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Systématique moléculaire et évolution caryologique du genre *Gentiana* (Gentianaceae)

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Seedcoat micromorphology and its systematic implications for Gentianaceae of Western China

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Abstract

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Scanning electron microscopy of the seedcoats of 46 species from 7 genera of Gentianaceae from mountainous regions of Western China reveals a high diversity of seed micromorphology. Two main types can be distinguished. The first comprises *Gentiana*, *Gentianopsis* and some species of *Swertia* with sculptured seedcoats. It may further be divided into several sub-types each of which corresponds to either a genus or a section. The second type is characterized by smooth seedcoats and is observed in *Comastoma*, *Gentianella*, *Halenia*, *Lomatogonium* and some species of *Swertia*.

Key words: China, *Gentiana*, Gentianaceae, micromorphology, seed, seedcoat, SEM.

Introduction

The family Gentianaceae has a world-wide distribution and comprises 80 genera with about 700 species. It is of great interest both to botanists and laymen be its large diversity and the beauty of the flowers of some species. Many studies have been devoted to its morphology, palynology, cytology and systematics. Some authors have also used micromorphology of seedcoats to try to improve its classification. Miège & Wüest (1984) studied the European species of *Gentiana* L. and *Gentianella* Moench extensively, using scanning electron microscopy (SEM). They showed that seedcoat micromorphology may be used to subdivide these genera into sections; they also provided a key based on seedcoat characters for these species. The seedcoats of some species of *Gentiana* sect. *Calathianae* (Froelich (=sect. *Cyclostima* Griseb.) have also been studied by Müller (1982) using SEM.

With its high number of gentianaceous species, the Southwestern mountainous region of China including the Gansu, Qinghai, Sichuan, Tibet and Yunnan provinces may represent one of the most important diversification centres of the family. But studies on the seed micromorphology of the family in this region are lacking, except for a few general taxonomic descriptions containing brief mentions of seed characters observed under the light microscope. Seed characters under the light microscope have been used by Ho (1988) to distinguish and genera. In a revision of the classification of the genus, Ho & Liu (1990) have recently recognized six types of seed micromorphology for the genus *Gentiana*. The study, however, consisted only of a very general description of the main types of seedcoats. Detailed observations on more species and a systematic overall evaluation of the seed micromorphology in the genus *Gentiana* and the other related genera are still necessary.

The present SEM observations on seeds of 46 species of 7 genera from Western China were carried out to elucidate the systematic value of seed micromorphological characters and, furthermore, to detect evolutionary trends among seed microcharacters and their correlation with other characters.

Materials and methods

The species examined are listed in the text. All the seeds examined were collected in the field at various localities, either in the Gansu, Shaanxi and Yunnan Province of Western China. Voucher specimens are deposited in the Herbarium of the Botanical Institute of the University of Neuchâtel (NEU).

Seeds suitable for scanning electron microscopy were selected and soaked in warm water until they reached their full size and were then fixed in 65% ethanol. After dehydration with ethanol and then acetone, the seeds were critical-point dried with CO_2, mounted on stubs and subsequently examined with a Philips PSEM-500 scanning electron microscope after being sputter-coated with ca. 40 nm gold.

Some of the terms defined by Barthlott (1981) were used.

Observations

1. Seedcoat types in the Gentianaceae

The seeds examined may be classified into two groups and several subdivisions, based principally on the shape of the epidermal ornamentations of the seedcoat, particularly the curvature of the outer periclinal wall, or so-called primary sculpture of the seedcoat.

The first is the *Gentiana* group, including species of the genera *Gentiana*, *Gentianapsis* Ma, and some of *Swertia* L. with varied sculpture, from fine reticulations to complicated honeycomb-like or finger-like types. This group may be further divided into the following subdivisions based on the nature of the primary sculpture:

a) fine reticulate type: the curvature of the outer periclinal wall of the seedcoat forms fine reticulate ornamentation with varied shapes and sizes of the meshes. This type is the commonest in *Gentiana* sect. *Cruciata* Gaudin (Fig. 1) and sect. *Chondrophyllae* Bunge (Fig. 9–16).

b) semi-winged type: the seeds have a narrow, oblique wing along one side, as in *G. pudica* Maxim. (Fig. 2).

c) compound lamellar type: the seeds are covered with supercellular membranous lamellae which form spongy and complex hexagonal pits as in *G. apiata* N. E. Br. (Fig. 3–4).

d) honeycomb type: the outer periclinal wall of the seedcoat forms honeycomb-like supercellular pits as in *G. callistantha* Diels et Gilg (Fig. 5–8).

e) undulate type: the epidermal cells rise up into undulation-like sculpture as in *G. expansa* H. Sm. (Fig. 17–19).

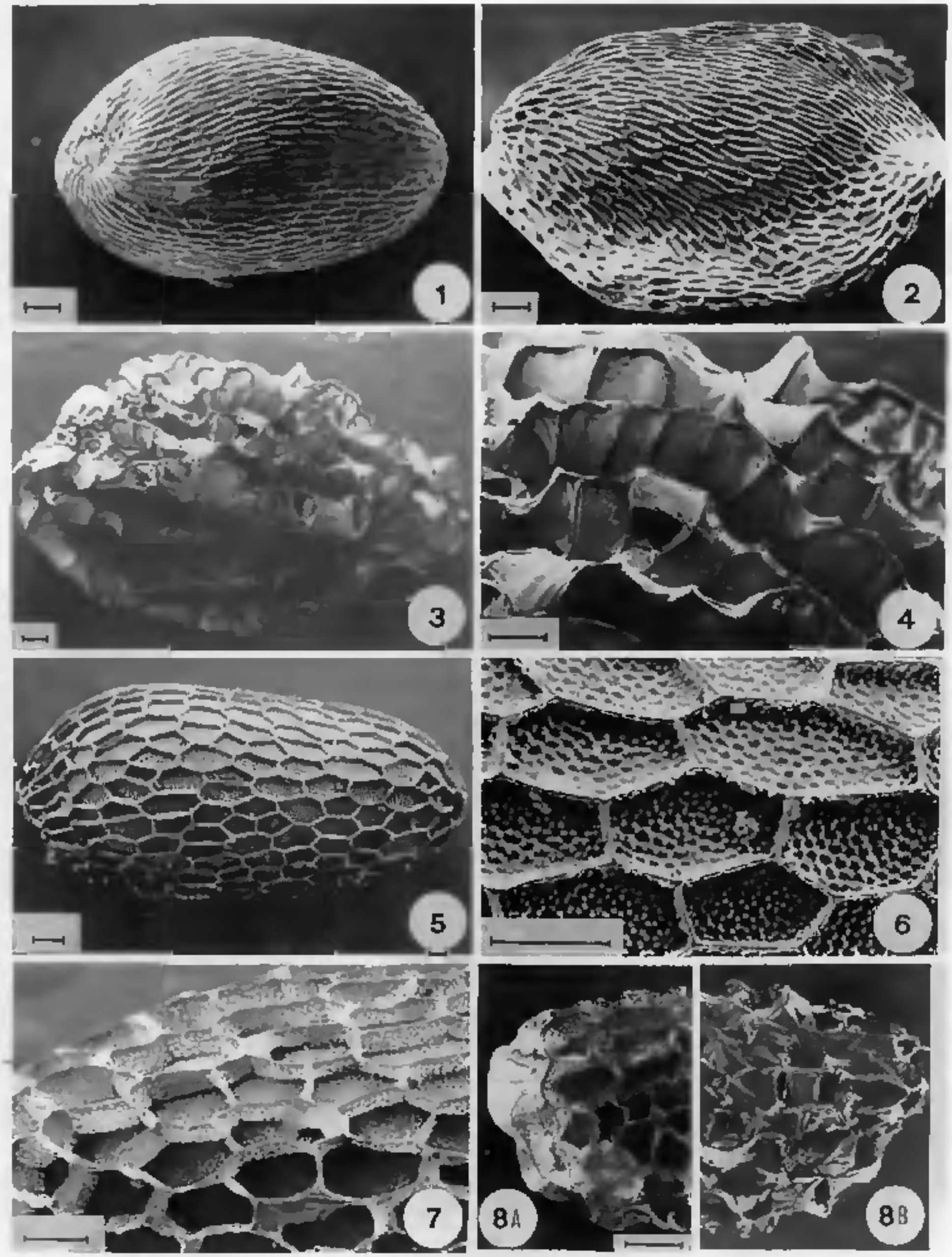

Fig. 1–8. SEM-micrographs of seedcoats of *Gentiana*. – 1: *G. straminea* (sect. *Cruciata*). – 2. *G. pudica* (sect. *Dolichocarpa*). – 3–4: *G. apiata* (sect. *Frigida*). – 5–6: *G. callistantha*; 7: *G. farreri*; 8: *G. regescens* (A. mature seed; B: immature seed with membranaceous cover on its seedcoat) (sect. *Monopodiae*). – Bars = 0.1 mm.

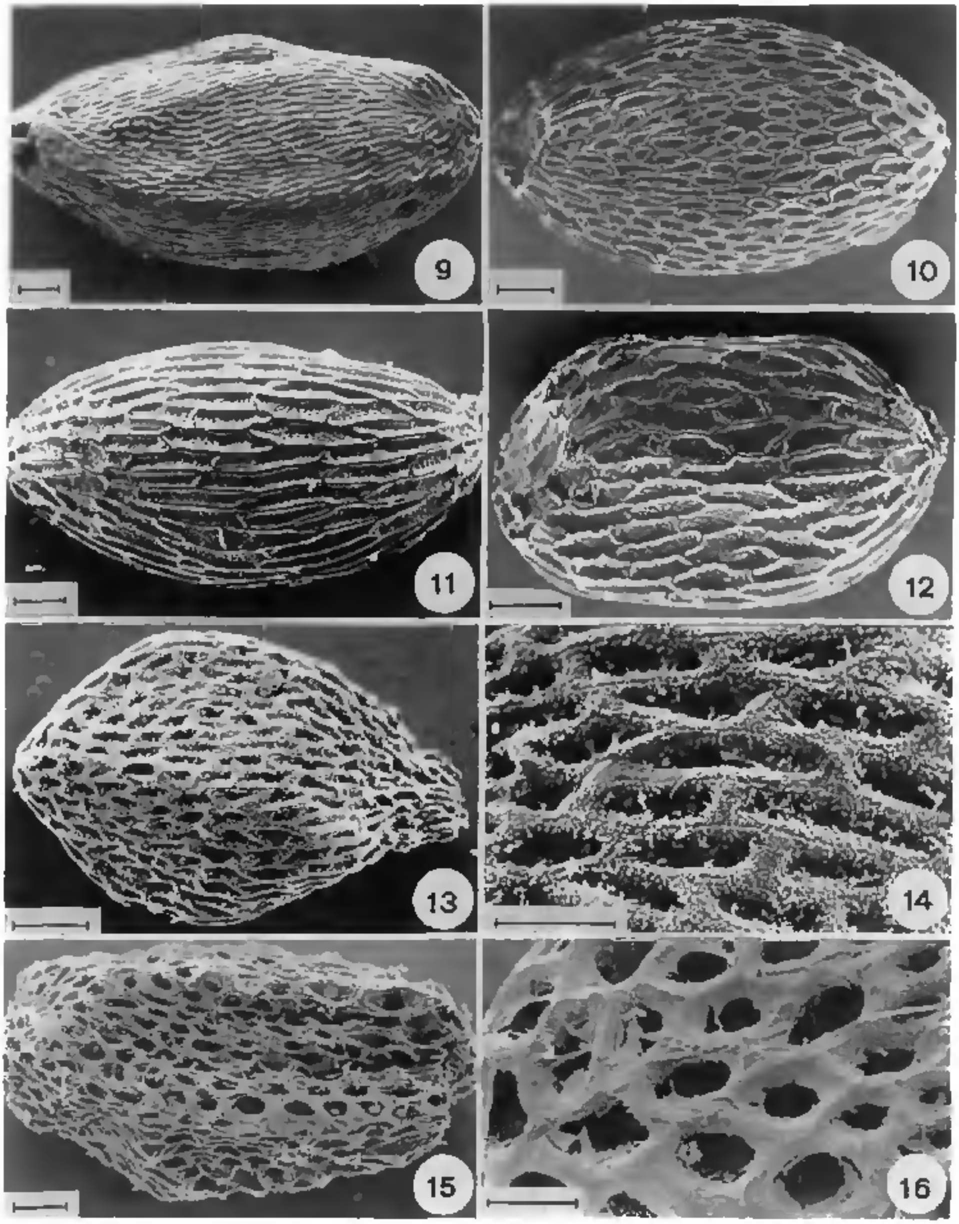

Fig. 9–16. SEM-micrographs of seedcoats of *Gentiana* sect. *Chondrophyllae*. – 9–10: group one: 9: *G. crassuloides*; 10: *G. pseudoaquatica*. – 11–12: group two 11: *G. leucomelaena*; 12: *G. squarrosa*. – 13–16: group three: 13–14: *G. alsinoides*; 15–16: *G. asterocalyx*. – Bars = 0.05 mm in Fig. 14 and 16; bars = 0.1 mm in others.

