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High paraphyly of *Swertia* L. (Gentianaceae) in the *Gentianella*-lineage as revealed by nuclear and chloroplast DNA sequence variation

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Abstract. The genus *Swertia* L., as currently defined, is polymorphic and mainly distributed in temperate regions of the northern hemisphere. Phylogenetic relationships between *Swertia* and the other genera of the Swertiinae sensu Struwe et al. (unpubl. data) are discussed based on cladistic analyses of DNA sequence data. The sequences used for this purpose include the *trn*L (UAA) intron, the intergenic spacers (IGS) between *trn*L (UAA) and *trn*F (GAA) exons, and between *trn*S (UGA) and *ycf*9 exons of cpDNA, as well as the ITS region of nrDNA. Although moderately resolved, the phylogenies resulting from the separate analyses of nuclear and chloroplast data are congruent, and the incongruence length difference test (Farris et al. 1995) detected no character incongruence. The phylogeny suggested by the analysis of combined data sets defines *Swertia* as strongly paraphyletic in relation to the other genera. This taxon may have acted as a stem group, giving rise to diverse lineages, some of which are morphologically distinct and have been recognised at the generic level. *Latouchea* and *Obolaria* are closely related and occupy the basalmost position in the molecular tree. *Swertia* species are distributed in 9 different clades, three of which share a basal polytomy with *Bartonia*, *Frasera*, *Gentianopsis*, *Halenia*, *Megacodon*, *Pterygocalyx* and *Veratrilla*. Two lineages have an intermediate position. The remaining 4 clades occupy a more derived position. Two of the latter clades show a close relation with species of *Gentianella* s. str., and one is included in a large clade comprising *Comastoma*, *Jaeschkea* and *Lomatogonium*. Selected character states and their proposed polarity, such as number and structure of nectaries, stylar and seedcoat characteristics, pollen morphology, fusion of floral parts and chromosome number are discussed in the context of molecular data. Rugose, spinose, or winged seeds are found mainly in basal lineages, while smooth ones are typical for derived species. Chromosome numbers follow a similar pattern with $x = 13$ restricted to basal lineages, while in more derived clades, x is always smaller than 13. With respect to the molecular phylogeny, taxonomic circumscriptions in the Swertiinae sensu Struwe et al. (unpubl. data) does not seem to reflect phyletic relationships.

Key words: Gentianaceae, *Swertia*, phylogeny, paraphyly, nectary, ITS, *trn*L, *trn*L-F, *trn*S, *ycf*9.

Introduction

Since the last infrafamilial classification of Gentianaceae published by Gilg (1895), the circumscription of the subtribe Gentianinae has remained quite stable. *Obolaria* and

Bartonia were included in Grisebach's (1845) tribe Swertieae by Bentham and Hooker (1876) but were subsequently removed from the Gentianinae by Gilg (1895). Both genera were reincluded therein following the recent molecular results of Struwe et al. (1998). The *Gentianella*-lineage (Gillett 1957; Toyokuni 1962, 1963; Ho and Liu 1990), in opposition to the *Gentiana*-lineage, was defined to include those genera of the subtribe Gentianinae sensu Gilg (1895) that have corolline nectaries, corolla lobes with 5–9 vascular bundles, corollas without plicae or folds and calyces without an intracalycular membrane (present in *Gentianopsis* but of a different nature than in *Gentiana* (Gillett 1957)). The genera that fall into this category and that are dealt with in this study are *Comastoma*, *Frasera*, *Gentianella*, *Gentianopsis*, *Halenia*, *Jaeschkea*, *Lomatogonium*, *Swertia* and *Veratrilla*. The description of the *Gentianella*-lineage was a first step towards the recognition of two distinct evolutionary lineages in the Gentianinae Gilg. Previous molecular studies have confirmed these two lineages (Yuan and Küpfer 1995, Struwe et al. 1998). *Pterygocalyx* and *Megacodon* have whorls of nectaries at the base of the ovary (Ho and Pringle 1995) but were found to be distinct from *Gentiana* and its allied genera by the molecular studies of Yuan and Küpfer (1995) and therefore also included in the *Gentianella*-lineage. *Latouchea* also has whorled nectaries at the base of the ovary but has not been sequenced prior to this study. Struwe et al. (unpubl. data) consider that Gilg's subtribe Gentianinae should be raised to a tribal status and further divided into two subtribes. These are subtribe Gentianinae (*Crawfurdia*, *Gentiana*, *Tripterospermum*) and subtribe Swertiinae (*Bartonia*, *Comastoma*, *Frasera*, *Gentianella*, *Gentianopsis*, *Halenia*, *Jaeschkea*, *Latouchea*, *Lomatogonium*, *Megacodon*, *Obolaria*, *Pterygocalyx*, *Swertia*, *Veratrilla*). Therefore, the *Gentianella*-lineage, as used in the text and figures, is herewith defined to include all the aforementioned genera and corresponds to the subtribe Swertiinae sensu Struwe et al.

The genus *Swertia* L. is cosmopolitan in distribution, although its ca. 150 species mainly occur in temperate regions of the northern hemisphere. The genus is, however, represented in tropical regions and in the southern hemisphere, and its highest species diversity is in the Himalayas and in south-western China (Meusel et al. 1978). Owing to the highest species diversity and the occurrence of taxa with the presumed ancestral characters (i.e. tall perennial plants, pentamery, few-flowered inflorescences, rugose seeds), Ho et al. (1994) argued that south-western China is the centre of origin of the genus. From there, *Swertia* has perhaps diversified and dispersed to south-east Asia as well to Africa and North America, where they have formed two secondary centres of diversification. There are two taxa in the Arabian Peninsula and one species in Madagascar. The genus is absent from Australia, New Zealand and South and Central America.

Swertia was described by Linnaeus (1753), in honour of Emanuel Swert, a botanical author of the 17th century. The circumscription of the genus has often been debated, resulting in disagreements among researchers of the family. Part of this debate is due to the morphological similarities (i.e. nectariferous and rotate corolla lobes) of the species of *Swertia* and related genera to one another, namely, *Halenia*, *Lomatogonium* and *Veratrilla*. Many species of these genera were described under *Swertia*, e.g. *Lomatogonium gamosepalum* (as *S. gamosepala*), *Veratrilla baillonii* (as *S. mekongensis*), *Halenia corniculata* (as *S. corniculata*). These genera are now widely accepted as distinct from and perhaps more advanced in evolution than *Swertia* (Allen 1933, Liu and Ho 1992). *Lomatogonium* was established as a distinct genus by Braun (1830) based on *Gentiana carinthiaca* Froehl. on grounds of its decurrent stigma. *Veratrilla* is dioecious and *Halenia* has spurred corolla lobes except in sect. *Swertiella* (Allen 1933) where the corolla lobes have short protuberances.

In addition to the similarities with the aforementioned genera, the definition of

Swertia proper has varied significantly since it was first described by Linnaeus. Many other genera were segregated from it, while others were redundantly described from Asia, North America, Europe and Africa. The following taxa are now recognised as synonyms of *Swertia* (Shah 1990, 1992; Pringle 1993; Ho and Pringle 1995; Garg 1987): *Frasera* Walter (1788), *Tesseranthium* Kellogg (1862) and *Leucocraspedum* Rydb. (1917) from North America; *Anagallidium*, *Ophelia*, *Agathotes* Griseb. (1839, 1845) and *Kingdon-Wardia* C. Marquand (1929) from the Himalayas; *Sczukinia*, *Stellera* and *Rellesta* Turcz. (1840, 1849) from eastern Asia; *Monobothrium* Hochst. (1844) from Africa; *Swertopsis* Makino (1891) from Japan; *Blepharaden* Dulac (1867) from the Pyrénées. *Henricea* Lem. (1824) is not valid due to its earlier use in Asteraceae. For Grisebach (1845), the generic concept of *Swertia* was narrow, that is, *Anagallidium*, *Stellera* and *Ophelia* were segregated from *Swertia*, and *Frasera* was accepted as a distinct genus. Bentham and Hooker's (1876) generic concept of *Swertia* was basically similar to that of Grisebach. Gilg (1895) listed the species of *Frasera* under his section *Euswertia*. *Frasera* is perhaps the most controversial of the variously described synonyms of *Swertia*. Kuntze (1891) discussed its generic relationships and reduced the taxon under *Swertia*. Card (1931) revised *Frasera* and recognised it as a distinct genus. *Frasera* was restored to *Swertia* by St. John (1941) and extensively dealt with by Pringle (1979, 1990) where evidence was presented in favour of its inclusion in *Swertia*. On the other hand, Toyokuni (1965) even went beyond recognising *Frasera* as a distinct genus and has moved some species of *Swertia*, e.g. *S. tashiroi*, *S. pseudochinensis* and *S. bimaculata* to *Frasera* and made new combinations of names accordingly. However, Toyokuni's systematic treatment of *Frasera* has not been widely accepted. It is evident from this brief account that the generic concept of *Swertia* has never been stable, mainly because macromorphological characters do not exhibit clear enough patterns to unambiguously justify taxonomic units.

