiaceae) inferred from ITS and *rbcL* sequence data. Systematic Botany (In press).

NICKRENT, D. L., K. P. SCHUETTE, and E. M. STARR. 1994. A molecular phylogeny of *Arceuthobium* (Viscaceae) based on nuclear ribosomal DNA internal transcribed spacer sequences. American Journal of Botany 81: 1149–1160.

NIEUWLAND, J. A. and R. M. KACZMAREK. 1914. Studies in *Viola*. I. Proposed segregates of *Viola*. American Midland Naturalist 3: 207–217.

OLMSTEAD, R. G., B. BREMER, K. M. SCOTT, and J. D. PALMER. 1993. A parsimony analysis of the Asteridae *sensu lato* based on *rbcL* sequences. Annals of the Missouri Botanical Garden 80: 700–722.

OLSEN, G. J., H. MATSUDA, R. HAGSTROM, and R. OVERBEEK. 1994. *fastDNAml 1.0.6*. Urbana: Univ. of Illinois.

OXELMAN, B. and M. LIDÉN. 1995. Generic boundaries in the tribe *Sileneae* (Caryophyllaceae) as inferred from nuclear rDNA sequences. Taxon 44: 525–542.

PAGE, R. D. M. 1993. On islands of trees and the efficacy of different methods of branch swapping in finding most-parsimonious trees. Systematic Biology 42: 200–210.

PORTER, J. M. 1993. Phylogeny of the Polemoniaceae based on nuclear ribosomal internal transcribed spacer sequences. American Journal of Botany (supplement) 80: 171.

RUSSELL, N. H. 1965. Violets (*Viola*) of eastern United States: An introductory survey. Sida 1: 1–113.

SANDERSON, M. J. 1989. Confidence limits on phylogenies: the bootstrap revisited. Cladistics 5: 113–129.

———and M. F. WOJCIECHOWSKI. 1996. Diversification rates in a temperate legume clade: are there "so many species" of *Astragalus* (Fabaceae)? American Journal of Botany 83: 1488–1502.

SANG, T., D. J. CRAWFORD, and T. F. STUESSY. 1995. Documentation of reticulate evolution in peonies (*Paeonia*)

using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. Proceedings of the National Academy of Sciences, U.S.A. 92: 6813–6817.

SMITH, J. F., K. J. SYTSMA, J. S. SHOEMAKER, and R. L. SMITH. 1991. A qualitative comparison of total cellular DNA extraction protocols. Phytochemical Bulletin 23: 2–9.

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

SUH, Y., L. B. THIEN, H. E. REEVE, and E. A. ZIMMER. 1993. Molecular evolution and phylogenetic implications of internal transcribed spacer sequences of ribosomal DNA in Winteraceae. American Journal of Botany 80: 1042–1055.

SUSANNA, A., N. G. JACAS, D. E. SOLTIS, and P. S. SOLTIS. 1995. Phylogenetic relationships in tribe *Cardueae* (Asteraceae) based on ITS sequences. American Journal of Botany 82: 1056–1068.

SYTSMA, K. J. and D. A. BAUM. 1996. Molecular phylogenies and the diversification of angiosperms. Pp. 314–340 in *Flowering plant origins, evolution and phylogeny*, eds. D. W. Taylor and L. J. Hickey. New York: Chapman and Hall.

———and W. J. HAHN. 1996. Molecular systematics: 1994–1995. Progress in Botany 58: 470–499.

VALENTINE, D. H. 1962. Variation and evolution in the genus *Viola*. Preslia 34: 190–206.

WENDEL, J. F., A. SCHNABEL, and T. SEELANAN. 1995a. Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). Proceedings of the National Academy of Sciences, U.S.A. 92: 280–284.

———, ———, and———. 1995b. An unusual ribosomal DNA sequence from *Gossypium gossypioides* reveals ancient, cryptic, intergenomic introgression. Molecular Phylogenetics and Evolution 4: 298–313.

WHITE, T. J., T. BIRNS, S. LEE, and J. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315–322 in *PCR protocols: a guide to methods and applications*, eds. M. Innis, D. Gelfand, J. Sninsky, and T. White. San Diego: Academic Press.

WOJCIECHOWSKI, M. F., M. J. SANDERSON, B. G. BALDWIN, and M. J. DONOGHUE. 1993. Monophyly of aneuploid *Astragalus* (Fabaceae): evidence from nuclear ribosomal DNA internal transcribed spacer sequences. American Journal of Botany 80: 711–722.

YUAN, Y.-M. and P. KUPFER. 1995. Molecular phylogenetics of the subtribe *Gentianinae* (Gentianaceae) inferred from the sequences of internal transcribed spacers (ITS) of nuclear ribosomal DNA. Plant Systematics and Evolution 196: 207–226.

YUZEPCHUK, S. V. and M. V. KLOKOV. 1974. Violaceae. Pp. 262–360 in *Flora of the U.S.S.R.*, ed. B. K. Shishkin. Jerusalem: Israel Program for Scientific Translations.