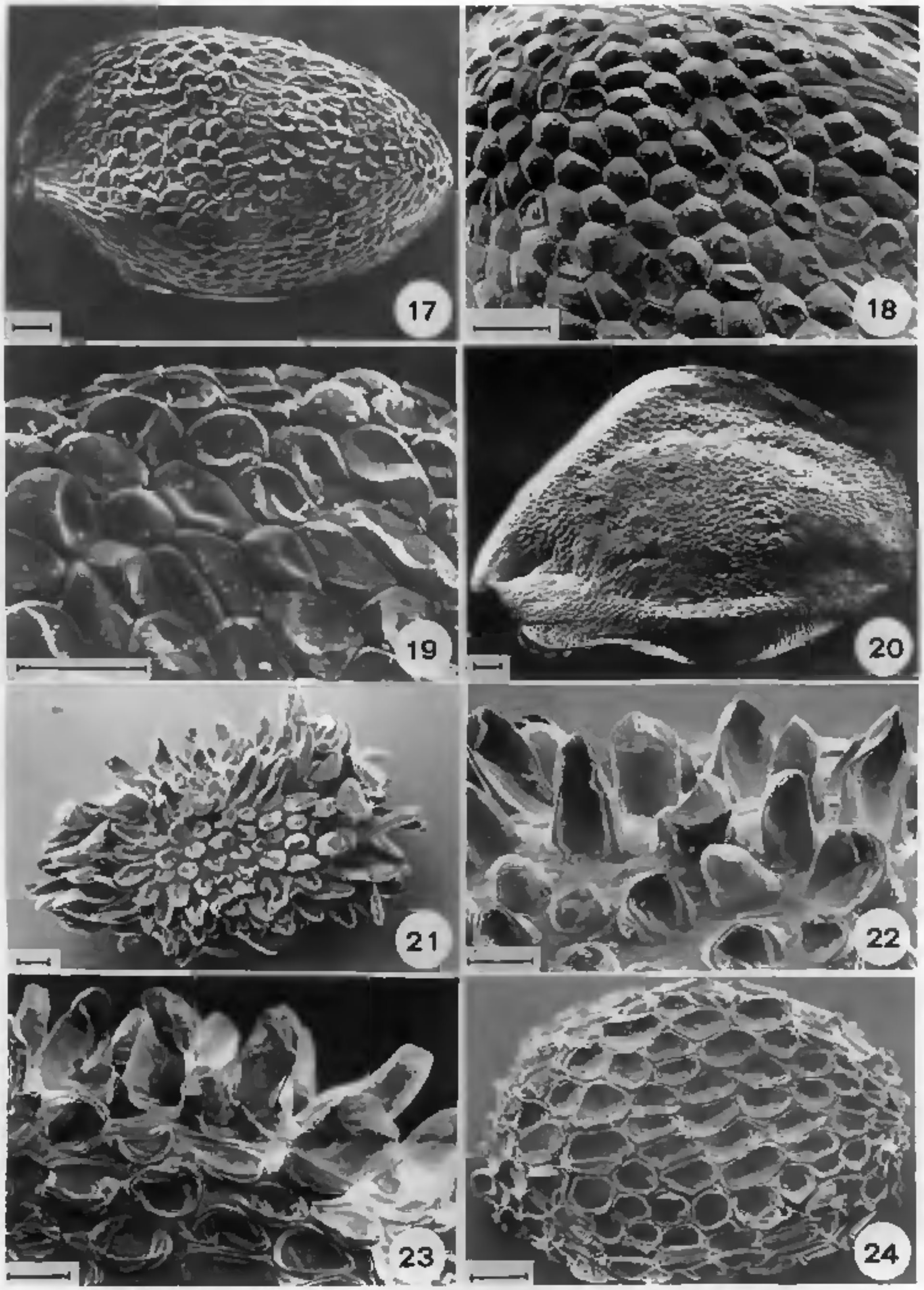

Fig. 17–24. SEM-micrographs of seedcoats of *Gentiana* sect. *Stenogyne*. (17–20) and *Gentianopsis* (21–24). – 17–18: *G. expansa*; 19: *G. primuliflora*; 20: *G. striata*. – 21: *Gentianopsis paludosa* var. *ovato-deltoidea*; 22: *Gentianopsis paludosa* var. *paludosa*; 23–24: *Gentianopsis barbata*. – Bars = 0.05 mm in Fig. 19, 22, 23; bars = 0.1 mm in others.

f) winged type: the seeds have peripheral wings. They are either triquetrous, with wings along three edges as in *G. striata* Maxim. (Fig. 20), or discoid, with wings along the periphery, as in *S. erythrosticta* Maxim. (Fig. 29). The sculpture on the wings and seedcoats is of undulate type.

g) pitted type: the seedcoats have irregular pits on their surface as in *S. diluta* (Turcz.) Bentham et Hooker fil. (Fig. 31) and *Gentianopsis barbata* (Froelich) Ma (Fig. 24).

h) finger-like type: the epidermal cells of the seedcoat protrude to form finger-like projections. This type is confined to the genus *Gentianopsis* (Fig. 21–23).

The second group includes the species of *Gentianella, Comastoma* (Wettst.) Toyokuni, *Lomatogonium* A. Braun, *Halenia* Borkh. and some of *Swertia*, with smooth or almost smooth seedcoats. The outer periclinal wall never forms projections or any other supercellular appendages (Fig. 25–28 and 30). The only diversity seems to be in the very fine dissimilarities of the epidermal cells and in the cuticle morphology of the cell wall, the so-called tertiary sculpture. It is, therefore, unnecessary to create any subdivision.

2. *Seedcoat characters in Gentian*

Just as the highest diversity of species occurs in the genus *Gentiana*, so does the highest diversity of seedcoat micromorphology. As demonstrated in sections *Calathianae, Pneumonanthe* (Gled.) Gaudin, and *Ciminalis* (Adanson) Dumort. by Miège & Wüest (1984) and Müller (1982), the seed micromorphology can provide a taxonomically significant character useful in evaluating intrageneric relationships within the genus. Our present studies on Chinese species mostly confirmed this, but some variations were also noted. The followings are details given section by section.

In sect. *Cruciata* six species were observed: *G. crassicaulis* Duthie ex Burkill, *G. dahurica* Fischer, *G. fetissowii* Regel et Winkler, *G. macrophylla* Pallas, *G. officinalis* H. Smith and *G. straminea* Maxim. The results show a very uniform micromorphology with fine reticulate sculpture on the seedcoat surfaces of all species. The veins forming the reticulations are smooth and much less marked than those of another similar group, sect. *Chondrophyllae*. The meshes are smooth and irregular in shape. No distinct interspecific differences were observed (Fig. 1).

Dolichocarpa T. N. Ho is a new section separated from sect. *Chondrophyllae* by Ho (1875) and based on *G. prostrata* Haenke. Only one of its species, *G. pudica*, was investigated. Each seed has a very narrow, oblique wing along one side, so it was considered a semi-winged type in the present paper. However, the ornamentations on the seedcoat and wing are similar to that of sect. *Chondrophyllae*, i.e. reticulate (Fig. 2).

In section *Frigida* Kusn. only one species, *G. apiata* N. E. Br., endemic to Mt. Taibaishan in Central China, was studied. It has a compound lamellar type of seedcoat with supercellular membranaceous lamellae covering the seeds. The lamellae are formed of a complex, sponge-linke structure in which the veins are usually polygonal with a very thin membranous cover (Fig. 3–4). This type seems to be the most complicated in the genus and is confined to this section.

Section *Monopodiae* T. N. Ho is also a new section, segregated from section *Frigida by* Ho (1985). Three species, *G. farreri* Balf. f., *G. callistantha* and *G. regescens* Hemsley, were investigated here. The primary sculpture patterns of the species from honeycombs with membranous cells (Fig. 5–8). The cells are penta-, hexa- and heptagonal, mainly hexagonal. A unique feature of the seeds of all species examined in this section is the special secondary sculpture they posses. These secondary sculpture patterns, such as the hexagonally raised papillae in *G. callistantha* (Fig. 5–6) or the hexagonal nets in *G.*

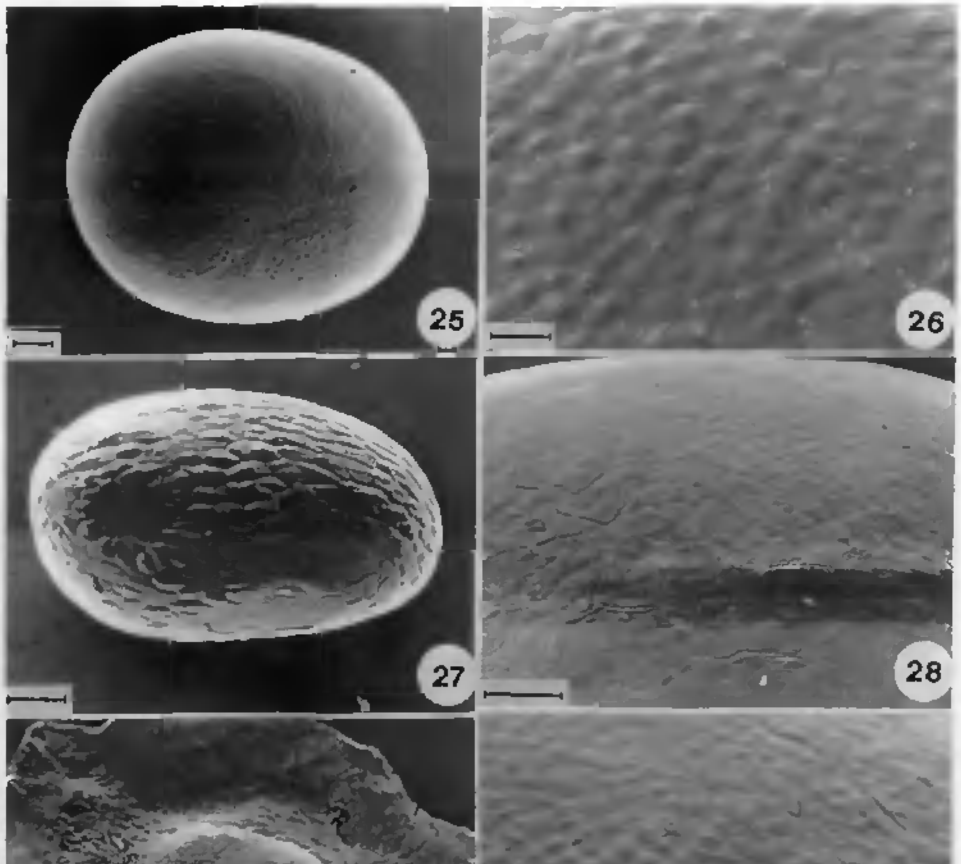

Fig. 25–32. SEM-micrographs of seedcoats. – 25–26: *Comastoma pulmonarium*; 27: *C. polycladum*. – 28: *Gentianella azurea*. – 29: *Swertia erythrosticta*; 30: *S. tetraptera*; 31–32: *S. diluta* (note the membranaceous cover layer in Fig. 32). – Bars = 0.01 mm in Fig. 26, 28 and 30; bar = 0.1 mm in others.

farreri (Fig. 7) and *G. regescens* (Fig. 8 A), are restricted to this section and are, therefore, good diagnostic characteristics. Interspecific differentiation is provided by the shape of the secondary sculpture and the height of the primary sculpture, that is, of the cells. In addition, some immature seeds of *G. rigescens* have a membranous cover over their honeycomb sculpture which may represent a developmental variation (Fig. 8 B).

Sect. *Stenogyne* Franchet is heterogeneous in seed micromorpbology. Four species, *G. striata*, *G. souliei* Franchet, *G. primuliflora* Franchet and *G. expansa*, were examined. Two different types of seeds were found. The former two species have triquetrous seeds with three winged edges (Fig. 20), but the latter two have more or less elliptical unwinged seeds (Fig. 17–19). The sculpture in all these species is of undulate type, protrusious by the raising of the epidermal cells (Fig. 17–20).

We paid the most attention to section *Chondrophyllae*: 18 species were examined; *G. alsinoides* Franchet, *G. anisostemon* Marquand, *G. aperta* Maxim., *G. aristata* Maxim., *G. asterocalyx* Diels, *G. crassuloides* Bureau et Franchet, G. *exigua* H. Sm., *G. heleonastes* H. Sm. ex Marquand, *G. intricata* Marquand, *G. leucomelaena* Maxim., *G. piaszekii* Maxim., *G. pratii* Kusn., *G. praticola* Franchet, *G. pseudoaquatica* Kusn., *G. spathulifolia* Kusn., *G. squarrosa* Ledeb., *G. tricolor* Diels et Gilg, and *G. vandellioides* Hemsley. The seedcoat sculpture patterns mostly belong to the fine reticulate type. The raised striations of the epidermal cell boundaries interconnect to form the reticulation. The width of the gaps between striations, the size and the shape of the meshes vary from species to species. However, three groups can be recognized. The first, represented by *G. crassuloides* (Fig. 9), has straight and smooth, or almost smooth, rather narrowly spaced striations. Most of the species examined, including *G. aperta*, *G. aristata*, *G. crassuloides*, *G. piaszekii*, *G. pseudoaquatica*, *G. spathulifolia* and *G. tricolor*, belong to this group (Fig. 9–10). The second, represented by *G. squarrosa* (Fig. 12), has more or less pronounced anticlinal undulations and micropapillate secondary sculpture on the periclinal walls and the raised boundaries of the seedcoat cells. *G. leucamelaena*, *G. pratii* and *G. vandellioides* also belong to this group (Fig. 11–12). The third, represented by *G. asterocalyx* and *G. alsinoides*, has rather rough striations with some fine irregular, floccose structures (Fig. 13–16). This group also includes *G. anisostemon*, *G. exigua*, *G. intricata* and *G. praticola*. It is interesting to note that all species of the first and second group were collected from Gansu and Shaanxi, the northernmost part of the area investigated, but the species of the third group were collected from Yunnan, the southernmost part of the area.

3. *Seedcaat characters in Gentianella, Comastama, Lomataganium, Halenia, Swertia and Gentianopsis*

In the genus *Gentianella* the seed of two populations of *Gentianella azurea* (Bunge) Holub were examined. Both have very smooth seedcoats with no obvious sculpture (Fig. 28). This results confirmed the previous, more detailed observations by Miège & Wüest (1984) on the European species of the genus.

In the genus *Comastoma*, *C. pulmonarium* (Turcz.) Toyokuni and *C. polycladum* (Diels et Gilg) T. N. Ho were observed, both showing smooth seedcoats very similar to that of *Centianella*, but granulate on the surface. A few differences can be found between the above two species: the former has a granulate surface, the latter a somewhat undulate one (Fig. 25–27).

Two species of *Lomatogonium* were examined, namely *L. rotatum* (L.) Fries ex Nyman and *L. macranthum* (Diels et Gilg) Fernald. They also have smooth seedcoats similar to those of *Gentianella* and *Comastoma*. No interspecific difference was found.

Only one species of *Halenia, H. elliptica* D. Don, was thoroughly studied. It is also similar to *Gentianella* in having smooth type seedcoats.

Observations on four species of *Swertia*, namely *S. erythrosticta, S. diluta, S. dichotoma* L., and *S. tetraptera* Maxim., revealed a variable situation in the genus. *S. erythrosticta* has peripherally winged seeds. The seedcoats are usually smooth or irregularly wrinkled (Fig. 29). The seeds of *S. diluta* have irregularly pitted surfaces, a feature unique among the materials examined (Fig. 31). However, some seeds of the species have membranous undulation-like covers over these pits (Fig. 32). This we suppose to represent a developmental variation and the pitted sculpture may be formed from the peeling off of the membranous cover layer. The seeds of *S. dichotoma* and *S. tetraptera* are similar to those of *Comastoma* and *Halenia*, with very finely granulate (or occasionally smooth) surfaces (Fig. 30).