Nilsson (1964, 1967, 1970) initiated extensive pollen micromorphological studies in the Gentianinae to shed a new light on possible relationships. *Bartonia* and *Obolaria* were examined by Nilsson and Skvarla (1969). African species of *Swertia* were extensively investigated by Jonsson (1973). Pollen morphology in the subtribe was found to be variable although its utility for taxonomic purpose seems limited. A few genera or sections are clearly discriminated by their palynological features, but the authors also report that similarities between other genera and sections are too confusing to allow a comparison. In the case of *Obolaria*, pollen morphology does not even support its inclusion in the Gentianinae preferably to the Chironiinae (as Erythraeinae in Nilsson and Skvarla 1969). Nevertheless, Nilsson (1967) described palynological affinities between *Frasera* and *Halenia*, and between *Lomatogonium* and *Comastoma*, and also noted that the pollen of these four genera shares common aspects. The pollen of *Swertia* is morphologically heterogeneous and variable. African species could be categorised into three groups, but no such clear distinction could be made for Asiatic species. The genera of the subtribe, thus, exhibit reticulate relationships with regard to their palynological data.

Some authors have used karyological data to attempt to establish natural genera and phyletic hypotheses in the *Gentianella*-lineage. Löve and Löve (1956) for example reduced *Comastoma* to a section of *Lomatogonium* on the grounds of shared base numbers ($x = 5$). Toyokuni (1965) on the other hand recognised three sub-groups based on morphological similarities in the *Gentianella*-lineage and, following Favarger (1962), favoured the hypothesis of parallel evolution of karyotypes to account for the co-occurrence of identical base numbers in the three sub-lineages (*Halenia* with $x = 11$, *Swertia-Frasera* with $x = 5, 12, 13$, *Gentianella*, *Gentianopsis*, *Comastoma*, *Lomatogonium*, *Jaeschkea* with $x = 5, 9, 11, 13$). Karyological considerations were also brought up by Post (1956) to justify the generic rank of *Frasera*. Unfortunately, relatively few species were cytologically documented at that time

(i.e. 6 for *Swertia* s. l., and even less for the other genera), and these views were not widely adopted.

Previous molecular results (Yuan and Küpfer 1995, von Hagen and Kadereit 2001) revealed possible polyphyly or paraphyly in *Swertia*, but the limited number of species sampled prohibited estimating to which extent. In view of the summary of the systematic problems involved with regard to the generic concept of *Swertia* and the lack of a rigorous molecular phylogeny of the members of the *Gentianella*-lineage, the present study mainly addresses 1) the circumscription of *Swertia*, i.e., to test the monophyly of *Swertia* in regard to its current taxonomic definition, 2) the systematic position and affinities of *Swertia* to related genera in the *Gentianella*-lineage and 3) the phylogeny of the genera of the *Gentianella*-lineage.

Materials and methods

Taxon sampling. The species included in this study are listed in Table 1. Those sequences retrieved from Genbank are indicated with an asterisk following their accession numbers. All the genera included in the Swertiinae sensu Struwe et al. (unpubl. data) are represented. Sampling of *Swertia* species has taken the following parameters into consideration: geographical distribution, morphological variation such as number and features of nectaries on corolla lobes, seed surface morphology (smooth and rugose), palynology, habit (annual, monocarpic and polycarpic perennials) and existing infrageneric classifications (Ho et al. 1994; Shah 1990, 1992). All the geographical and morphological groups in this taxon are sampled, except for a few monotypic groups for which material was not available. A total of 48 operational taxonomic units (OTUs) are included in this study as representatives of the ingroup taxa. Three *Gentiana* species were used as outgroup, following the latest molecular studies of the Gentianaceae – Gentianinae (Yuan and Küpfer 1995, Struwe et al. 1998). They were selected from 26 other species of the *Gentiana*-lineage (including *Crawfurdia* and *Tripterospermum*) following a preliminary analysis of ITS sequences such as to minimise long-branch attraction between ingroup and outgroup taxa.

Molecular markers. Five molecular markers were used in this study, i.e. internal transcribed spacers of nrDNA (ITS1 and ITS2), and noncoding regions of chloroplast DNA (*trn*L intron, *trn*L-F and *trn*S-*ycf*9 intergenic spacers). Primers ITS5 (5′-GGAAGTAGAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990) were used for the amplification of the internal transcribed spacers of the nuclear ribosomal DNA. The name and sequences of primers used for the amplification of *trn*L intron and IGS *trn*L-F follow Taberlet et al. (1991). Primers "c" (5′-CGAAATCGGTAGACGCTACG-3′), "d" (5′-GGGGATAGAGGGGACTTGAAC-3′) were used for the amplification of *trn*L (UAA) intron and "e" (5′-GGTTCAAGTCCCTCTATCCCC-3′) and "f" (5′-ATTTGAACTGGTGACACGAG-3′) were used for the spacer between *trn*L- (UAA)- 3′-exon and *trn*F-(GAA) gene. The spacer between *trn*S- (UGA) and *ycf*9 genes was amplified with primers *trn*S (5′-GAGAGAGAGGGATTCGAACC-3′) and *trn*fM (5′-CATAACCTTGAGGTCACGGG-3′) (Demesure et al. 1995).

DNA extraction. DNA was extracted from silica gel dried leaf tissue (Chase and Hills 1991). Total DNA extraction was made using the CTAB procedure of Doyle and Doyle (1987).

Polymerase chain reaction (PCR). Double-stranded DNA was directly amplified by PCR of all markers. Reaction volumes were 25 µl and contained 2.5 µl 10X PCR buffer, 1 µl 25mM Mg^{++}, 0.5 µl 10mM dNTPs, 0.5 µl of 10mM primers, 0.2 µl HotstarTaq polymerase (5u/µl) (QIAGEN, Basel), and 17.05 µl ddH_2O. About 10–20 ng genomic DNA was added to the PCR cocktail. PCR was performed in a Biometra® Tgradient thermal cycler and consisted of 15 min at 95 °C for the activation of the Hotstar polymerase, followed by 30 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 1 min 30 sec at 72 °C with a final extension period of 4 min at 72 °C.

PCR purification and sequencing. The quality and quantity of the PCR products were checked on 0.8% agarose gel using a mini-gel applying a low voltage. There was always a single sharp band resolved on the gel for ITS, *trn*L intron and IGS *trn*L-F after each polymerase chain reaction. PCR products were purified using QIAquick™

Table 1. Origin of plant material, voucher information and Genbank accession numbers. Sequences retrieved from Genbank for the purpose of this study are marked with an asterisk

Taxon	Collector	Voucher	Locality	Genbank number *trn*L(UAA) intron	*trn*S(UGA)-*ycf*9 spacer	*trn*L(UAA)-F(GAA) spacer	ITS1	ITS2
Bartonia virginica (L.) Britton Stearns & Poggenb.	Strong	2394	USA, Virginia, Sussex country	AJ315185	AJ315279	AJ315231	AJ318533	AJ410312
Comastoma pulmonarium (Turcz.) Toyok.	Yuan & Küpfer	NEU 92-279	China, Sichuan, Ganzi	AJ315225	AJ315319	AJ315271	Z48108*	Z48121*
Comastoma traillianum (Forrest) Holub	Yuan & Küpfer	NEU 92-197	China, Yunnan, Zhongdian	AJ315186	AJ315280	AJ315232	AJ318534	AJ410313
Frasera albomarginata S. Watson	Schweich	NEU 00-23	USA, California, San Bernardino county	AJ315187	AJ315281	AJ315233	AJ318535	AJ410314
Frasera speciosa Griseb.	Yuan	NEU 91-52	USA, Colorado, Boulder	AJ315230	AJ315324	AJ315276	Z48146*	Z48124*
Gentiana frigida Haenke	Yuan	NEU 93-17	Bulgaria, Mt. Rila	X77883*	AJ315325	AJ315277	Z48063*	Z48084*
Gentiana lutea L.	Yuan	NEU Y91-S5	Switzerland, Neuchâtel	X75702*	AJ315326	AJ315278	Z48122*	Z48119*
Gentiana phyllocalyx C. B. Clarke	Chassot	NEU 97-30	Nepal, Makalu	AJ315189	AJ315283	AJ315235	AJ318537	AJ410316
Gentianella foliosa (Kunth.) Fabris	Chassot	NEU 00-3	Ecuador, Pichincha. Volcán Illiniza	AJ315190	AJ315284	AJ315236	AJ318538	AJ410317
Gentianella umbellata (M. Bieb.) Holub	Küpfer	NEU 91-G3	Georgia, Mt. Caucasus	AJ315226	AJ315320	AJ315272	Z48102*	Z48132*
Gentianopsis contorta (Royle) Ma	Chassot	NEU 97-69	Nepal, Rara lake	AJ315191	AJ315285	AJ315237	AJ318539	AJ410318
Gentianopsis grandis (Harry Sm.) Ma	Yuan & Küpfer	NEU 92-222	China, Yunnan, Lijiang	AJ315227	AJ315321	AJ315273	Z48105*	Z48130*
Halenia brevicornis G. Don	Mansion & Zeltner	NEU 990115	Mexico, Las Piedracitas	AJ315192	AJ315286	AJ315238	AJ318540	AJ410319

Table 1 (continued)

Taxon	Collector	Voucher	Locality	Genbank number				
				*trn*L(UAA) intron	*trn*S(UGA)-*ycf*9 spacer	*trn*L(UAA)-F(GAA) spacer	ITS1	ITS2
Halenia elliptica D. Don	Yuan & Küpfer	NEU 93-52	China, Sichuan, Shiqü	AJ315193	AJ315287	AJ315239	AJ318541	AJ410320
Halenia weddeliana Gilg	Chassot	NEU 00-5	Ecuador, Pichincha. Volcán Illiniza	AJ315194	AJ315288	AJ315240	AJ318542	AJ410321
Jaeschkea microsperma C. B. Clarke	Yuan & Küpfer	NEU 92-107	China, Tibet, Nyalam	AJ315195	AJ315289	AJ315241	AJ318543	AJ410322
Latouchea fokienensis Franch.	Yuan	NEU cn2k-14	China, Fujian, Wuyishan	AJ315196	AJ315290	AJ315242	AJ318544	AJ410323
Lomatogonium bellum (Hemsl.) H. Smith	Yuan & Küpfer	NEU 92-236	China, Yunnan, Lijiang	AJ315197	AJ315291	AJ315243	AJ318545	AJ410324
Lomatogonium brachyantherum (C. B. Clarke) Fernald	Chassot	NEU 97-13	Nepal, Langtang	AJ315198	AJ315292	AJ315244	AJ318546	AJ410325
Lomatogonium macranthum (Diels & Gilg) Fernald	Yuan & Küpfer	NEU 93-91	China, Sichuan, Ganzi	AJ315228	AJ315322	AJ315274	Z48108*	Z48135*
Lomatogonium perenne T. N. Ho & S. W. Liu	Chassot & Yuan	NEU 99-78	China, Yunnan, Deqin	AJ315199	AJ315293	AJ315245	AJ318547	AJ410326
Megacodon stylophorus (C. B. Clarke) Harry Sm.	Chassot & Yuan	NEU 99-36	China, Yunnan, Zhongdian	AJ315200	AJ315294	AJ315246	AJ318548	AJ410327
Obolaria virginica L.	Nicolson	24-IV-00	USA, Virginia	AJ315201	AJ315295	AJ315247	AJ318549	AJ410328
Pterygocalyx volubilis Maxim.	Chassot & Yuan	NEU 99-100	China, Yunnan, Deqin	AJ315202	AJ315296	AJ315248	AJ318550	AJ410329
Swertia angustifolia Ham. ex D. Don	Chassot & Yuan	NEU 99-172	China, Yunnan, Binchan	AJ315203	AJ315297	AJ315249	AJ318551	AJ410330
Swertia bimaculata Hook. f. & Thomas. ex C. B. Clarke	Chassot	NEU 97Z-22	Nepal, Makalu	AJ315204	AJ315298	AJ315250	AJ318552	AJ410331
Swertia binchuanensis T. N. Ho & S. W. Liu	Chassot & Yuan	NEU 99-160	China, Yunnan, Binchan	AJ315205	AJ315299	AJ315251	AJ318553	AJ410332
Swertia calycina Franch.	Yuan & Küpfer	NEU 92-232	China, Yunnan, Lijiang	AJ315206	AJ315300	AJ315252	AJ318554	AJ410333

Swertia chirayita Karst.	Chassot	NEU 97-2	Nepal, Langtang	AJ315207	AJ315301	AJ315253	AJ318555	AJ410334
Swertia ciliata (D. Don ex G. Don) B. L. Burtt	Chassot	NEU 97-6	Nepal, Langtang	AJ315208	AJ315302	AJ315254	AJ318556	AJ410335
Swertia cordata (Wall. ex D. Don) C. B. Clarke	Chassot	NEU 97-17	Nepal, Langtang	AJ315209	AJ315303	AJ315255	AJ318557	AJ410336
Swertia crassiuscula Gilg	Wohlhauser	NEU 98-5	Kenya, Mt. Kenya	AJ315210	AJ315304	AJ315256	AJ318558	AJ410337
Swertia cuneata Wall. ex D. Don	Chassot	NEU 97-14	Nepal, Langtang	AJ315211	AJ315305	AJ315257	AJ318559	AJ410338
Swertia decora Franch.	Chassot & Yuan	NEU 99-176	China, Yunnan, Dali	AJ315212	AJ315306	AJ315258	AJ318559	AJ410339
Swertia delavayi Franch.	Chassot & Yuan	NEU 99-66	China, Yunnan, Zhongdian	AJ315213	AJ315307	AJ315259	AJ318561	AJ410340
Swertia engleri Gilg	Sileshi Nemomissa	ETH 990517-8/3	Ethiopia, Simen mts.	AJ315214	AJ315308	AJ315260	AJ318562	AJ410341
Swertia hispidicalyx Burkill	Yuan & Küpfer	NEU 92-72	China, Tibet, Nagarze	AJ315215	AJ315309	AJ315261	AJ318563	AJ410342
Swertia kilimandscharica Engl.	Sileshi Nemomissa	ETH 990725-2/1	Ethiopia, Bale mts.	AJ315216	AJ315310	AJ315262	AJ318564	AJ410343
Swertia macrosperma C. B. Clarke	Yuan & Küpfer	NEU 92-213	China, Yunnan, Lijiang	AJ315217	AJ315311	AJ315263	AJ318565	AJ410344
Swertia perennis L.	Küpfer	NEU 98-10	Switzerland	AJ315218	AJ315312	AJ315264	AJ318566	AJ410345
Swertia aff. *pseudohookeri* Harry Sm.	Chassot	NEU 97-28	Nepal, Makalu	AJ315219	AJ315313	AJ315265	AJ318567	AJ410346
Swertia pubescens Franch.	Yuan & Küpfer	NEU 92-224	China, Yunnan, Lijiang	AJ315220	AJ315314	AJ315266	AJ318568	AJ410347
Swertia punicea Hemsl.	Chassot & Yuan	NEU 99-63	China, Yunnan, Zhongdian	AJ315221	AJ315315	AJ315267	AJ318569	AJ410348
Swertia tashiroi (Maxim.) Makino	Chassot	NEU 99-7b	Taiwan, Hsinch	AJ315222	AJ315316	AJ315268	AJ318570	AJ410349
Swertia tetraptera Maxim.	Yuan & Küpfer	NEU 92-315	China, Gansu, Magu	AJ315229	AJ315323	AJ315275	Z48115*	Z48139*
Swertia volkensii Gilg	Wohlhauser	NEU 98-4	Kenya, Mt. Kenya	AJ315223	AJ315317	AJ315269	AJ318571	AJ410350
Swertia yunnanensis Burkill	Chassot & Yuan	NEU 99-200	China, Yunnan, Lijiang	AJ315224	AJ315318	AJ315270	AJ318572	AJ410351
Veratrilla baillonii Franch.	Chassot & Yuan	NEU 99-37	China, Yunnan, Zhongdian	AJ315188	AJ315282	AJ315234	AJ318536	AJ410315

purification kit (QIAGEN AG, Base1) following the manufacturer's protocol prior to sequencing.