Three taxa of *Gentianopsis, G. paludosa* (Hooker fil.) Ma var. *paludosa, G. paludosa* var. *ovato-deltoidea* (Burkill) T. N. Ho, and *G. barbata*, were examined in this genus. Characteristically, all materials show finger-like sculpture on the outer surfaces of the seedcoats. The finger-like projections are usually longer along the periphery and shorter on both the dorsal and ventral surfaces (Fig. 21–23). Some interspecific variations of sculpture were observed. The seeds of *G. paludosa* possess finger-like projections throughout their ripening process (Fig. 21–22), while the seeds of *G. barbata* usually have finger-like projections only along the periphery (Fig. 23), or have only pits instead of this type projections on their whole surface at maturity (Fig. 24). This may represent a developmental variation where the pits were formed from the shedding of the finger-like projections.

Discussion

Seed morphology has been used for a sectional classification of the genus *Gentiana*. The present examinations of more species and genera from China under SEM further confirmed that seeds of the family show considerably diversity but also relative stability in certain taxa. Among the closely related genera in the subtrible *Gentianinae*, two groups have been recognized, based on the position of floral glands: the *Gentiana* group, including *Gentiana, Crawfurdia* Wall., *Tripterospermum* Blume and *Megacodon* (Hemsley) H. Sm., and the *Gentianella* group, including *Gentianella, Comastoma, Gentianopsis, Lomatogonium, Lomatogoniopsis* T. N. Ho et S. W. Liu, *Swertia* and *Halenia* (Ho & Liu 1990). The present studies on seedcoats confirms this division to a certain extent. Although only the largest genus *Gentiana* was studied here, the former group has sculptured seedcoats, while the latter has mostly more or less smooth seedcoats, except for the genus *Gentianopsis* and some species of *Swertia. Gentianopsis* was based on *Gentiana* sect. *Crossopetalum* Froelich ex Griseb. (Ma 1951). Both gross and micromorphology confirm the division. The specific finger-like sculpture on the seedcoats also justify its isolated position in the *Gentianella* group mentioned above. It is also far from the *Gentiana* group from the point of view of seedcoat sculpture.

In the genus *Gentiana*, different patterns of seedcoat sculpture generally distinguish sections and may be diagnostic characters, except that sections *Cruciata* and *Chondrophyllae* have somewhat similar reticulate seedcoat sculpture even though they are quite different in gross morphology. Section *Monopodiae* was split off from section *Frigida* on the basis of their different branching patterns (Ho 1985). The seedcoat micromorphology confirms this division, since sect. *Frigida* has primary sculpture of the compound lamellar type and no obvious secondary sculpture, while sect. *Monopodiae* has honeycomb-like primary sculpture with special hexagonally raised secondary sculpture. In sect. *Chondrophyllae* three different groups of seedcoat sculpture with subtle differences were distinguished. This seems to be correlated with the geographical distribution as mentioned above. In *Swertia* and *Gentiana* sect. *Stenogyne* seed micromorphology is not always congruent with the gross morphology, especially as regards the presence of wings and the shape of the seeds. Since only limited numbers of species were studied, it is necessary to check more species before any valid conclusion can be drawn.

The developmental variations of seed micromorphology are worthy to be taken into account, no only because the give a better comprehension of sculpture development but also because they supply indications for understanding both the evolution of the sculpture patterns and the relationships between the different types of the sculpture. They also remind us of the importance of avoiding the confusion between developmental and phylogenetic variations of sculpture when we compare different taxa: *Gentianopsis barbata* and *Swertia diluta*, for example, have similar seedcoat sculpture patterns at maturity, but these patterns have very different origins.

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Karyological studies on *Gentiana* section *Cruciata* Gaudin (Gentianaceae) from China

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SUMMARY — Chromosome numbers and karyotype structures were determined for 12 populations of 6 species of *Gentiana* L. sect. *Cruciata* Gaudin from China. The basic chromosome number $x = 13$ was confirmed for all the taxa. *G. straminea* Maxim. is tetraploid ($2n = 52$); while *G. crassicaulis* Duthie ex Burkill, *G. dahurica* Fischer, *G. fetissowii* Regel et Winkler, *G. macrophylla* Pallas and *G. officinalis* H. Sm. are diploid ($2n = 26$). The chromosome numbers of *G. crassicaulis* and *G. officinalis* are reported here for the first time. The karyotypes of the species belong to the 1A and 2A types according to STEBBINS' asymmetry classification of karyotypes. The existing karyological studies on the genus *Gentiana* are concisely reviewed and the differentiation of species and its mechanisms are discussed.

INTRODUCTION

1. *Karyological background of the genus «Gentiana» L.*

Gentiana (Gentianaceae) is a subcosmopolitan genus with 15 sections and 361 species, according to HO and LIU (1990). 312 species are known in Asia, of which 247 occur in China. The mountain regions of SW China and NE Burma (between 25°-34° N and 91°-105° E) are rich in species and may represent one of the most important centers of origin and diversification (HO and LIU 1990).

There is no doubt that cytological characters, especially chromosome numbers and karyotypes, play an important role in modern plant taxonomy. But, referring to the genus *Gentiana*, chromosome data have contributed little towards resolving the problems of classification and phylogeny, because for many species, especially some key species from Asia, the existing chromosome data are extremely incomplete. Among the total of 361 species, about 95 have been studied cytologically so far (Table 1). All the European species (ca. 29) and almost all the American species (ca. 30) of the genus have thus been investigated. But, although a high specific diversity occurs in China, very few species (ca. 5) have been observed cytologically. Some beautiful Chinese ornamental species such as *G. sino-ornata* Balf. f. and *G. farrerii* Balf. f. were introduced in Europe more than 50 years ago, but, surprisingly, still no chromosome number reports exist for them.

TABLE 1 - Gametic chromosome numbers known for the sections of the genus *Gentiana* (data upto 1991, our data not included).

Section	a/b	Number of species in a section for which each gametic chromosome number has been reported *																	
		n = 7	8	9	10	11	12	13	14	15	16	18	19	20	21	22	24	26	48
Calathianae	14/14	1			1	1			2	7	1		1						
Chondrophyllae	23/158			3	10		1	4				4		1		1	2		1
Ciminalis	7/7											7							
Cruciata	8/21		1?				1?	3							1?			5	
Dolichocarpa	3/12											2		1					
Fimbricorna	0/4																		
Frigida	6/18						3	2							1				
Gentiana	5/5													5					
Isomeria	0/18																		
Microsperma	0/10																		
Monopodiae	2/37						1	1											
Otophora	0/12																		
Phyllocalyx	0/1																		
Pneumonanthe	27/38							26								1			
Stenogyne	0/14																		
Total	95/369	1	1?	3	11	1	5	36	2	7	1	13	1	7	2	2	2	5	1

a: the number of species for which a chromosome number has been reported.

b: total number of species belonging to each section according to Ho and LIU (1990), except for the sections *Calathianae* (after MÜLLER, 1982) and *Ciminalis* (after TUTIN, 1972). There are therefore 8 more species here than in the text.

* Species with different chromosome number reports were calculated repeatedly.

Actually, some pioneer karyological observations have been made on *Gentiana* (e.g. SAKAI 1934, 1940; WOYCICKI 1933), but their results were either indefinite or were mentioned only briefly in regional studies. The genus was not properly known till the studies of RORK (1949) and FAVARGER (1949, 1952). RORK (1949) studied a wide geographical range, including species from Asia, Europe and America, while FAVARGER (1949, 1952) mainly studied the European species. These contemporary contributions permitted a significant advance in our cytotaxonomical knowledge of *Gentiana*. Since then, chromosome number counts on the genus have been accumulating (see for example, the observations of MEHRA and GILL (1968), MALLIKARJUNA *et al.* (1987), WADA (1967) and VASUDEVAN (1975) on the Himalayan species, SKALINSKA (1951), KÜPFER (1974, 1980), LÖVE and LÖVE (1975, 1986) and MÜLLER (1982) on European species, ZHUKOVA (1967, 1980, 1982), KRASNOBROV *et al.* (1980) and KROGULEVICH (1978) on Russian species, SHIGENOBU (1982, 1984) on Japanese species, POST (1983) and WEAVER and RÜDENBERG (1975) on American species, BORGMANN (1968) on New Guinea species).

From the chromosome data available, one finds that most of the sections (they were defined mainly using gross morphological criteria) are polybasic, except for the two European sections *Ciminalis* (Adanson) Dumorr, and *Gentiana* whose members all have $2n = 36$ and $2n = 40$ chromosomes respectively. So far, chromosome numbers of $2n = 14$, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 38, 40, 42, 44, 48, 52 and 96 have been recorded for the genus (cf. Table 1). The cytotype with the lowest chromosome number of $2n = 14$ was reported only for *G. nivalis* L. The chromosome number $2n = 26$ is the most widely distributed both geographically and specifically, e.g. in the sections *Pneumonanthe* (Gled.) Gaudin and *Cruciata*. The numbers $2n = 24$, 26, 42 have been recorded for the section *Frigida* Kusn. The greatest diversity of chromosome numbers occurs in the sections *Calathianae* Froelich (= sect. *Cyclostigma* Griseb.) and *Chondrophyllae* Bunge. The first is well represented in Europe, the second in Asia. MÜLLER (1982) published rhe numbers $2n = 14$, 20, 22, 28, 30, 32, and 38 for the section *Calathianae*. Although the chromosome data available are quite incomplete and the majority of its species has still not been investigated cytologically, the section *Chondrophyllae* also shows a great variation with $2n = 18$, 20, 24, 26, 36, 40, 44, 48, 96-98. Intraspecific polyploidy was recorded for *G. carinata* Griseb. with $n = 10$ and 20 from the W Himalaya (VASUDEVAN 1975), and *G. nipponica* Maxim. with $2n = 36$, 96, 97 and 98 from Japan (SHIGENOBU, 1982). There are some sections such as *Isomeria* Kusn., *Microsperma* T.N. Ho, *Monopodiae* (H. Sm.) T.N. Ho and *Stenogyne* Franchet for whom no chromosome data are as yet available.

The geographical distribution of the chromosome numbers shows that in N America the number most commonly found was $2n = 26$. Two cytological diversity centers can be recognized based on the existing chromosome data. One is in European Alps and the other in the Himalaya. But one may also expecr to find high diversity in the mountain regions of W China which is rhe area of the highest specific diversity for this genus.

The mechanisms of chromosome variation in the genus *Gentiana* are not well understood. MÜLLER (1982) suggested centric fission from an ancestral number of $x = 5$ to explain the origin of the number $n = 7$ in sect. *Calathianae*. But it is still difficult to explain the relationships among so many different numbers and the existence of a real relarionship of chromosomal centric fission and Robertsonian rranslocation (centric fusion) still remains to be confirmed. It is obviously impossible to draw a valid conclusion concerning chromosomal evolution without considering the high specific diversity occurring in W China.

HOLUB (1973) and LÖVE and LÖVE (1972, 1975, 1986) have advocated the splitting of the genus *Gentiana* into several smaller so called monobasic genera, based on limited morphological criteria and basic chromosome numbers. The proposed genera are named *Calathiana* Delarbre (= sect. *Calathianae*), *Chondrophylla* A. Nelson (= sect. *Chondrophyllae* p.p., based on *G. prostrata* Haenke), *Ciminalis* Adanson (= sect. *Ciminalis*), *Dasystephana* Adanson (= sect. *Pneu-*

monanthe p.p., based on *G. asclepiadea* L.), *Favargera* Löve et Löve (=sect. *Frigida* p.p., based on *G. froehlichii* Jan ex Reichenb.), *Gentiana* L. s. str. (=sect. *Gentiana*), *Gentianodes* Löve et Löve (=sect. *Frigida* p.p. + sect. *Isomeria* p.p. + sect. *Microsperma* p.p. + sect. *Monopodiae* p.p., based on *G. frigida* Haenke), *Holubogentia* Löve et Löve (=sect. *Chondrophyllae* p.p., based on *G. pyrenaica* L.), *Kuepferella* Lainz (=sect. *Chondrophyllae* p.p., based on *G. boryi* Boiss.), *Pneumonanthe* Gled. (=sect. *Pneumonanthe* excl. *G. asclepiadea*) and *Tretorhiza* Adanson (=sect. *Cruciata*). However, these further divisions of *Gentiana* have rarely been accepted (PRINGLE 1978; HO and LIU 1990). In fact, the split groups do not necessarily possess a unique basic number, e.g. in *Calathiana* at least 7 different basic numbers have been shown to exist. What's more, basic numbers are still not always congruent with morphology in these groups. The majority of species of the genus is uninvestigated cytologically and new basic numbers are still being found, one after another. Thus, splitting might well simply add to the confusion, while the real cytological mechanism influencing the variation of chromosome numbers is not yet well understood. More studies on the chromosomes and karyotypes of the whole genus are needed before considering recognizing these groups. MÜLLER's (1982) study on the section *Calathianae* is an example which should be followed. He studied the cytotaxonomy and cytogeography of 14 species of the section extensively, throughout their whole distribution area. This kind of study is absolutely needed for the other sections.

The present studies were carried out in order to answer some of the questions corcerning chromosome data of Chinese species, especially the species from the high altitude regions of W China. We attempted, by observing as many species as possible, to find the key chromosome numbers and the links between them, in order to explain the cytotaxonomical and cytogeographical relationships among different species of the genus, and to understand the evolutionary history of the genus better. On this basis we hoped to then establish a natural and useful classification. Since the existing chromosome data suggest that the combination of dysploidization and polyploidization play a great role in speciation of the genus, we paid special attention to the analysis of karyotype structure, in order to evaluate karyotype repatterning and to elucidate the basic chromosome numbers for each section. The results obtained will be published successively, the first part presented here concerning the karyology of the section *Cruciata*.