Cycle sequencing was performed using the dideoxy chain termination method using an ABI PRISM™ BigDye™ Terminator cycle sequencing kit with AmpliTaq DNA polymerase FS (Applied Biosystems) in a Biometra® Tgradient thermal cycler (5 µl reaction volumes). Cycling parameters were 25 cycles of 20 sec at 96 °C for denaturation, 10 sec at 54 °C for primer annealing and 4 min at 60 °C for primer extension. The cycle sequencing products were cleaned by ethanol precipitation and applied to the ABI 310 automated sequencer (Applied Biosystems). Basecalling was checked on the chromatograms using Sequence Navigator (Applied Biosystems) and edited manually when necessary. Sequences have been deposited in Genbank with their accession numbers indicated in Table 1.

Sequence alignment. The boundaries of ITS and *trn*L intron in the study material were determined by comparison with *Gentiana* sequences (Yuan and Küpfer 1995, Gielly et al. 1996). The limits of IGS *trn*S-*ycf*9 and IGS *trn*L-F were established by comparison with sequences of *Arabidopsis thaliana*, *Nicotiana tabacum* and *Zea mais* (Genbank numbers: AP000423, ZOOO44, X86563). ITS sequences were preliminarily aligned with ClustalX (Tompson et al. 1997) and subsequently hand-adjusted. Chloroplast sequences were aligned manually in order to respect indel events otherwise not recognised. Potentially informative indels of more than 1 base were recoded (1 for presence, 0 for absence) and added to the data matrix. The data matrix is available from the authors on request.

Phylogenetic analyses. All data sets were analysed with heuristic parsimony searches using PAUP*, v.4.0b4 (Swofford 2000). In all analyses characters were equally weighted and unordered (Fitch 1971). Gaps were treated as missing data. All searches consisted of 1000 random taxon addition sequences with TBR branch swapping, MULPARS and ACCTRAN options on; branches with minimum lengths of zero (''amb-'') were collapsed to form polytomies.

For the results based on both separate and combined data, the robustness of individual clades was evaluated using jackknife with 37% deletion (Farris 1996) as implemented in PAUP*, and decay index (Bremer 1988, Donoghue et al. 1992). Decay analyses were performed with AutoDecay (Eriksson and Wikström 1996) and trees were viewed with TREEVIEW (Page 1996).

A monophyletic *Swertia* topology was used as constraint tree in PAUP*. Most parsimonious trees found under this condition were compared with the unconstrained topologies using Templeton's significantly less parsimonious (SLP) test (Templeton 1983).

Data combination and congruence testing. By increasing the number of characters in an analysis, phylogenetic signal may assert itself over the noise (coincidental similarity due to homoplasy) from each individual data set, resulting in a more accurate estimate of the true phylogeny (Barrett et al. 1991, Mishler 1994, Olmstead and Sweere 1994). However, heterogeneity among data sets other than sampling error, such as different stochastic processes acting on the characters or different branching histories, can lead to erroneous phylogenetic inferences. Under such a circumstance it is justified to keep the data sets separate or to use a consensus method (Bull et al. 1993, de Queiroz et al. 1995). Following the conditional data combination approach (Huelsenbeck et al. 1996), we have used the incongruence length difference (ILD) test of Farris et al. (1995) (with 100 replicates and same settings as for other analyses) as implemented in PAUP* to assess the character congruence between the different data partitions (ITS1, ITS2, *trn*L, IGS *trn*L-F, IGS *trn*S-*ycf*9).

Results

Characteristics of ITS sequences. The spacer length ranges from 173 to 237 bases for ITS1 and from 226 to 232 bases for ITS2. When aligned, the consensus sequences have 268 and 267 sites for ITS1 and ITS2, respectively. Out of these, 6 were excluded from ITS1 and 16 from ITS2 because of alignment ambiguities, leaving respectively 116 and 113 informative sites. ITS1 sequences were found to be slightly less divergent (1%–25%) than ITS2 (1%–28%). Among species of *Swertia*, sequence divergence ranges from 0.5% up to 16%, whereas this difference can be much lower than between some *Swertia* species and species of markedly different genera like *Gentianella* and *Megacodon*. At 152 sites, gaps were needed to accommodate this relatively high

divergence, and only one potentially informative indel larger than 1 nucleotide was found.

ITS phylogeny. The parsimony analysis carried out using PAUP* resulted in 70 equally parsimonious phylogenetic trees of 980 steps (CI = 0.50, RI = 0.53 including autapomorphies). Their strict consensus is presented in Fig. 1a. The single possibly informative indel situated near the end of ITS2 has no influence on the resulting trees. Although relatively low, the ILD test value (p = 0.1) indicates that ITS1 and ITS2 are not significantly incongruent. Separate analysis of ITS1 and ITS2 offered a very poor resolution, high homoplasy indices and received no jackknife support. Some branches are exclusively found in the analysis of one or the other data set only and, in view of their lack of support, are likely to be due to

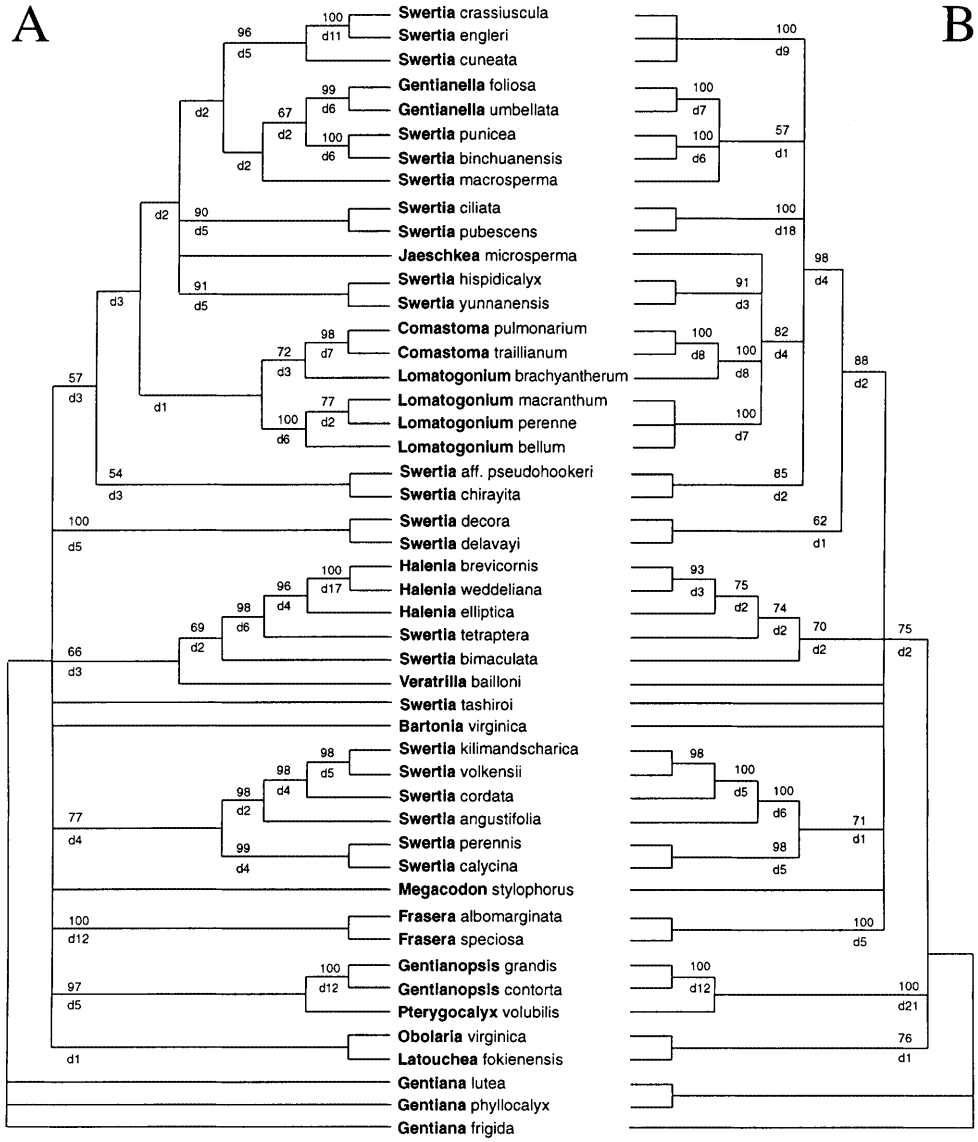

Fig. 1. Strict consensus trees of **A** 70 most parsimonious trees of 980 steps, CI = 0.50, RI = 0.53 (including autapomorphies) from nuclear DNA analysis; **B** 24 most parsimonious trees of 659 steps, CI = 0.75, RI = 0.77 (including autapomorphies) from chloroplast DNA analysis. Above branches: jackknife values if greater than 50%. Below branches: decay index

random error and may in turn account for the low ILD test value.

The ITS phylogeny presents an important polytomy at its base, that consists of several well supported clades. *Latouchea* forms a sister-group relationship with *Obolaria*. *Gentianopsis* and *Pterygocalyx* are strongly associated, as well as the two sampled species of *Frasera*, that join in an independent clade with high support. *Megacodon* forms an isolated branch, as does *Bartonia*. *Veratrilla* shows a sister group relationship to the *Halenia* – *Swertia* clade with moderate jackknife value (66%) and decay index (d = 3). *Swertia* is present pro parte in this basal polytomy, notably in the well-supported clade containing the type of the genus, *S. perennis*. Two species (*S. bimaculata* and *S. tetraptera*) show a close link with *Halenia*. *Swertia tashiroi* does not group with any other *Swertia* species present at the base of the tree. *Swertia decora* and *S. delavayi* also form a highly supported clade.

The upper part of the tree is better resolved, although jackknife support is below 50% for deeper nodes, with at its base the moderately supported clade of *S.* aff. *pseudohookeri* – *S. chirayita*. The *Lomatogonium* – *Comastoma* clade lacks support as a whole, but *L. brachyantherum* is associated to *Comastoma* with good jackknife and decay values. *Jaeschkea* is placed in a polytomy with the *S. ciliata* – *S. pubescens* clade and the *S. yunnanensis* – *S. hispidicalyx* clade. *Gentianella* species are more closely related to *Swertia* than to *Comastoma*, *Lomatogonium* or *Gentianopsis*. Within the uppermost *Swertia* clade, they formed a monophyletic sister taxon to *S. punicea* and *S. binchuanensis* with a jackknife and decay index support of 67% and 2, respectively.

Characteristics of chloroplast sequences. The chloroplast sequence length ranges from 361 to 393 bases for the *trn*L intron, from 234 to 403 bases for the IGS *trn*L-F and from 271 to 350 bases for the IGS *trn*S-*ycf*9. This variation is mainly due to long A/T repeats in *trn*L, and to indels in the two IGS's. When aligned, the sequences have 496, 678 and 474 sites for *trn*L, *trn*L-F and *trn*S-*ycf*9, respectively. Out of these, 90 were excluded from *trn*L, 245 from *trn*L-F and 21 from *trn*S-*ycf*9 because of alignment ambiguities, leaving respectively 59, 83 and 76 informative sites. *trn*L-F sequences were found to be slightly more divergent (0%–19%) than *trn*L (0%–12%) and *trn*S-*ycf*9 (0%–11%). When the three data sets are combined, the sequence divergence ranges from 0% to 14%. As noticed in ITS, chloroplast sequence divergence between *Swertia* species and other genera can be much lower than between two different species of *Swertia*. 32 potentially informative indels of more than 1 nucleotide were recoded and added to the data matrix (5 from *trn*L, 14 from IGS *trn*L-F and 13 from IGS *trn*S-*ycf*9).

***Trn*L intron, *trn*L-F and *trn*S-*ycf*9 intergenic spacer phylogeny.** The parsimony analysis of the chloroplast data resulted in 24 trees of 659 steps (CI = 0.75, RI = 0.77 including autapomorphies). The three chloroplast partitions were found to be congruent with p = 0.42. Figure 1b presents the strict consensus of the 24 most parsimonious trees, which offers a moderate degree of resolution. The basal polytomy suggested by the ITS data is partly resolved by the chloroplast sequences. The *Gentianopsis* – *Pterygocalyx* clade and the *Obolaria* – *Latouchea* clade appear at the base of the tree, with a jackknife value of 75% and a decay index of 2 supporting their segregation from the remaining taxa. Also, the *Swertia decora* – *S. delavayi* clade is excluded from this polytomy with good support. For the remaining taxa, the branching is identical to the one found by the analysis of ITS data, with the exception of *Veratrilla*, that is not placed inside the *Swertia* – *Halenia* clade.

The upper part of the tree consists of a large pentatomy, in which the individual clades are well supported. Here again, but with less resolution, the branching is similar to the ITS phylogeny, with the exception of *Jaeschkea* and the *S. yunnanensis* – *S. hispidicalyx* clade, that are included in a well supported clade along with *Lomatogonium* and *Comastoma*. As in the

ITS phylogeny, *L. brachyantherum* forms a sister group relationship with *Comastoma* species, although this clade does not group with the remainder of *Lomatogonium* species.

Combined nuclear and chloroplast DNA phylogeny. The ILD test probability was 0.54 in favour of homogeneity between nuclear and chloroplast data. All branches whose position differs across the trees received low statistical support in at least one tree, or formed a polytomy.

The analysis of equally weighted characters yielded 48 trees of 1649 steps (CI = 0.60, RI = 0.62). This represents only 10 more steps than the sum of the most parsimonious trees obtained by separate analysis of nuclear and chloroplast data. Figure 2 presents their strict consensus.

Statistical support is globally increased, not only for clades recognised independently from each data set, but also for the deeper nodes in the upper half of the tree. The phylogeny inferred from the combined data sets presents a different branching pattern than the ones obtained from separate analysis. In the combined tree, *Obolaria* and *Latouchea* are the basalmost taxa, as suggested by the chloroplast tree, whereas *Gentianopsis* and *Pterygocalyx* are placed in the large basal polytomy. Data combination has brought no further resolution of this polytomy, except for the grouping of *Bartonia* and *S. tashiroi*. The position retained for *Veratrilla* is the same as in the ITS tree. *Swertia decora* and *S. delavayi* are placed according to the chloroplast tree, whereas the position of *S. chirayita* and *S.* aff. *pseudohookeri* follows the ITS tree. *Jaeschkea* and the *S. hispidicalyx – S. yunnanensis* clade group with *Comastoma* and *Lomatogonium* as it is the case in the chloroplast phylogeny. The position of *Gentianella* species as sister group to *S. binchuanensis S. punicea* remains unchanged. The *S. crassiuscula – S. macrosperma* clade, present but poorly supported in the ITS tree, and not detected in the chloroplast tree, has a good jackknife support in the combined phylogeny.

The phylogenetic relationships resolved in these analyses confirm previous molecular studies suggesting the paraphyly of *Swertia* (Yuan and Küpfer 1995, von Hagen and Kadereit 2001). This idea has been further investigated by searching for trees compatible with a constrained monophyletic *Swertia* clade. Such trees are 31 steps longer (ITS), 16 steps longer (chloroplast data), and 46 steps longer (combined data) than the unconstrained most parsimonious trees, clearly departing from the notion of sub-optimality ($p < 0.1$ in SLP test for all data sets).

Discussion

Phylogeny of Swertiinae and *Swertia*. The potential difficulties inherent to the distinction between a gene tree and species phylogeny were explored in detail (e.g. Doyle 1992). Combining data sets increases the phylogenetic signal, which may in turn increase support for the "correct" phylogenetic tree (Bremer et al. 1999, Hardig et al. 2000, von Hagen and Kadereit 2001). The resolution of the internal clades and high jackknife values due to the analyses of the combined data sets in the *Gentianella*-lineage suggests that this is also the case in our study. The tree derived from the analysis of combined data sets was thus used as final phylogenetic hypothesis.

Swertia, as currently defined in regional floras or otherwise (Pringle 1990, 1993; Shah 1990, 1992; Ho and Pringle 1995), is not a monophyletic taxon when considering the molecular data. It is a strongly paraphyletic stem group, and the data suggest that different ancestral taxa or lineages of *Swertia* account for the origin of several morphologically different genera of the *Gentianella*-lineage. Nevertheless, despite the use of 5 different markers, parsimony analysis of our data still leaves two major polytomies unresolved.