2. *Karyological studies on «Gentiana» sect. «Cruciata» Gaudin*

The section *Cruciata* comprises 21 species distributed in the Eurasian temperate area, with a specific concentration center in W China and the Himalayan region. Among the 21 species, 16 occur in China (HO and LIU

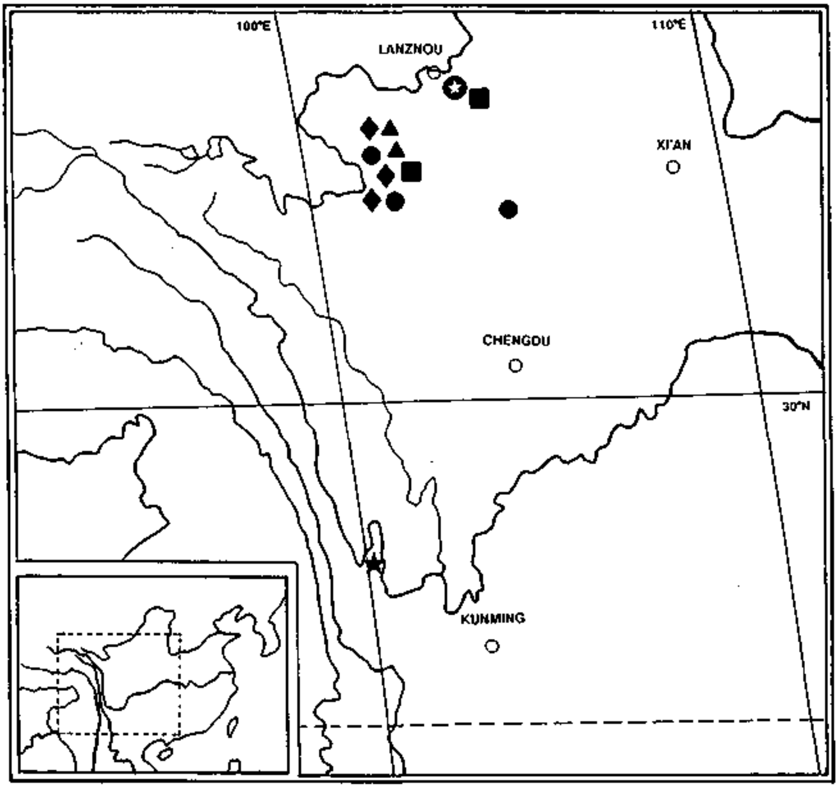
1990). Chromosome numbers have been published previously for 8 species, but no detailed karyotype was given. WOYCICKI (1933) was the first to give an approximate gametic chromosome number of n = ca. 16-18 for *G. fetissowii* Regel et Winkler from this section. RORK (1949) did the most extensive investigation on chromosomes of the section. She counted chromosome numbers of $2n = 26$ and 52 for five taxa of the section (see Table 2) which were confirmed by later workers, except the count of $2n = 42$ for *G. macrophylla* Pallas which was found to be $2n = 26$ by KROGULEVICH (1978) and $2n = 24$ by BELAEVA and SIPLIVINSKY (1975, 1977).

ZHUKOVA (1967) gave $2n = 52$ for *G. walujewii* Regel et Schmalh. and *G. tibetica* King ex Hooker fil., and $2n = 16$ for *G. phlogifolia* Schott et Kotschy (= *G. cruciata* ssp. *phlogifolia* (Schott et Kotschy) Tutin) for which RORK (1949) and LÖVE and LÖVE (1986) counted $2n = 52$. MATVEEVA and TIKHONOVA (1968) published $2n = 26$ for *G. olgae* Regel et Schmalh. KROGULEVICH (1978) counted $2n = 26$ for *G. decumbens* L. but LÖVE and LÖVE (1986) found $2n = 52$ for this taxon under the name *Tretorhiza decumbens* (L.) LÖVE et LÖVE. The present paper gives a primary karyoilogical observation on the section with detailed karyotype analyses.

MATERIALS AND METHODS

The taxa analyzed were *Gentiana crassicaulis* Duthie ex Burkill, *G. dahurica* Fischer, *G. fetissowii* Regel et Winkler, *G. macrophylla* Pallas, *G. officinalis* H. Sm. and *G. straminea* Maxim. The species along with their origins are listed in Table 2. Seeds were collected in the field. The sampling sites are shown in Map 1. Vouchers were collected at the same places and were deposited both in the herbarium of the University of Neuchâtel, Switzerland (NEU) and in that of Lanzhou University, China (LZU). Seeds were germinated in petri dishes on wet filter papers at room temperature. Root tips were pretreated with a saturated water solution od α-bromonaphthalene for 110 minutes at room temperature and then fixed with Carnoy 3:1 for at least four hours. After washing out the fixer completely with water, the root tips were stained in 1% aceto-orcein for one hour, and then heated gently for 2-3 minutes over a flame, before, being squashed in 45% acetic acid. This procedure mainly follows that of KRÄHENBÜHL and KÜPFER (1992). Photographs were taken with a Leitz-Diaplan microscope and drawings were made with the camera lucida apparatus from temporary and permanent slides.

The well spread chromosomes were measured from at least five cells. The terminologies used for centromere positions were those defined by LEVAN *et al.* (1964). STEBBINS' (1971) asymmetry classification of karyotype, and the intrachromosomal asymmetry index (A_1) and interchromosomal asymmetry index (A_2) introduced by ROMERO ZARCO (1986) were followed.



Map. 1. — The sampling sites of the populations of *Gentiana* sect. *Cruciata* examined. ★ *G. crassicaulis*, ▲ *G. dahurica*, ✪, *G. fetissowii*, ● *G. macrophylla*, ■ *G. officinalis*, ♦ *G. straminea*.

RESULTS

1. *Chromosome number.*

All the populations of *G. crassicaulis*, *G. dahurica*, *G. fetissowii*, *G. macrophylla* and *G. officinalis* investigated were uniformly diploid, with $2n=26$ (Table 2 and Figs. 1-4,6). However, one individual of *G. fetissowii* was found to have a chromosome number of $2n=26+2B$. Along wirh rhe two very small B-chromosomes, we discovered two chromosomes showing telomeric secondary constrictions which did not appear in other individuals. The populations of *G. straminea* were tetraploid with $2n=52$ (Table 2 and Fig. 5). The chromosome numbers for *G. crassicaulis* and *G. officinalis* are reported here for the first time. The chromosome numbers for the other species were confirmed. The basic number of the section *Cruciata* is then undoubtedly $x=13$.

2. *Karyotype structures.*

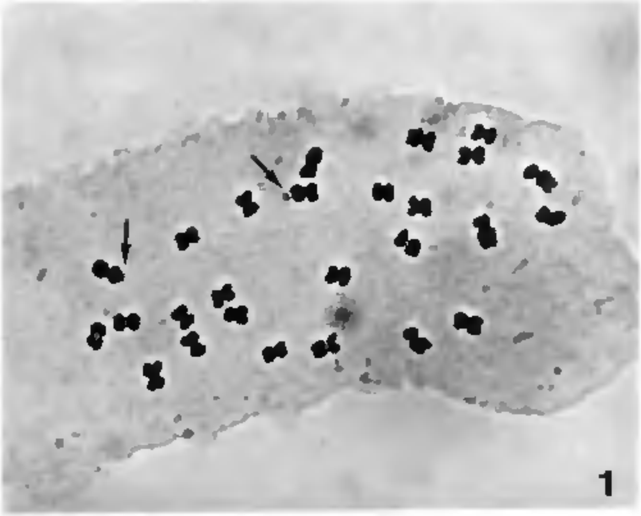
The karyotype of the species investigated are illustrated in Figs. 7-12. Karyotype structure, chromosome length range, the ratio between longest and shortest chromosomes, the proportion of the chromosomes where the arm ratios are higher than two, karyotype classification after Stebbins (1971) and karyotype asymmetry indices (A_1 and A_2) after Romero Zarco (1986) are listed in Table 3. The chromosomes of the section are small, and the mean

Table 2 · Chromosome numbers of *Gentiana* sect. *Cruciata*.

Taxon	Coll. no.	Locality and altitude	Chrom. no.($2n$)	Author
G. crassicaulis	G129	Lijiang, Yunnan, 3250m	26	present
G. cruciata	—	—	52	Rork (1949); Majovsky & *al.* (1974); Homel & Wieffering (1979); Semerenko (1985); Löve & Löve (1986)
G. phlogifolia	—	—	52	Rork (1949); Löve & Löve (1986)
			16	Zhukova (1967)
G. dahurica	G153	Xiahe, Gansu, 2950m	26	present
	G183	Hezuo, Gansu, 2900m	26	present
	—	—	26	Rork (1949)
G. decumbens	—	—	26	Krogulevich (1978)
	—	—	52	Löve & Löve (1986)
G. fetissowii	G168	Lanzhou, Gansu, 2460m	26	present
	—	—	32-36*	Woycicki (1933)
G. macrophylla	G176	Maqu, Gansu, 3350m	26	present
	G184	Hezuo, Gansu, 2900m	26	present
	G195	Dangchang, Gansu, 2650m	26	present
	—	—	42	Rork (1949)
			24	Balaeva & Siplivinsky (1975, 1977)
			26	Krogulevich (1978)
G. officinalis	G165	Lanzhou, Gansu, 2600m	26	present
	G.175	Hezuo, Gansu, 2900m	26	present
G. olgae	—	—	26	Matveeva & Tikhonova (1968)
G. straminea	G154	Xiahe, Gansu, 2950m	52	present
	G179	Maqu, Gansu, 3350m	52	present
	G189	Luqu, Gansu, 3300m	52	present
	—	—	52	Rork (1949)
G. tibetica	—	—	52	Zhukova (1967)
G. walujewii	—	—	52	Zhukova (1967)

* the number originally given was gametic, $n =$ ca. 16-18.

Figs. 1-6. — Metaphase chromosomes of *Gentiana* spp. 1. *G. macrophylla* (G 176) $2n = 26$, 2. *G. crassicaulis* (G 129) $2n = 26$, 3. *G. fetissowii* (G 168) $2n = 26$, 4. *G. dahurica* (G 153) $2n = 26$, 5. *G. straminea* (G 154) $2n = 52$, 6. *G. officinalis* (G 175) $2n = 26$. Arrows indicate the satellited chromosomes.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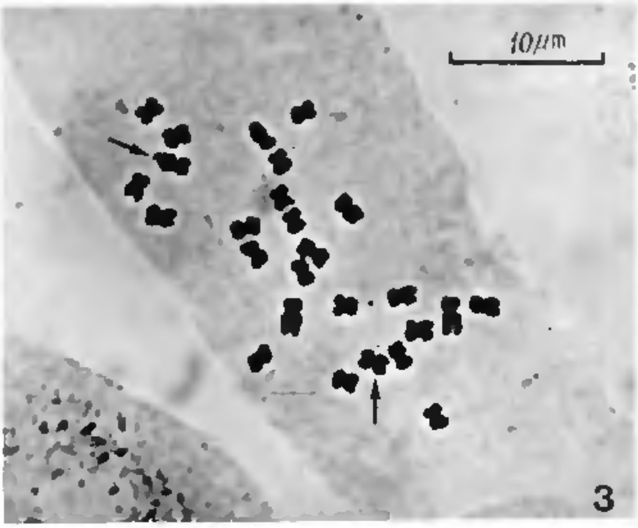

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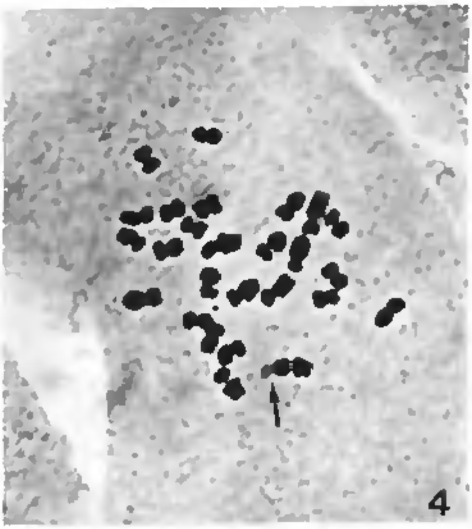
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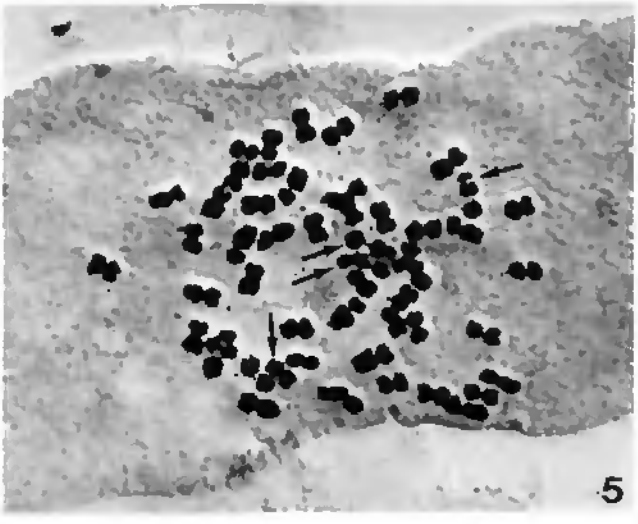
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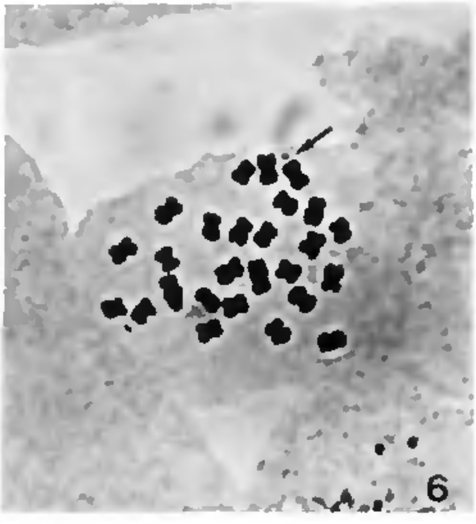
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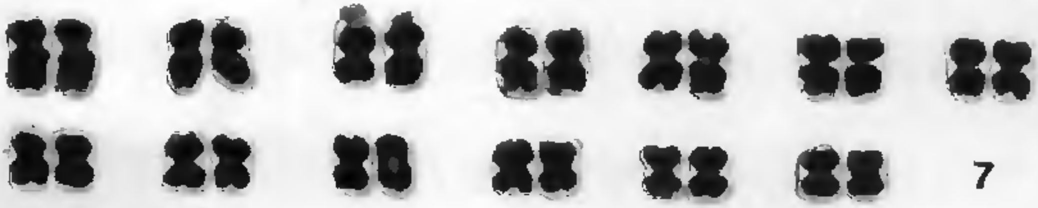
Figs. 7-12. — Karyograms of *Gentiana* spp. 7. *G. macrophylla* (G 176), 8. *G. crassicaulis* (G 129), 9. *G. fetissowii* (G 168), 10. *G. dahurica* (G 153), 11. *G. officinalis* (G 175), 12. *G. straminea* (G 154).