Phylogenetic relationships in the *Gentianella*-lineage have been addressed previously (Yuan and Küpfer 1995, Struwe et al. 1998, von Hagen and Kadereit 2001), yet, only few of the concerned taxa were included, and the phylogenies were based on fewer markers. These studies differ from our results notably

by proposing additional resolution for some branches included in the polytomies of our tree, although these relationships receive only low statistical support. Whether the lack of synapomorphies that would allow resolving these polytomies is due to a rapid geographic isolation of these lineages is not clear. Nevertheless, it can be noted that many of the basal

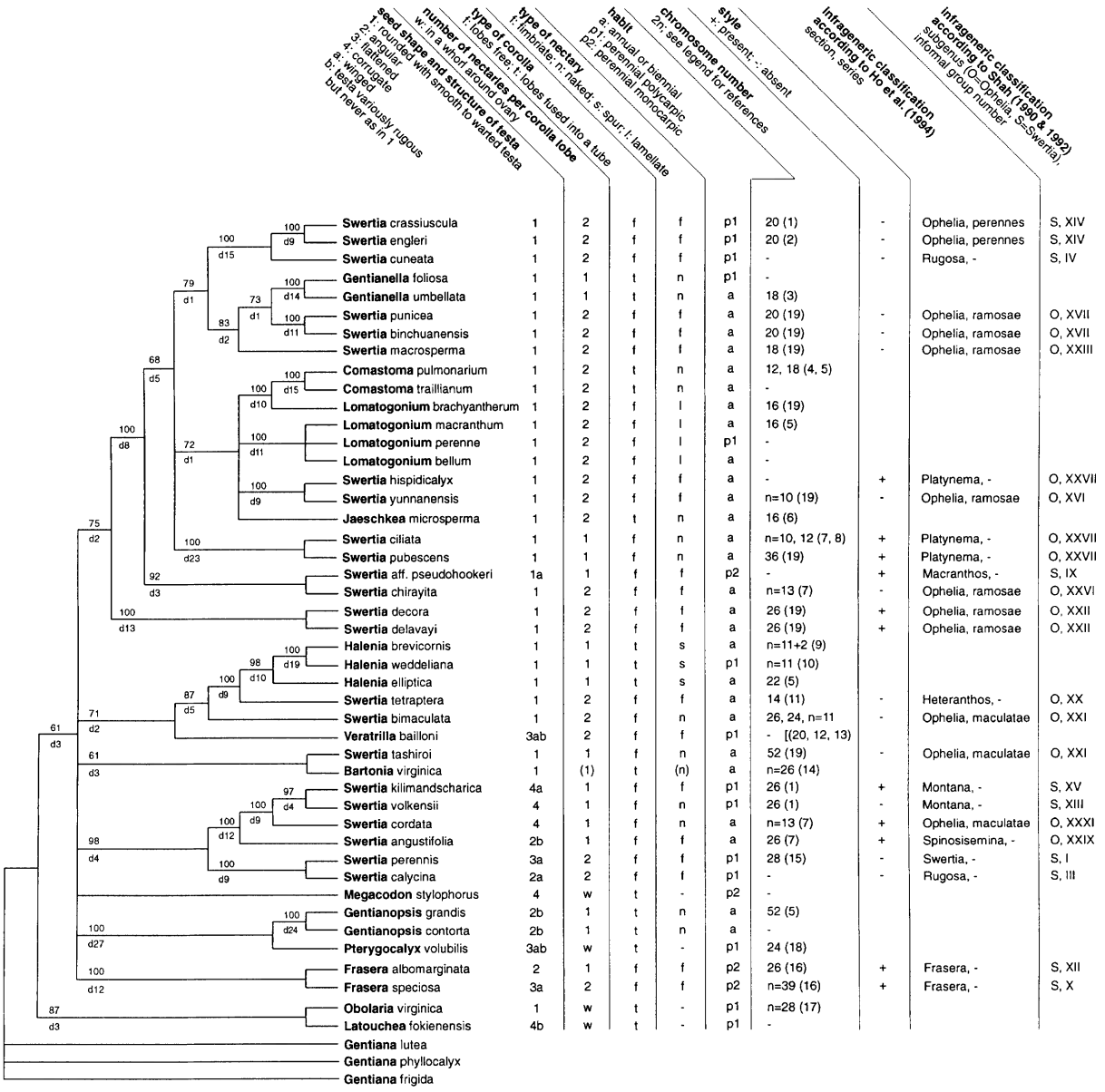

Fig. 2. Strict consensus tree of 48 most parsimonious trees of 1649 steps, CI = 0.60, RI = 0.62 (including autapomorphies) from analysis of combined data. Above branches: jackknife values if greater than 50%. Below branches: decay index. In regard: states for the principal characters discussed in the text. References for chromosome numbers: (1) Hedberg and Hedberg (1977), (2) Nemomissa (1994), (3) Gagnidze et al. (1992), (4) Krogulevitch (1978), (5) Yuan and Küpfer (1993), (6) Yuan et al. (1998), (7) Khoshoo and Tandon (1963), (8) Mehra and Gill (1968), (9) Weaver and Rudenberg (1975), (10) Pringle (1981), (11) Ho et al. (1999), (12) Shigenobu (1983), (13) Roy et al. (1988), (14) Rork (1949), (15) Favarger (1952), (16) Post (1956), (17) Kondo (1970), (18) Yuan and Küpfer (unpublished), (19) present study, (20) Wada (1954)

clades consist of geographically isolated taxa, and/or genera represented by only a few species. *Swertia tashiroi* is an insular endemic in Taiwan and the south-western islands of Japan. Its fleshy habit and chromosome number ($2n = 52$) distinguish it from all the other *Swertia* species included in the basal polytomy. *Megacodon* is a distinct genus comprising only two species restricted to the eastern Himalayas. *Veratrilla* is morphologically very close to *Swertia* but its two species are clearly dioecious, a very rare trait in the *Gentianella*-lineage. *Latouchea* (1 species) is endemic to south-eastern and south-western China. *Bartonia* (4 species) and *Obolaria* (1 species) are North American saprophytes. *Frasera* (14 species) is also restricted to North America and, despite an indubitable resemblance with *Swertia*, seems to have differentiated independently from the remaining *Swertia* species.

As suggested in Yuan and Küpfer's (1995) ITS tree and von Hagen and Kadereit's (2001) *mat*K/ITS tree, *Comastoma* and *Lomatogonium* also group together in our phylogeny, but in a broader clade including *Swertia* pro parte and *Jaeschkea*. *Lomatogonium* was found to be polyphyletic by von Hagen and Kadereit (2001) and our results also suggest its paraphyly in relation to *Comastoma*. *Lomatogonium brachyantherum* has none of the characteristics of *Comastoma* (corolla lobes fused in a tube with fringed scale at its base). The scenario that such an observed clustering of *L. brachyantherum* with *Comastoma* has its root in an erroneous definition of the taxon is ruled out. The *Gentianella* species form a clade corresponding to the *Gentianella* s. str. (uninectariate) clade of von Hagen and Kadereit (2001) with the same *Swertia* species as sister clade. Binectariate *Gentianella* species are absent from our taxon sample but were placed clearly outside of the *Gentianella* s. str. clade and close to *Lomatogonium* species in von Hagen and Kadereit's (2001) study. Note that *Jaeschkea microsperma* is included in the broad *Lomatogonium – Comastoma – Swertia* clade in our combined phylogeny, whereas *J. oligosperma* was found to be clearly separated from the former two genera in von Hagen and Kadereit's (2001) *mat*K/ITS tree.

Morphology and molecular data. The *Gentianella*-lineage is an assemblage of genera that exhibit intrageneric polymorphism on one hand, and unprecedented morphological ties with each other on the other. The presence of corolline nectaries, regardless of the fine details, is one major feature shared by nearly all genera and has been emphasised as representing a different line of evolution in subtribe Gentianinae (Meszaros 1994). According to Ho and Pringle (1995), nectaries are sometimes absent in *Pterygocalyx*. *Megacodon*, *Latouchea* and *Obolaria* are different by bearing nectaries in a whorl at the base of the ovary like in *Gentiana*. Regarding *Obolaria*, Holm (1897) states that "the corolla bears nectaries", and describes a scale halfway between the base of the filament and the base of the corolla tube. He then adds that "the grooves are very imperfect". However, Lindsey (1940) reports glandular material at the base of the ovary. Wood and Weaver (1982) believe that the latter are more likely the nectaries. Concerning *Bartonia*, neither Wood and Weaver (1982) nor Holm (1907) mention nectaries. A recent collection of *B. Virginica* was examined by the authors. The basal part of the corolla lobes was found to be thickened and of brownish-green colour. It was not possible to assess if this tissue is glandular but it is reminiscent of the nectariferous tissue found in *S. tashiroi* or *S. bimaculata*.