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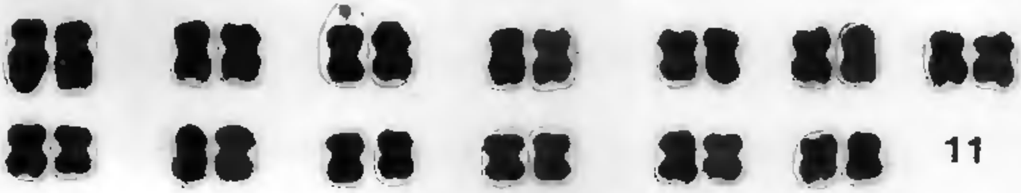

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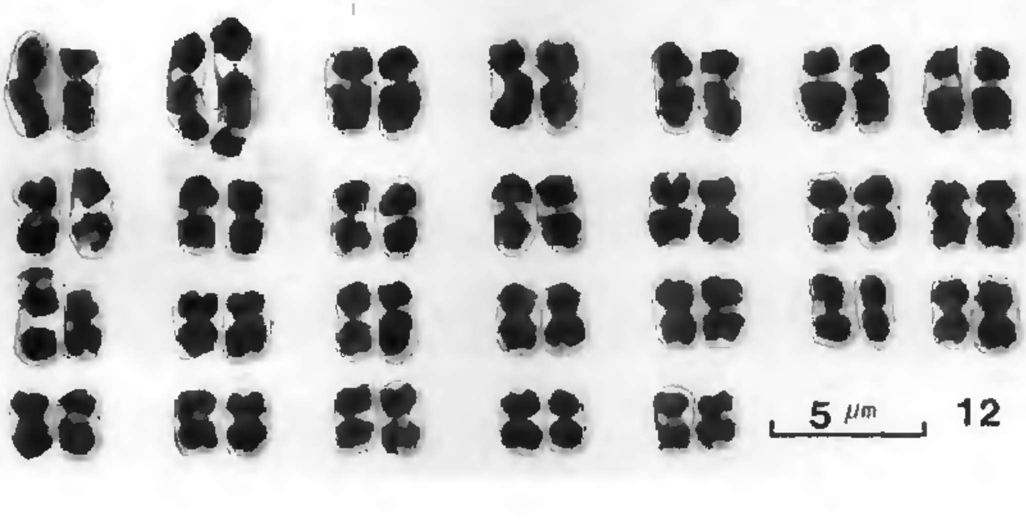

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TABLE 3 · Karyotype structure of *Gentiana* sect. *Cruciata*.

Taxon	Coll. No.	Karyotype structure	Length range (μm)	L/S	P	Type	A_1	A_2
G. crassicaulis	G129	2*n* = 2m(SAT) + 22m + 2sm	1.4-2.6	1.79	0.08	2A	0.235	0.169
G. dahurica	G153	2*n* = 2m(SAT + 20m + 4sm	1.4-2.6	1.88	0.00	1A	0.286	0.171
	G183	2*n* = 2m(SAT) + 22m + 2sm	1.3-2.2	1.73	0.08	2A	0.183	0.198
G. macrophylla	G176	2*n* = 2m(SAT) + 22m + 2sm	1.4-2.2	1.70	0.00	1A	0.297	0.131
	G184	2*n* = 2m(SAT) + 24m	1.6-2.5	1.55	0.00	1A	0.228	0.119
	G195	2*n* = 2m(SAT) + 20m + 4M	1.6-2.6	1.69	0.00	1A	0.189	0.148
G. fetissowii	G168	2*n* = 2m(SAT) + 20m + 4M	1.5-2.2	1.82	0.00	1A	0.184	0.165
G. officinalis	G165	2*n* = 2m(SAT) + 21m + 3sm	1.5-2.6	1.71	0.04	2A	0.237	0.157
	G175	2*n* = 2m(SAT) + 22m + 2sm	1.4-2.2	1.57	0.00	1A	0.154	0.124
G. straminea	G154	2*n* = 4m(SAT) + 44m + 4sm	1.6-3.0	1.92	0.04	2A	0.223	0.158
	G179	2*n* = 2m(SAT) + 42m + 2M + + 6sm	1.6-2.9	1.82	0.04	2A	0.214	0.166
	G189	2*n* = 2m(SAT) + 50m	1.2-2.1	1.74	0.00	1A	0.205	0.122

L: length of the longest chromosome in a karyotype.
S: length of the shortest chromosome in a karyotype.
P: proportion of the chromosomes of which the arm ratioes are more than 2 in a karyotype.
Type: referring to the classification of karyotypes of STEBBINS (1971).
A_1: the intrachromosomal asymmetry index defined by ROMERO ZARCO (1986).
A_2: the interchromosomal asymmetry index defined by ROMERO ZARCO (1986).

lengths range from 1.2 to 3.0 μm. According to STEBBINS' (1971) classification, the karyotypes of the populations belong either to type 1A or 2A and have very uniform metacentric chromosomes. Only a few submetacentric chromosomes (usually involving the longest pair) were found, with the arm ratios ranging from 1.7 to 2.2 in each karyotype. Some chromosomes are sometimes so similar morphologically that it is difficult to recognize homologous pairs, except for the four longest, two satellited and two shortest ones. However, on the whole, the karyotypes of all the populations are very symmetrical. The commonest karyotype of the diploid species is 2*n* = 2m(SAT) + 22m + 2sm. The tetraploid species *G. straminea* has chromosomes similar to the diploid species. We noticed that two or occasionally four satellites were visible.

The variations of karyotype mainly involved the changes of satellite number and position, the appearance of additional secondary constrictions and the changes of arm ratios of some chromosomes. Heterozygous chromosome pairs were found in some populations of *G. officinalis*. The longest pair of chromosomes of the population G165 was heterozygous with one submetacentric chromosome (arm ratio = 2.16) and another metacentric (arm ratio = 1.18), but in population G175, the heterozygous pair were the satellited chromo-

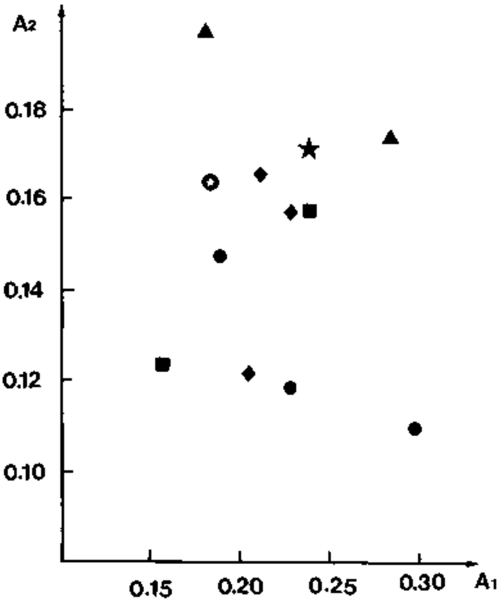

Fig. 13. — Scatter diagram showing the intrachromosomal asymmetry index (A_1) against interchromosomal asymmetry index (A_2) of the karyotypes of the populations examined. The symbols of the species are the same as those used in Map 1.

somes with different sizes and arm ratios. Usually there is a pair of chromosomes bearing satellites in every karyotype, but exceptions with no visible satellite or with only one satellite exist. Additional secondary constrictions were observed in G168, G129, and G183. All the variations mentioned above may be of little importance for classification, but their presence at least revealed that species and even populations are heterogeneous and that some structural changes of chromosomes exist which accumulate the fundamental material necessary for karyotype repatterning.

According to STEBBINS' (1971) classification, the asymmetry of the karyotypes in this section seems very conservative. The 2A type does not differ significantly from the 1A type among the species of the section. In fact, in the 2A type, usually only one pair of chromosomes is submetacentric with an arm ratio a little higher than 2. The asymmetry indices are quite close to one another; the differences between populations of the same species and between different species are also on the same scale. We can see in the scatter diagram (Fig. 13) that species overlap and that no clear disjunction exists between them, except however for populations of *G. dahurica* (G153 and G183) and *G. crassicaulis* (G129) which have an asymmetry level slightly higher than the others.

DISCUSSION

Our karyological observations clearly established that the basic chromosome number of $x = 13$ is fairly stable in this section. The only chromosome number variation, $2n = 26 + 2B$, observed in *G. fetissowii*, seems to be the result of chromosome structural changes. Considering the chromosome numbers previously reported for the section *Cruciata*, one realizes that most reports were either diploid ($2n = 26$) or tetraploid ($2n = 52$) (cf. Table 2). The number $2n = 16$ reported by ZHUKOVA (1967) for *G. phlogifolia* is very different from the basic number and needs to be confirmed. For the same taxon, both RORK (1949) and LÖVE and LÖVE (1986) always counted $2n = 52$. For *G. macrophylla*, a widely distributed and polymorphic species, we always found $2n = 26$, but BALAEVA and SIPLIVINSKY (1975, 1977) reported $2n = 24$, which could be the result of structural changes of the chromosomes. In addition, RORK (1949) reported $2n = 42$ for the same species introduced in Ottawa. This we suppose could be caused by natural hybridization in the botanical garden.

The diploid species known for the section *Cruciata* are mainly distributed in the northern and western parts of China, in Siberia, the Far East and Central Asia. The most widespread and the only species of the section native to Europe, *G. cruciata*, is always tetraploid. A few other tetraploid species are regionally sympatrical with diploid species in W. China. *G. straminea* especially may be found on the same sites as the diploid species *G. macrophylla* and *G. dahurica* in SW Gansu (Map 1). The southernmost species of the section, *G. crassicaulis*, distributed in the high mountain meadows (usually above 3000m) of the Yunnan, Sichuan, Tibet and Gansu Provinces in SW China is similar to *G. tibetica* ($2n = 52$) but much bigger than the latter. It was suspected to be a polyploid species, but our observation revealed that it is diploid, with $2n = 26$ chromosomes. The distribution pattern of the cytotypes, in accordance with the high diversity of species, indicates that the western and southwestern highland of China represents the most important diversification and probably origin center of the section.

G. macrophylla, G. fetissowii and *G. officinalis* form a very closely related complex. Some authors treated them as intraspecific variations (e.g. MA *et al* 1964). They can be distinguished by some trivial characters such as the ratio of the length of the corolla and calyx, corolla colour and shape. They are parapatrically distributed with a contact zone in SW Gansu where our materials were collected. The karyotype analysis indicates that their karyotypes are also similar to each other. We suppose that they might have been differentiated from a common ancester by structural changes of chromosomes and that up till now, the reproductive barrier has not become effective between them, therefore permitting occasional hybridization and backcrossing between the populations. This could explain why it is difficult to differentiate them morphologically, as well as why the variations between their karyotypes are so small. The other diploid species, *G. crassicaulis* and *G. dahurica*, may have followed a similar differenciation process, but they are now well isolated genetically, and have also become well differentiated ecologically from the other diploid species, *G. crassicaulis* occupying higher altitudes and *G. dahurica* drier and sunnier places. Karyologically both species have a greater karyotype asymmetry than the others (Fig. 13).

The mechanisms of speciation in the section could be both rapid and gradual. Polyploidization supplies the possibility of rapid speciation, but at the diploid level the main cause of speciation is probably structural changes of chromosomes and gene mutations, reinforcing the barriers between populations and between species gradually. We suppose that equal or occasionally unequal reciprocal translocation is the main mechanism of chromosome structural changes in the section, because in the diploid species all the populations have the same number and a similar karyotype with a high proportion of metacentric chromosomes. Therefore the breakages probably often happen within or near the centromeres and easily result in an equal reciprocal translocation. Obviously this will not influence the asymmetry of the karyotype. However, analyses of chromosome pairing during meiosis and studies of chromosome banding might supply some more direct evidence and are therefore necessary to complete our knowledge of these questions.

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KARYOLOGICAL STUDIES ON *GENTIANA* SECT. *FRIGIDA* S. L. AND SECT. *STENOGYNE* (*GENTIANACEAE*) FROM CHINA

by

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WITH 19 FIGURES AND 3 TABLES

INTRODUCTION

Section *Frigida* Kusn. s. l. refers to the circumscription of the section by SMITH (1961) and PRINGLE (1978). They lumped section *Isomeria* Kusn. with the section *Frigida* Kusn. both of which were recognized by KUSNEZOW (1895) in his first comprehensive monograph of the genus. But some other authors split the group into several sections or even genera; for examples, LÖVE & LÖVE (1972) established two genera for this group, mainly according to the basic chromosome numbers (see below): the monotypic *Favargera* Löve et Löve based on *Favargera froelichii* (Jan ex Reichenb.) Löve et Löve (= *Gentiana froelichii* Jan ex Reichenb.) and *Gentianodes* Löve et Löve which included all the other members of this group. But, in their very recent revision of the classification of *Gentiana* L., HO (1985) and HO & LIU (1990) adopted a rather narrower concept for the section and rejected the splitting of the group into different genera. They recognized the sections *Isomeria* and *Frigida* of KUSNEZOW (1895) and established three additional sections: *Monopodiae* T. N. Ho and *Phyllocalyx* T. N. Ho, split from the section *Frigida* sensu Kusnezow, and *Microsperma* T. N. Ho split from *Isomeria* sensu Kusnezow. The diagnostic characters of these newly established sections were mainly the branching patterns, habits and seed characters. For the sake of convenience, we follow their narrower sections in the following discussion.

According to HO & LIU (1990), the section *Frigida* sensu Ho & Liu consists of eighteen species distributed in the northern temperate area, from Europe to Asia and North America, with a high concentration in the mountainous regions of Southwestern China and Northeastern Burma (15 species). Two species, *G. frigida* Haenke and *G. froelichii*, are endemic to the Alps, Carpathians and Southwestern Bulgaria. North America shares 2 species, *G. algida* Pallas and *G. glauca* Pallas with Northeastern and Eastern Asia. The sections *Monopodiae* and *Microsperma*, consisting of 37 and 10 species respectively, are restricted to Eastern and Southeastern Asia and the

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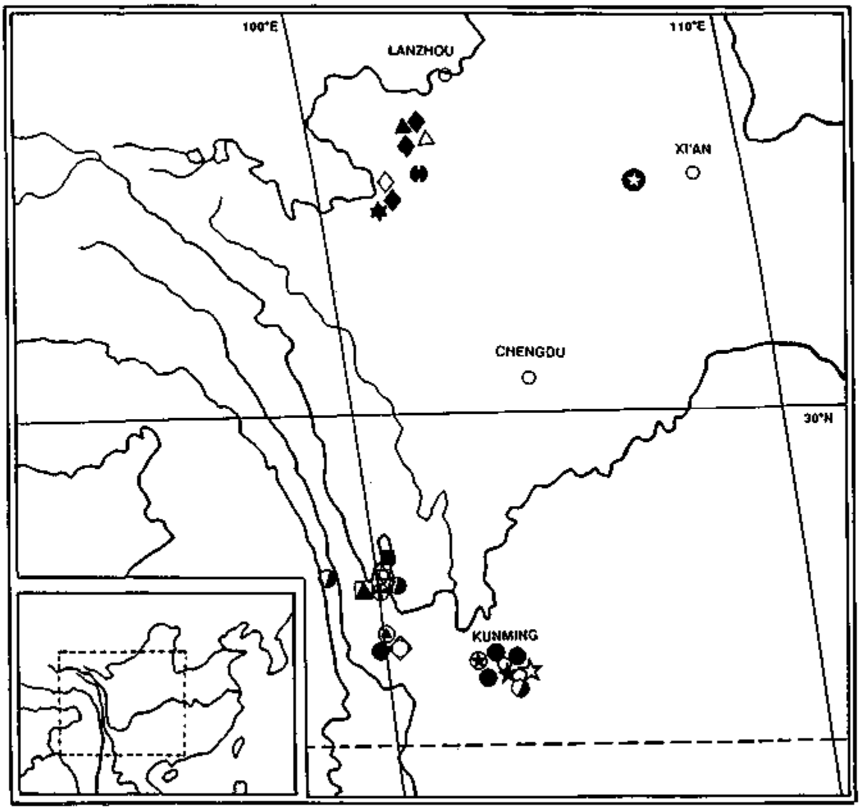

Fig. 1. The sampling sites of the populations of *Gentiana* species examined and their corresponding chromosome numbers.

▲	*G. callistantha* $2n = 26$;	◬	*G. cephalantha* $2n = 24$;	⍟	*G. duclouxii* $2n = 24$;
◆	*G. farreri* $2n = 48$;	◈	*G. melandrifolia* $2n = 24$;	●	*G. regescens* $2n = 24$;
◇	*G. sino-ornata* $2n = 48$;	△	*G. veitchiorum* $2n = 24$;	✡	*G. delavayi* $2n = 26$;
⊕	*G. yunnanensis* $2n = 24$;	○	*G. apiata* $2n = 24$;	✶	*G. nubigena* $2n = 24$;
■	*G. expansa* $2n = 34$;	★	*G. gentilis* $2n = 42$;	⍓	*G. pterocalyx* $2n = 34$;
☆	*G. primuliflora* $2n = 42$;	◑	*G. rhodantha* $2n = 46$;	◍	*G. striata* $2n = 46$.

adjacent Himalayan area. The highest diversity occurs in the southwestern provinces of China and the eastern Himalayan region. The monotypic section *Phyllocalyx* includes only *G. phyllocalyx* C. B. Clarke found in the Himalaya and Southwestern China.

Chromosome numbers have been reported for seven species and one variety of this group, from Europe, Northeastern and Eastern Asia, and North America (cf. Table 1). Among them, *G. algida*, the most widespread species of this group, and *G. glauca* have been well studied. The numbers reported for the whole group are of $2n = 24$ for most species. Nevertheless, a few species differ. Thus *G. froelichii* was always counted as having $2n = 42$ chromosomes (FAVARGER 1965; LOVKA *et al.* 1971, 1972). It is precisely based on this distinct number that LÖVE & LÖVE (1972) esta-

TABLE 1.

The previous reports of chromosome numbers on *Gentiana* sect. *Frigida* s.l.

Taxon	*Origin*	*Chrom. no. (2n)*	*Reference*
sect. *Frigida* s.s.			
G. algida	Siberia	24	KRASNOBOROV & ROSTOVTSEVA (1975)
	Siberia	24	KRASNOBOROV *et al.* (1980)
	Tunkinsky Mt.	24	KROGULEVICH (1976)
	Sayanskiy Mt.	24	KROGULEVICH (1978)
	Colorado	24	LÖVE & LÖVE (1975)
	Japan	24	SHIGENOBU (1984)
	Altai	26	SOKOLOVSKAYA & STRELKOVA (1938)
	NE Yakutsk	24	YURTSEV & ZHUKOVA (1982)
	W Chukotka	24	ZHUKOVA (1967a)
	unkown	26	ZHUKOVA (1967b)
	Anyuy Mt.	24	ZHUKOVA (1980)
	W Chukotskiy	24	ZHUKOVA & PETROVSKY (1976)
G. algida var. *igaraskii*	Japan	24	SHIOENOBU (1984)
G. frigida	Czechoslovakia	24	MURIN (1974)
	Tatry Mt.	24	SKALINSKA (1951)
G. froelichii	Slovenia	42	FAVARGER (1965)
	Slovenia	42	LOVKA *et al.* (1971, 1972)
G. glauca	Alaska	24	DAWE & MURRAY (1979)
	Alaska	24	JOHNSON & PACKER (1968)
	Yukon	24	MULLIGAN & PORSILD (1969)
	Kamchatka	24	SOKOLOVSKAYA (1963, 1968)
	W Chukotskiy	24	ZHUKOVA (1966)
	Chukotskiy Yuzhnyy	24	ZHUKOVA (1969)
	Anyuyskiy Mt.	24	ZHUKOVA (1980)
	Chukotka	24	ZHUKOVA (1982)
	Chukotskiy	24	ZHUKOVA & TIKHONOVA (1971)
G. romanzowii	Kamchatka	24-26	SOKOLOVSKAYA (1963)
sect. *Monopodiae*			
G. formosana	Taiwan	26	HSU (1968)
G. yakushimensis	Japan	26	SHIGENOBU (1984)

blished the genus *Favargera* typified on *G. froelichii*. In addition, *G. formosana* Hayata (= *G. davidii* Franchet var. *formosana* (Hayata) T. N. Ho) from Taiwan and *G. yakushimensis* Makino from Japan were found to have $2n = 26$ chromosomes (HSU 1968; SHINGENOBU 1984). Although there is a high diversity of species, no observation has been made on the

Chinese and the Himalayan species, not even on some of the beautiful ornamental species such as *G. sino-ornata* Balf. f. and *G. farreri* Balf. f. which were introduced in Europe more than 50 years ago.

Stenogyne Franchet is a poorly known section both taxonomically and cytologically; nevertheless it has often been accepted (PRINGLE 1978; HO & LIU 1990). According to the current circumscription of HO & LIU (1990), the section contains fourteen species: ten are completely restricted to Southwestern China, two are relatively widespread in Northwestern and Central China, one is extending to Eastern Burma from Southwestern China and one is endemic to Thailand. None of them has been studied cytologically. Chromosome numbers are completely unkown for this section.

Following our reports on sections *Cruciata* Gaudin (YUAN, in press) and *Chondrophyllae* Bunge (KÜPFER & YUAN, submitted), this paper contributes additional chromosome data from China on the genus *Gentiana*.

MATERIALS AND METHODS

25 populations of 18 species were observed in this investigation. The species names and populations, along with their origins and chromosome numbers are listed in Table 2. The sampling sites are shown in Fig. 1. All the voucher specimens were deposited in the herbaria of the University of Neuchâtel, Switzerland (NEU) and of Lanzhou University, China (LZU). Seeds and flower buds were collected in the field and the flower buds were fixed with Carnoy. Chromosomes were observed either from mitosis in young ovaries and root tips germinated from seeds, or from meiosis of pollen mother cells, as indicated in Table 2. For observations on mitosis of root tips, the aceto-orcein squashing method was used and the procedure is the same as in our previous reports (YUAN, in press). SNOW's (1963) method was employed for the observations of meiosis and ovary mitosis.

The terminologies for centromeric positions introduced by LEVAN *et al.* (1964), the karyotype classification of STEBBINS (1971) and the karyotype asymmetry indices defined by ROMERO ZARCO (1986) were followed.

RESULTS

1. Sect. *Frigida* s.l.

Most species of the section *Frigida* s. l. have $2n = 24$ or $n = 12$ chromosomes. This confirms their diploid level and the basic number of $x = 12$ (Table 2). This, for example, is the case for *G. cephalantha* Franchet ex Hemsley, *G. duclouxii* Franchet, *G. melandrifolia* Franchet ex Hemsley, *G. regescens* Franchet ex Hemsley, *G. veitchiorum* Hemsley, *G. yunnanensis* Franchet, *G. apiata* N. E. Br. and *G. nubigena* Edgew. (Fig. 3-4, 6-7, 9 and 11-13). Tetraploid numbers were found for the first time for *G. farreri* and *G. sino-ornata* with $n = 24$ (Fig. 5) and $2n = 48$ (Fig. 8). Furthermore, the basic number of $x = 13$ was also found in this group, in *G. callistantha* Diels et Gilg and in *G. delavayi* Franchet, which were diploid with $2n = 26$ (Fig. 2 and 10). According to Ho (1988), these two species belong respectively to the sections *Monopodiae* and *Microsperma* (cf. Table 2).

TABLE 2.

Origins of the materials examined and their chromosome numbers

Taxon	*Collection number*	*Locality and altitude*	*Examined organs*	*Chromosome number*
sect. *Monopodiae*				
G. callistantha	G173	Xiahe, Gansu, 2950m	root	$2n = 26$
G. cephalantha	G135	Dali, Yunnan, 2800m	ovary	$2n = 24$
G. duclouxii	G142	Kunming, Yunnan, 2050m	ovary	$2n = 24$
G. farreri	G045	Xiahe, Gansu, 2950m	anther	$n = 24$
	G156	Xiahe, Gansu, 2950m	root	$2n = 48$
	G194	Maqü, Gansu, 3200m	root	$2n = 48$
G. melandrifolia	G137	Dali, Yunnan, 2100m	anther	$n = 12$
G. regescens	G090	Kunming, Yunnan, 2000m	anther	$n = 12$
	G136	Dali, Yunnan, 2300m	anther	$n = 12$
	G144	Kunming, Yunnan, 2150m	anther	$n = 12$
	G150	Kunming, Yunnan, 2200m	ovary	$2n = 24$
G. sino-ornata	G177	Maqü, Gansu, 3500m	root	$2n = 48$
G. veitchiorum	G200	Xiahe, Gansu, 2950m	root	$2n = 24$
sect. *Microsperma*				
G. delavayi	G112	Lijiang, Yunnan, 2850m	anther	$n = 13$
			ovary	$2n = 26$
G. yunnanensis	G107	Lijiang, Yunnan, 2500m	anther	$n = 12$
sect. *Frigida* s.s.				
G. apiata	G077	Taibaishan, Shaanxi, 3700m	root	$2n = 24$
G. nubigena	G030	Maqü. Gansu, 3800m	ovary	$2n = 24$
sect. *Stenogyne*				
G. expansa	G117	Lijiang, Yunnan, 2850m	root	$2n = 34$
G. gentilis	G152	Kunming, Yunnan, 2200m	anther	$n = 21$
G. pterocalyx	G106	Lijiang, Yunnan, 2500m	anther	$n = 17$
G. primuliflora	G151	Kunming, Yunnan, 2200m	root	$2n = 42$
G. rhodantha	G091	Bijiang, Yunnan, 1500m	ovary	$2n = 46$
	G098	Lijiang, Yunnan, 2500m	ovary	$2n = 46$
	G143	Kunming, Yunnan, 2200m	ovary	$2n = 46$
G. striata	G188	Lüqu, Gansu, 3050m	root	$2n = 46$

Observations on meiosis in some species (cf. Table 2) indicated that both the diploid species, e.g. *G. yunnanensis, G. regescens,* and the tetraploid species sucb as *G. farreri* have regular pairing and segregation of homologous chromosomes. Only bivalents were observed in the diakinesis of these species. However, the meiosis of some species such as *G. melandrifolia* and *G. cephalantha* are not synchronous, contrary to *G. regescens* and *G. yunnanensis* where the meiosis are fairly synchronous.

Karyotype analysis of selected species indicated that their chromosomes were dominantly metacentric and therefore the karyotypes were rather symmetrical, which can be seen from both the karyotype classification (1A or 2A) and the asymmetry indices (A_1 and A_2). The sizes of chromosomes were small to medium (Table 3).

2. Sect. *Stenogyne*

In section *Stenogyne*, three different numbers were discovered (Table 2). *G. expansa* H. Sm. and *G. pterocalyx* Franchet ex Hemsley had $2n = 34$ and $n = 17$ chromosomes (Fig. 14 and 16); their basic number therefore should be $x = 17$. *G. gentilis* Franchet and *G. primuliflora* Franchet had $n = 21$ and $2n = 42$ chromosomes respectively (Fig. 15 and 17); their basic number is therefore probably $x = 21$. Whereas *G. rhodantha* Franchet ex Hemsley and *G. striata* Maxim. had $2n = 46$ and $x = 23$ (Fig. 18 and 19). All these numbers are new for the section. Among them, $x = 17$ and 23 are also recorded for the first time for the genus *Gentiana*. In addition, *G. rhodantha* shows very specific heteropycnosis. Its chromosomes form very obvious chromocentres which scatter in late prophase nuclei (Fig. 18).

The karyotypes of the section were more asymmetrical with a higher proportion of submetacentric and acrocentric chromosomes. The classification of karyotype was of 2A and 3A types. The karyotype asymmetry indices further indicated that the asymmetry was mainly intrachromosomal, that is, due to the difference between the arms of each individual chromosome. The intrachromosomal asymmetry indices (A_1) of *G. expansa* and *G. primuliflora* were as high as 0.459 and 0.503 respectively, while their interchromosomal asymmetry indices (A_2) were equal to or even slightly lower than those of the members of other sections. Chromosome sizes of the species of this section were smaller (Table 3).

DISCUSSION

Our results have documented chromosome numbers for the first time for all the 18 species of *Gentiana* investigated and revealed two new basic numbers, $x = 17$ and 23, for the genus. These two newly found numbers fill the only gaps of the spectrum of chromosome numbers of *Gentiana* (YUAN, in press; KÜPFER & YUAN, submitted); thus a continuous series of gametic chromosome numbers from 6 to 26 can be found in the genus, which suggests rather complicated and reticulate relationships among the different cytotypes. Both dysploidization and polyploidization were probably important processes in the chromosome evolution of this genus. There is no simple relationship between the chromosome numbers and classification, because each basic chromosome number is not simply confined to a single infrageneric group.