Corolline nectaries are either fimbriate or naked, with or without well-developed marginal scales, or even sunken in spurs as in *Halenia*. The nectary features such as number, as well as their nakedness or fimbriation were used for the segregation of genera. But in regard to the molecular phylogeny, nectaries cannot be considered as reliable synapomorphic characters at the generic level. Their number may be identical between two distantly related clades, and, similarly, naked nectaries characterise distantly related genera like *Bartonia* (?), *Comastoma*, *Gentianella*, *Gentianopsis*, *Pterygocalyx* as well as some species of *Swertia* included in this study, e.g.

S. bimaculata, *S. volkensii* and *S. tashiroi*. Fimbriate nectaries are found in *Frasera*, *Veratrilla* and *Swertia*, where the fimbriae can also occur in association with a more or less developed marginal scale. Spurs however are restricted to *Halenia* sect. *Haleniastrum* (Allen 1933). At the infrageneric level however, von Hagen and Kadereit (2001) have shown that nectary number and fimbriation are representative of phylogenetic relationships in *Gentianella*. Such a pattern is not found for *Swertia* species.

Little has been published on the pollination biology of *Swertia* and allied genera. In their observations, Khoshoo and Tandon (1963) note that even in mixed populations of cross-pollinated *Swertia*, nearly each studied species has its specific pollinator and that the shape and fimbriation of the nectaries seem to play an important role in the pollinator specificity. Under such pollinator-mediated selective pressure, both fimbriate and naked nectaries may have evolved several times and been acquired independently in the different lineages of the *Gentianella*-lineage from plesiomorphic whorled nectaries situated around the ovary as is seen in *Latouchea*, *Megacodon* and *Obolaria*. To be noted, is that in a majority of cases, the nectary itself is devoid of fimbriae in those taxa where it is not readily accessible, i.e. when the corolla lobes are fused (*Bartonia* (?), *Comastoma*, *Gentianella*, *Gentianopsis*, *Jaeschkea*), or when hidden inside a spur as in *Halenia* sect. *Haleniastrum* (Allen 1933). Similarly, a parallel tendency towards conspicuous fimbriation can be seen with the vascularised fimbriae at the base of the corolla lobes in *Gentianella* and the comparable but nonvascularised structure in *Comastoma*. In *Gentianopsis*, fimbriae are absent, but the petals are fringed in many species.

When viewed in the context of the molecular phylogeny, the character state of seed shape and ornamentation doesn't appear as representative of relationships between genera. Many unrelated taxa have smooth rounded seeds, and, similarly, *Veratrilla bailloni*, *Swertia perennis* and *Frasera speciosa* for example all have flat annular winged seeds. Nevertheless, seeds with well-developed ridges, wings or coarsely papillose, and not rounded in shape occur only in basal lineages, i.e. *Latouchea*, *Pterygocalyx*, *Gentianopsis*, *Frasera*, *Megacodon*, *S. perennis – S. kilimandscharica* clade, *Veratrilla*. Concerning the sampled species of African *Swertia*, this character seems to be congruent with the molecular data and confirms the grouping proposed by Nemomissa (1994). For *Swertia* as a whole, it can be noted that seeds in the *S. perennis – S. kilimandscharica* clade are different from those of all other species. They are either corrugate-cristate (*S. volkensii*, *S. calycina*, *S. cordata*), polyhedral with spinose projections (*S. angustifolia*) or flat with an annular wing (*S. perennis)*. All other species of *Swertia* have seeds more or less rounded, with a smooth to warted testa (rarely small wings in the *S.* aff. *pseudohookeri* group). Future investigation of a broader taxon sample will allow assessing with more precision the reliability of this character.

Molecular results were also compared with the detailed pollen studies carried out by Nilsson (1964, 1967, 1970), Nilsson and Skvarla (1969) and Jonsson (1973). According to Nilsson, pollen morphological data are difficult to interpret due to similar pollen types in unrelated taxa throughout the *Gentianella*-lineage, and also to transitional sexine patterns when more material is investigated. Only few suggestions regarding inter-generic pollen morphological affinities were made. Nilsson's remark that the pollen structure of *Comastoma* and *Gentianopsis*/*Pterygocalyx* is clearly different from that of *Gentianella* is supported by our tree, as well as the affinity of *Comastoma* with *Lomatogonium*. Likewise, the pollen of *Frasera* is different from that of most other *Swertia* species. On the other hand, the similarity between the pollen of *Halenia* and *Lomatogonium*/*Comastoma* is not congruent with the molecular data. Concerning *Swertia*, Nilsson treated the species according to their geographic distribution and only made comments on a few interesting taxa. The African species are divided in 3 groups. In the *S. crassiuscula* type, the exine is beset with spinules.

The distinction between the other two groups (*S. kilimandscharica* and *S. volkensii* types) is less marked, and there are a number of species with transitional pollen morphology (Jonsson 1973), but both types are devoid of spinules. This is congruent with the molecular tree. Regarding the Asiatic species, such a clear correlation between Nilsson's five pollen types and our tree cannot be found. Spinuliferous exine seems to be a good character in discriminating the two African clades, but in the context of the whole *Gentianella*-lineage, this trait appears to be homoplasious. Beside the African species of the *S. crassiuscula* type, spinules are found in *S. cuneata*, the only Himalayan species belonging to the African *S. crassiuscula* clade, *S. tashiroi* and *Lomatogonium* p.p.

The presence of a style has been used as a distinguishing feature in the segregation of *Frasera* from *Swertia* by Bentham and Hooker (1876) and has been commonly retained by later authors as a diagnostic character of the taxon. At that time, little was known of the global variability of *Swertia*, but it is now evident that styled stigmata are found in many *Swertia* species. Besides, as Pringle (1990) mentions for *Swertia perennis* and *Frasera*, stylar differences are "a matter of relative length rather than being qualitative". When viewed in regard to the molecular tree, it is clear that this trait does not reflect phylogenetic relationships in the genus.

Another morphological marker worth considering is the fusion of corolla lobes to form a tube. Although petals are fused in all the taxa of the *Gentianella*-lineage, the tube is nearly inconspicuous in *Frasera*, *Lomatogonium*, *Swertia* and *Veratrilla*, in contrast to the other genera where it is well developed. This floral trait has significantly contributed to the confusion in the taxonomy of the *Gentianella*-lineage. As shown by the phylogenetic tree, corolla tubes have appeared and disappeared several times in the group. Therefore, phylogenetic inferences and subsequent grouping based on the extent of corolla tube formation may be misleading.

Ho et al. (1994) have suggested a polarity in the evolution of morphological characters for the purpose of phylogeographic hypotheses in *Swertia*. They define ancestral characters as tall, perennial plants with a solitary stem, with few, rather large pentamerous flowers and corrugate-cristate seeds (sect. *Rugosae*). More advanced species would be rather small annuals with many tetramerous flowers borne on branched stems, with smooth to warted, rounded seeds. A comparison with our phylogeny calls for the following remarks. The (perennial versus) annual habit (of the species) seems to be polyphyletic and reflects more ecological requirements than phylogenetic relationships. There are two examples in the tree where perennials and annuals occur in the same clade. *Swertia calycina* and related species are all perennial and mainly of alpine ecology. These species are closely related to annual species of the *S. angustifolia* type that occur at lower elevations. The same pattern is valid for the *S.* aff. *pseudohookeri* (perennial) and *S. chirayita* (annual) groups of species. As discussed above, the character state of rugose or winged seeds does seem to be partly synapomorphic with respect to our phylogeny and proper to the basal *S. calycina* – *S. kilimandscharica* lineage of *Swertia*.

Cytological data and taxonomy. As pointed out by Pringle (1990), earlier taxonomic definitions and phyletic hypotheses based on chromosome counts of a restrained number of species of the *Swertia* complex have not been confirmed by the increasing number of counts of new taxa. Many species in the *Gentianella*-lineage have been recently studied cytologically, giving rise to new considerations (summarised by Yuan 1996 and Pringle 1990). Concerning *Swertia* s. l., Pringle notes that there are two prevailing base numbers, i.e. $x = 13$ and $x = 10$, with a few aneuploid and polyploid species in both groups. This is congruent with our results, where all species from *S. pubescens* upward in the tree have $n = 9$, 10, 18, all others sharing $n = 13$, 14, 26. Only *S. tetraptera* ($n = 7$) (Ho et al. 1999) departs from this picture. More generally, and despite the lack of data for

several genera, x = 13 seems to be common in the basal lineages, although there are a few variations around this number. The paucity of species investigated in the more derived clades (*S. pubescens* upward and particularly *Lomatogonium* and *Comastoma*), coupled with a greater variability of chromosome numbers still keeps us from understanding karyological relationships. Yuan and Küpfer's (1993) suggestion that *Comastoma* (x = 8, 9) shows more affinity with *Lomatogonium* (x = 8) than with *Gentianella* (x = 9) from a karyological and morphological point of view is also supported by our phylogeny. The position of *Jaeschkea* (n = 8) close to *Lomatogonium* and *Comastoma* is also congruent with the karyology, although different chromosome numbers have been reported for other *Jaeschkea* species (n = 9, 11 for *J. oligosperma* (as *J. gentianoides*) (Gohil et al. 1981, Koul and Gohil 1973) and n = 10 for *J. canaliculata* (as *J. latisepala*) (Mehra and Vasudevan 1972, Vasudevan 1975)).

The importance of polyploidy associated to dysploidy and/or hybridisation has been stressed in the explanation of the karyotype evolution of *Gentianopsis* and particularly of *Gentiana* (Yuan and Küpfer 1993, Yuan 1996). Several examples of polyploidy can be found in our phylogeny (e.g. *Bartonia*, *Frasera speciosa*, *Obolaria*, *Swertia tashiroi*, S. *pubescens*). Cases of up dysploidy are found in *S. perennis* and *Obolaria*. Down dysploidy seems to be more frequent, e.g. *Pterygocalyx*, *S. bimaculata*, *Halenia* spp., *S. pubescens*. The case of *S. tetraptera* illustrates the importance of this factor in the karyological evolution of the Swertiinae. Its position in the phylogeny suggests that it is derived from an ancestor with n = 13 and that dysploidy may have affected up to 12 chromosomes during the evolution of this taxon. In regard to the molecular phylogeny, it seems that parallel evolution of karyotypes due to the aforementioned factors may have led to similar chromosome numbers in unrelated lineages, e.g. between *Gentianella* spp. (n = 9, 18) and *S. pubescens* (n = 18), or between *Halenia* spp. (n = 11) and *Gentianopsis ciliata* (n = 22) (Favarger 1949), at different ploidy levels.

Systematics and the definition of *Swertia*. In regard of the molecular phylogeny, most of the genera in the *Gentianella*-lineage seem to be monophyletic and relatively well circumscribed. This is not the case for *Lomatogonium*, that could be paraphyletic towards *Comastoma*, or for *Swertia*. The latter seems to have acted as a stem group, giving rise to different lineages, some of which eventually differentiating enough from the typical swertioid aspect as to be recognised at a generic level, e.g. *Halenia*, *Gentianella*, while other lineages exhibit nearly no variation.

The absence of clear morphological differences between lineages of *Swertia*, combined with parallel evolution of traits commonly referred to in the taxonomy of the genus has contributed much to the profusion of speculative controversies among different authors regarding its generic segregates. In his monograph, Shah (1990, 1992) notes that many earlier synonyms were published by authors unaware of previous descriptions or unfamiliar with the global morphological variability exhibited by species on other continents. He further remarks that erroneous descriptions of type material has also contributed to misleading taxonomic treatments. Although most present day authors agree to consider *Swertia* as a large genus including all previous segregates, some synonyms are still debated and pertinent to the molecular phylogeny. These are *Anagallidium*, *Frasera* and *Ophelia*, and shall be briefly discussed here. Grisebach (1836) separated *S. dichotoma* and *S. tetrapetala* to the genus *Anagallidium*. Later, Ma (1976) also placed *S. tetraptera* in this genus. Even if there are no reliable characters in Grisebach's generic diagnosis, these three species are taxonomically closely related (Shah 1992). Embryological studies in *S. tetraptera* (Xue et al. 1999) reveal more affinities with *Halenia* than with *Swertia*, and the position of *S. tetraptera* in our phylogeny suggests that *Anagallidium* might be a justified taxon, although its proper rank and its relation to *S. bimaculata* may be difficult to determine. The status of *Frasera* has been widely debated,

although no reliable character has ever been brought up to justify its separation from *Swertia* (Pringle 1990). Nilsson's studies indicate that the species of *Frasera* may have a monophyletic origin. Only two species are included in our tree, but preliminary results including all 14 species (not shown) support the monophyly of this lineage without a close link to any other *Swertia* species, contrarily to Toyokuni's (1965) suggestions. At present, this taxon is generally regarded as a section of *Swertia* (Pringle 1990) but its position in our phylogeny at the same level as well differentiated genera might favour its recognition at a generic rank, a position also adopted by Struwe et al. (unpubl. data). The definition and taxonomic rank of *Ophelia* have varied depending on the authors. At present, the character referred to for treating *Ophelia* as a distinct taxon under *Swertia* is the annual habit of the plants (excepted sect. *Ophelia* ser. *perennes* in Ho et al. 1994). Our phylogeny clearly suggests that the annual or perennial habit of the species is not representative of relationships in *Swertia*, neither at a generic level, nor at a sectional level.

A number of local infrageneric classifications have been attempted for *Swertia* (see Nemomissa (1994) for a review), but only three publications offer a treatment of the whole genus (Shah 1990, 1992; Ho et al. 1994). Shah divided *Swertia* into two subgenera (*Swertia* and *Ophelia*) and proposed 35 informal groups according to 1) geographical distribution, and 2) morphological similarities. Ho et al. proposed 11 sections and 16 series based on the hypothetical evolutionary polarity of morphological characters. When compared, these two classifications are contradictory in many ways, that is, species in one of Shah's groups are placed in different series by Ho et al. and vice versa. Figure 2 shows the position of species in the subdivisions of Shah and Ho et al. respectively. Both classifications are not congruent with the molecular data. The subdivisions of *Swertia* according to the perennial versus annual habit of the species in Shah represents the main incongruence. For the rest, with 35 unranked groups for ca. 150 species, Shah was aware not only of the variability within *Swertia* but also of the hypothetical nature of relationships based on morphology alone. The classification of Ho et al. contrasts with our phylogeny in several ways. The sectional rank seems to be overestimated in some cases, especially in the *S. perennis* – *S. kilimandscharica* clade, with 5 sections represented. Another incongruence is to be found in sect. *Ophelia*, which is present in 8 different clades in our tree. Furthermore the sect. *Ophelia* itself is divided in three series, that are also not congruent with the molecular data. Besides, the hypothesis of Ho et al. that African species are monophyletic and derived from *Ophelia* is not supported. According to our results, the African species of *Swertia* represent two distinct lineages (*S. crassiuscula* clade, *S. kilimandscharica* clade). Problems concerning the definition and circumscription of *Ophelia* are also due to the absence of a clearly designated type by either D. Don (1836), or by Grisebach (1836). Garg (1987) proposed *S. ciliata* as lectotype. In the chinese edition of the Flora of China, Ho et al. (1988) give *S. angustifolia* as type of sect. *Ophelia*. Note that *S. angustifolia* perfectly fits the description of sect. *Spinosisemina* published later by Ho et al. (1994).

In order to elaborate a satisfactory classification of *Swertia* and allied genera, and to better understand the phylogeography of this group, it seems necessary to first establish clear relationships between taxa and to identify reliable morphological characters correlated with the paraphyletic *Swertia* clades identified in the phylogeny. Discussing biogeographic issues and character polarisation section by section as attempted by Ho et al. (1994) is not convincing in absence of the aforementioned conditions. A phylogenetic analysis of a much wider taxon sampling (ca. 150 species) is underway in our laboratory and should allow proposing a comprehensive taxonomic treatment reflecting evolutionary relationships

within the *Gentianella*-lineage as well as new phylogeographic hypotheses.

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