Referring to the section *Frigida* s.s., the basic number is dominantly $x = 12$, with the exception of *G. froelichii* with $x = 21$ or $x = 7$ ($2n = 42$). Moreover, *G. algida* was generally found to have $2n = 24$ chromosomes in North America, Northeastern and Eastern Asia (cf. Table 1), except for the two reports of $2n = 26$ by SOKOLOVSKAYA & STRELKOVA (1938) and

TABLE 3.
Karyotype structures of some species of *Gentiana* sect. *Frigida* s.l. and sect. *Stenogyne*

Taxon	*Coll. No.*	*Karyotype*	*Length range (µm)*	*L/S*	*P*	*Type*	A_1	A_2
G. callistantha	G173	$2n = 2m$(SAT) $+ 24m$	1.8-3.1	1.70	0.00	1A	0.176	0.143
G. sino-ornata	G177	$2n = 40m + 8sm$	2.0-3.1	1.52	0.13	2A	0.298	0.130
G. veitchiorum	G200	$2n = 18m + 6sm$	2.7-4.4	1.63	0.00	1A	0.306	0.131
G. apiata	G077	$2n = 20m + 4sm$	2.1-3.0	1.40	0.00	1A	0.282	0.103
G. expansa	G117	$2n = 2m$(SAT) $+ 8m + 24sm$	1.7-2.8	1.69	0.29	2A	0.459	0.134
G. primuliflora	G151	$2n = 12m + 24sm + 6st$	1.4-1.9	1.40	0.71	3A	0.503	0.095

L: length of the longest chromosome in a karyotype.
S: length of the shortest chromosome in a karyotype.
P: proportion of the chromosomes of which the arm ratio is higher than two in a karyotype.
Type: referring to the classification of karyotype of STEBBINS (1971).
A_1: the intrachromosomal asymmetry index defined by ROMERO ZARCO (1986).
A_2: the interchromosomal asymmetry index defined by ROMERO ZARCO l.c.

ZHUKOVA (1967b). In section *Monopodiae*, in addition to the common number for many species of $2n = 24$ that was revealed by our investigation, $2n = 26$ was also reported for *G. formosana* from Taiwan (HSU 1968), *G. yakushimensis* from Japan (SHIGENOBU 1984) and *G. callistantha* from Western China by our present investigation. So, both the basic number $x = 12$ and 13 are present and their relationships and taxonomic implications need to be confirmed by more studies. The situation in section *Microsperma* seems similar to that in section *Monopodiae*: the only two chromosome reports on this section contributed by our present study show that both $x = 12$ and 13 exist. Therefore, at least three different basic numbers, $x = 12$ (6?), 13, 21 (7?), exist in section *Frigida* s.l.

LÖVE & LÖVE (1972) simply divided this group into two genera according to their basic chromosome numbers: the monotypic *Favargera* with a basic number of $x = 7$ (21?) based on *G. froelichii*, and *Gentianodes* with a basic number of $x = 6$ (12?) including all the other members of sect. *Frigida* s.l. However, some species such as *G. delavayi* which they included in their $x = 6$ genus have in fact another basic number ($x = 13$). Additional careful and critical reconsiderations of these groups are therefore necessary.

Furthermore, HO (1985) recognized smaller sections in this group. The present investigation shows that these sections are also chromosomally polybasic. In particular, she recognized the section *Microsperma* mainly by its annual (or rather biennial) habit, but as demonstrated in section *Chondrophyllae* (KÜPFER & YUAN, submitted), plant habits are not congruent with chromosome numbers in the genus. The two annual species belonging to the section *Microsperma*, *G. delavayi* and *G. yunnanensis*, also have different basic numbers (x = 13 and 12 respectively).

Stenogyne is a poorly known section. Our present investigation took its chromosome number into account for the first time and revealed numbers very distinct from those of other members of the genus. In addition to the numbers $2n$ = 42 and n = 21 of *G. gentilis* and *G. primuliflora* which have been reported for the European *G. froelichii* of the section *Frigida*, the other numbers of $2n$ = 34 or n = 17 of *G. expansa*, *G. pterocalyx* and $2n$ = 46 of *G. rhodantha* and *G. striata* have not been recorded in the genus *Gentiana* before. The higher and obviously secondary basic numbers x = 17, 21 and 23 suggest a specialized and isolated position of this section in the genus. The karyotype data also supports that: all the species of the section analyzed have rather small chromosomes and more asymmetrical karyotypes; their intrachromosomal karyotype asymmetry indices are much higher than that of others (Table 3). According to STEBBINS (1971, p. 90), there is a predominant trend in flowering plants toward increasing asymmetry of the karyotype. Therefore, from the point of view of chromosome number and karyotype asymmetry, the section *Stenogyne* is a more advanced group than the other sections of the genus.

In addition, we demonstrated previously that the common chromosome numbers of *G. aristata* Maxim. and *G. nivalis* L. ($2n$ = 14), *G. squarrosa* Ledeb. and *G. terglouensis* Hacq. ($2n$ = 38) of sections *Chondrophyllae* and *Calathianae* Froelich may have derived independently (KÜPFER & YUAN, submitted). This could also be the case for the $2n$ = 42 of *G. primuliflora* and *G. gentilis* of the section *Stenogyne* revealed here and *G. froelichii* of section *Frigida* reported previously (FAVARGER 1968; LOVKA et al. 1971, 1972), because the former two species are very isolated both morphologically and geographically from the latter. These phenomena suggest that the same basic number may not necessarily indicate a monophyletic origin and therefore, chromosome number cannot become a good criterion for classification until the cytogenetic mechanism influencing the variation of chromosome numbers in the genus *Gentiana* is well understood.

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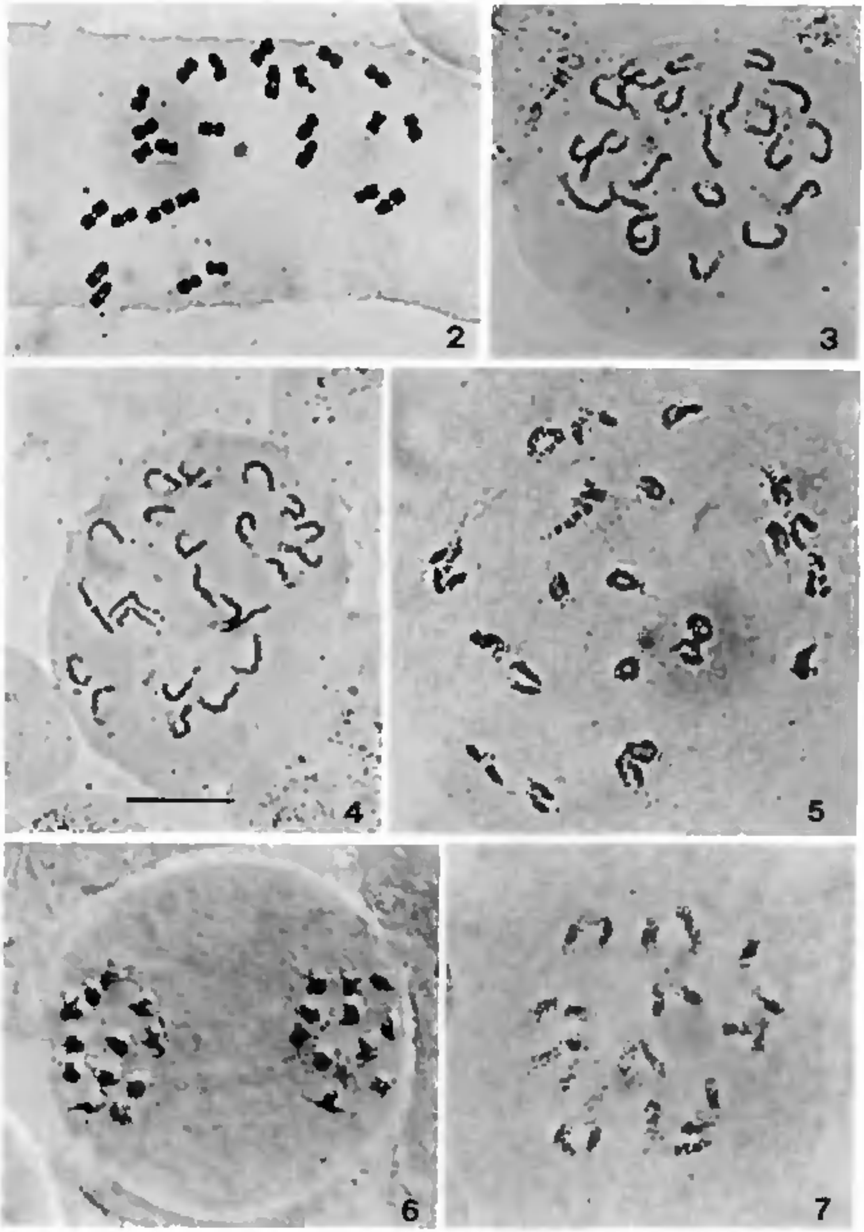
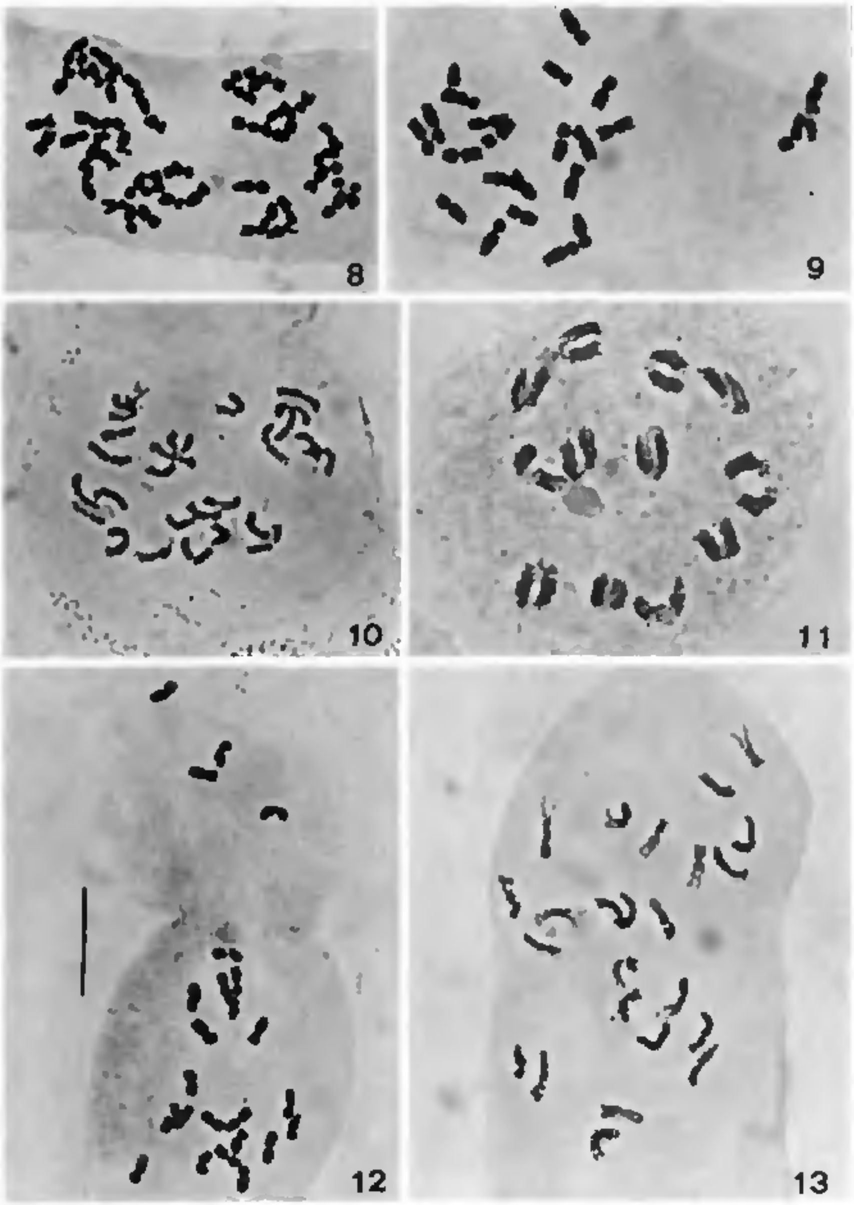
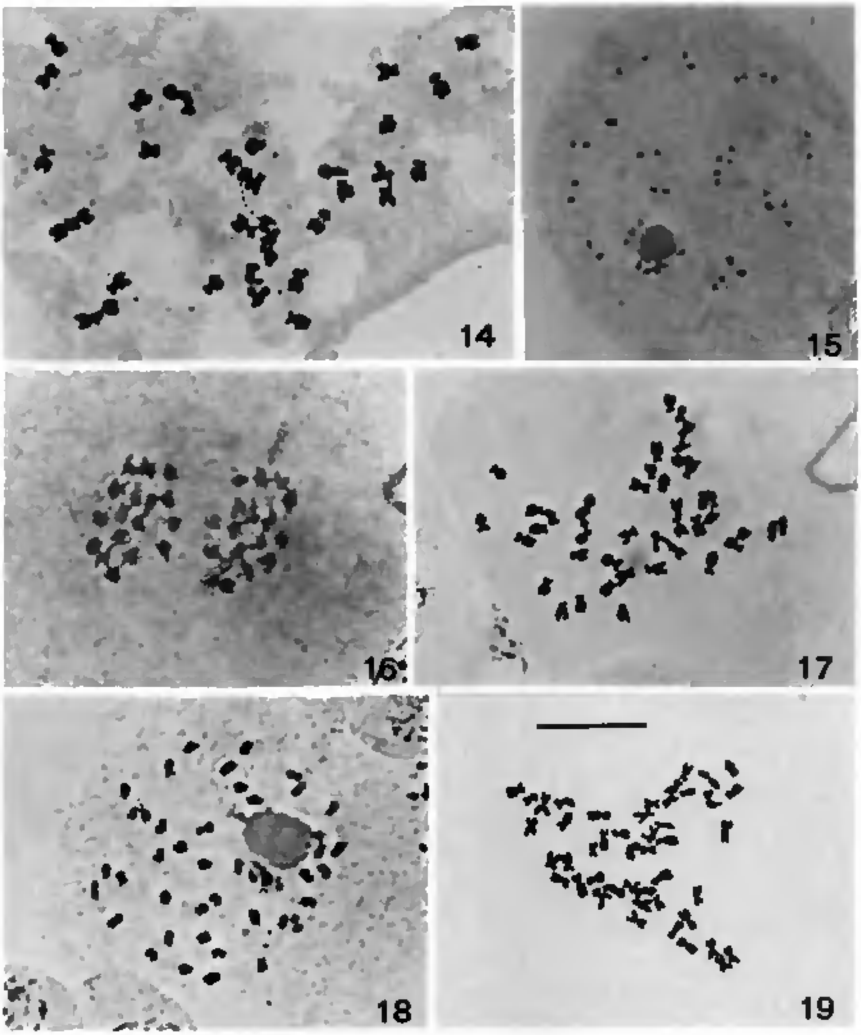


Figs. 2-7. Chromosomes of *Gentiana* sect. *Frigida* s.l. 2. *G. callistantha*, mitotic metaphase of root-tip, $2n = 26$; 3. *G. cephalantha*, mitotic prophase of ovary, $2n = 24$; 4. *G. duclouxii*, mitotic prophase of ovary, $2n = 24$; 5. *G. farreri*, diakinesis, $n = 24$; 6. *G. melandrifolia*, meiotic anaphase I, $n = 12$; 7. *G. regescens*, diakinesis, $n = 12$. — bar = 10 µm.



Figs. 8-13. Chromosomes of *Gentiana* sect. *Frigida* s.l. 8. *G. sino-ornata*, mitotic metaphase of root-tip, $2n = 48$; 9. *G. veitchiorum*, mitotic metaphase of root-tip, $2n = 24$; 10. *G. delavayi*, mitotic prophase of ovary, $2n = 26$; 11. *G. yunnanensis*, diakinesis, $n = 12$; 12. *G. aplata*, mitotic metaphase of root-tip, $2n = 24$; 13. *G. nubigena*, mitotic prophase of ovary, $2n = 24$. —bar = 10 μm.



Figs. 14-19. Chromosomes of *Gentiana* sect. *Stenogyne*. 14. *G. expansa*, mitotic metaphase of root-tip, $2n = 34$ (the dotted line indicates the chromosome which was broken by squashing); 15. *G. gentilis*, diakinesis, $n = 21$; 16. *G. pterocalyx*, meiotic anaphase I, $n = 17$; 17. *G. primuliflora*, mitotic metaphase of root-tip, $2n = 42$; 18. *G. rhodantha*, mitotic prophase of ovary, $2n = 46$; 19. *G. striata*, mitotic metaphase of root-tip, $2n = 46$; —bar = 10 µm except that in Fig. 15 the bar represents 20 µm.

Résumé

Les nombres chromosomiques de 18 espèces appartenant aux sections *Frigida* Kusn. s.l. (sect. *Monopodiae* T.N. Ho et sect. *Microsperma* T.N. Ho inclus) et *Stenogyne* Kusn. du genre *Gentiana* sont mentionnés pour la première fois. Les 25 populations étudiées proviennent des montagnes de l'ouest et du sud-ouest de la Chine. Dans la section *Frigida* s.l., *G. apiata* N. E. Br., *G. cephalantha* Hemsley, *G. duclouxii* Franchet, *G. melandrifolia* Hemsley, *G. nubigena* Edgew., *G. regescens* Hemsley, *G. veitchiorum* Hemsley et *G. yunnanensis* Franchet offrent toutes la même valence chromosomique à $2n = 24$ chromosomes; en revanche, *G. callistantha* Diels et Gilg and *G. delavayi* Franchet possèdent $2n = 26$ chromosomes; enfin, *G. farreri* Balf. f. et *G. sino-ornata* Balf. f. se sont révélés tetraploïdes à $2n = 48$ chromosomes. La polyploïdie est signalée pour la première fois dans la section. Le découpage de la section *Frigida* s.l. par HO & LIU (1990) n'a pas conduit à une meilleure adéquation des données caryologiques et morphologiques; chacune des petites sections reste polybasique. Le sect. *Frigida* s.s. paraît le plus homogène, toutes les espèces offrant $2n = 24$, à l'exception de l'espèce européenne *G. froehlichii* à $2n = 42$.

Les données relatives au sect. *Stenogyne* sont fragmentaires et hétérogènes. *G. expansa* H. Sm. et *G. pterocalyx* Hemsley ont $2n = 34$ chromosomes; *G. gentilis* Franchet et *G. primuliflora* Franchet offrent $2n = 42$ chromosomes alors que *G. rhodantha* Hemsley et *G. striata* Maxim. partagent un troisième nombre somatique, $2n = 46$. Tous ces nombres sont nouveaux pour la section, les nombres $2n = 34$ et $2n = 46$ sont même inédits pour le genre. Sur la base des données caryologiques, la section *Stenogyne* paraît relativement isolée au sein du genre. Elle contraste non seulement par ses nombres chromosomiques somatiques relativement élevés, aux relations phylétiques incertaines, mais aussi par ses chromosomes relativement petits et ses caryotypes particulièrement asymétriques.

L'ensemble de nos données rècentes (YUAN, in press; KÜPFER & YUAN, submitted) ont montré que le genre *Gentiana* possédait une série continue de nombres gamétiques de $n = 6$ à $n = 26$. La polyploïdie et la dysploïdie ont donc participé d'une manière particulièrement intense à l'évolution du genre. D'une manière générale, l'identité de nombres chromosomiques entre deux espèces du genre *Gentiana* n'indique pas nécessairement une parenté étroite mais relève sans doute, dans plusieurs cas, d'homoplasies. L'interprétation des données caryologiques nécessite donc une extrême prudence et implique l'étude parallèle des caractères morphologiques, biochimiques et phytochimiques.

Summary

Chromosome numbers are documented here for the first time for 18 species including 25 populations of *Gentiana* sect. *Frigida* Kusn. s.l. and sect. *Stenogyne* Kusn. from the high altitude regions of Western and Southwestern China. In the sect. *Frigida* s.l., *G. apiata* N. E. Br., *G. cephalantha* Hemsley, *G. duclouxii* Franchet, *G. melandrifolia* Hemsley, *G. nubigena* Edgew., *G. regescens* Hemsley, *G. veitchiorum* Hemsley and *G. yunnanensis* Franchet all had $2n = 24$ chromosomes; whereas *G. callistantha* Diels et Gilg and *G. delavayi* Franchet had $2n = 26$ chromosomes; *G. farreri* Balf. f. and *G. sino-ornata* Balf. f. were tetraploids with $2n = 48$ chromosomes. The tetraploid number $2n = 48$ was found for the first time for the section. In sect. *Stenogyne*, *G. expansa* H. Sm. and *G. pterocalyx* Hemsley had $2n = 34$ chromosomes; *G. gentilis* Franchet and *G. primuliflora* Franchet had $2n = 42$ chromosomes; *G. rhodantha* Hemsley and *G. striata* Maxim. had $2n = 46$ chromosomes. All these chromosome numbers were found for the first time for the section, among them $2n = 34$ and $2n = 46$ were new numbers for the genus. Systematic relationships of these species are discussed on the basis of their chromosome numbers.

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Karyological Studies of *Gentianopsis* Ma and Same Related Genera of Gentianaceae from China

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In the course of our karyological investigation on the genus *Gentiana* from China (Küpfer and Yuan, Yuan (a), Yuan and Küpfer, submitted), we paid attention to the other allied genera including *Gentianopsis* Ma, *Comastoma* (Wettst.) Toyokuni, *Lomatogonium* A. Braun., *Halenia* Borkh. and *Swertia* L.

The genus *Gentianopsis* was established by Ma (1951) to separate the Fringed Gentians from the genus *Gentiana* in which they were included as section *Crossopetalum* Froelich of the subgenus *Gentianella* Kusn. (Kusnezow 1895). Ma (1951) explained the full reason of his segregation carefully and was followed by Iltis (1965), Pringle (1978), Ho (1988) and Ho and Liu (1990), but was rejected by Gillett (1957), Smith (1977) and Pritchard and Tutin (1972) who usually treated this group as a section of the genus *Gentianella* Moench. However, both the macro- and micromorphology of the related species qualify its generic standing. Iltis (1965) has elucidated its morphological circumscription and nomenclatural aspects well. According to him, the genus comprises about 26 species distributed in the north temperate region. Chromosome numbers have been reported for 10 taxa in the genus (Table 1). The previously reported numbers are mainly 2n=78 with a few exceptions (Table 1). No observation has been made on Chinese materials.

The genus *Comastoma* was established from the homonymous section of the genus *Gentianella* (or *Gentiana* subgen. *Gentianella* as some authors suggested). It is characterized by a vascularly fimbriate corolla. Pringle (1978), Ho (1988) and Ho and Liu (1990) recognized the segregation, but some authors (e.g. Smith 1977, Gillet 1957, Pritch and Tutin 1972) considered it as a section or a subgenus of the genus *Gentianella*. This genus comprises about 15 species distributed in the north temperate region. Among them, *C. tenellum* (Rottb.) Toyokuni has been well studied karyologically, and was always found to have 2n=10 chromosomes (Favarger 1949, Krogulevich 1978, Löve D. 1953, Johnson and Packer 1968, Skalinska *et al.* 1968, Zhukova 1966); whereas *C. falcatum* (Turcz. ex Kar. et Kir.) Toyokuni from Siberia had 2n=28 or 30 chromosomes and *C. pulmonarium* (Turcz.) Toyokuni from Siberia had 2n=12 chromosomes (Krugolevich 1978); *C. nanum* (Wulfen) Toyokuni from Italy had 2n=30 chromosomes (Löve and Löve 1986). The other species and all the Chinese material have still not been studied karyologically.

Lomatogonium comprises 24 species distributed in Europe, Asia and North America with a higher concentration in Asia with about 20 species (Ho 1988). Only two species, *L. rotatum* (L.) Fries ex Nym. and *L. carinthiacum* (Wulfen) Reichenb. have been observed karyologically. 2n=10 (Löve and Löve 1982) and 2n=16 chromosomes (Löve and Löve 1986) have been reported for the former species; 2n=40 (Fürnkranz 1965), n=24 (Vasudevan 1975) and 2n=32 chromosomes (Krogulevich 1978, Löve 1986) for the latter.

Swertia is a highly diversified and widely distributed genus. It comprises about 170 species. Among them, 41 species have been observed karyologically. Chromosome numbers of 2n=14 (1sp.), 16 (1sp.), 18 (2spp.), 20 (9spp.), 24 (3spp,). 26 (24 spp.), 28 (2spp.), 52 (1sp.)

and 60 (1sp.) have been revealed and some species exhibited several different chromosome numbers (Goldblatt 1981–1990, Moore 1973–1977, Ornduff 1967–1968).

Halenia is mainly distributed in southwestern North America and northwestern South America. Only a few species are found in Eurasia. 14 species have been studied karyologically and all possess 2n=22 (or n=11) chromosomes (Goldblatt l.c., Moore l.c., Ornduff l.c.).

The present paper contributes more karyological data from China to these genera.

Table 1. Chromosome numbers of *Gentianopsis* species*

Taxon	Chrom. No.	Origin	References
Previous reports			
G. barbata	2n=ca. 50	Siberia	Belaeeva and Siplivinsky (1977)
	2n=78	Yakuta	Zhukova *et al.* (1977)
	2n=78	Chukotkiy Mt.	Zhukova and Petrovsky (1977)
	2n=78	South Ural	Löve & Löve (1986)
G. barbata form. *simplex*	2n=ca. 70	Tunkinsky	Krogulevich (1976)
G. ciliata	n=22	Jura	Favarger (1949)
	2n=44	Morocco	Quézel (1957)
	2n=44	Tatra	Skalinska *et al.* (1959)
	2n=44	Central Pyrénées	Löve and Löve (1975)
	2n=78	Central Pyrénées	Löve and Löve (1986)
G. ciliata ssp. *doluchanovii*	2n=78	East Ukraine	Löve and Löve (1986)
G. crinita	2n=78	Forest Home, New York	Rork (1949)
	2n=78	Castile, New York	Rork (1949)
	2n=78	Manitoba	Löve and Löve (1982)
G. crinita ssp. *macounii*	2n=78	Manitoba	Löve and Löve (1982)
G. detonsa	2n=44	Iceland	Löve D. (1953)
	2n=78	Iceland	Löve A. (1983)
	2n=78	Iceland	Löve and Löve (1986)
	n=13	Kashmir	Vasudevan (1975)
G. holopetala	n=39	California	Post (1983)
G. procera	2n=ca. 80	unknown	Denniston (1913)
	n=39	Wisconsin	Rork (1949)
	2n=78	Manitoba	Löve and Löve (1982)
G. vvedenskyi	2n=26	unknown	Matveeva and Tikholova (1968)
Present results			
G. barbata	n=13	Lijiang, Yunnan	**Yuan G097
	2n=26	Lijiang, Yunnan	**Yuan G097
	2n=52	Xiahe, Gansu	**Yuan G163
G. grandis	n=26	Lijiang, Yunnan	**Yuan G111
	2n=52	Lijiang, Yunnan	**Yuan G111
G. lutea	n=13	Lijiang, Yunnan	**Yuan G113
	2n=26	Lijiang, Yunnan	**Yuan G113
G. paludosa var. *paludosa*	2n=26	Hezuo, Gansu	**Yuan G008
G. paludosa var. *ovato-deltoideo*	2n=26	Dangchang, Gansu	**Yuan G198

* Some of the previous reports of chromosome numbers were given using the names under the genus *Gentiana* instead of the genus *Gentianopsis* in the original literatures.

** Yuan Gxxx refers to the collection number of the voucher specimen.

Materials and methods

The species examined are listed in Table 1 (*Gentianopsis*) and Table 2 (other genera). All the voucher specimens are deposited in the herbaria of Lanzhou University, China (LZU) and the University of Neuchâtel, Switzerland (NEU).

Chromosome observations were made either from the meiosis of pollen mother cells of anthers or from the mitosis of young ovaries fixed in the field with absolute alcohol and glacial acetic acid (3: 1), or from the mitosis of root-tips. For the observations on root-tips, seeds collected in the field were germinated on wet filter papers in petri dishes. The suitable root-tips, pretreated with a saturated aqueous solution of α-bromonaphthalene for 1 hr and 20 min, were stained and squashed with the aceto-orcein method previously described (Yuan (a), submitted). Snow's (1963) method was followed for the observations on meiosis of the pollen mother cells and the mitosis of young ovaries.

Drawings were made with a camera lucida apparatus using temporary slides. Each chromosome number was determined from at least 10 different preparations.

Table 2. Chromosome numbers and origins of materials of examined species of *Comastoma*, *Lomatogonium*, *Swertia* and *Halenia*

Taxon	Origin and altitute	Coll. No.	Chrom. No.	Previous report
C. polycladum	Xiahe, Gansu; 2900 m	G161	$2n=16^{*}$	
C. pulmonarium	Luqü, Gansu; 3000 m	G193	$2n=18^{**}$	$2n=12$ (Krogulevich 1978)
	Hezuo, Gansu; 2950 m	G182	$2n=18^{**}$	
L. rotatum	Lanzhou, Gansu; 2800 m	G166	$2n=16$	$2n=10$ (Löve and Löve 1982), 16 (Löve and Löve 1986)
L. macranthum	Xiahe, Gansu; 2900 m	G157	$2n=16^{*}$	
S. diluta	Dangchang, Gansu; 2600 m	G199	$2n=20^{*}$	
H. elliptica	Hezuo, Gansu; 2980 m	G185	$2n=22$	$2n=22$ (Favarger 1952, Weaver and Rudenberg 1975)
	Lanzhou, Gansu; 2600 m	G170	$2n=22$	

* First chromosome number report for the species.

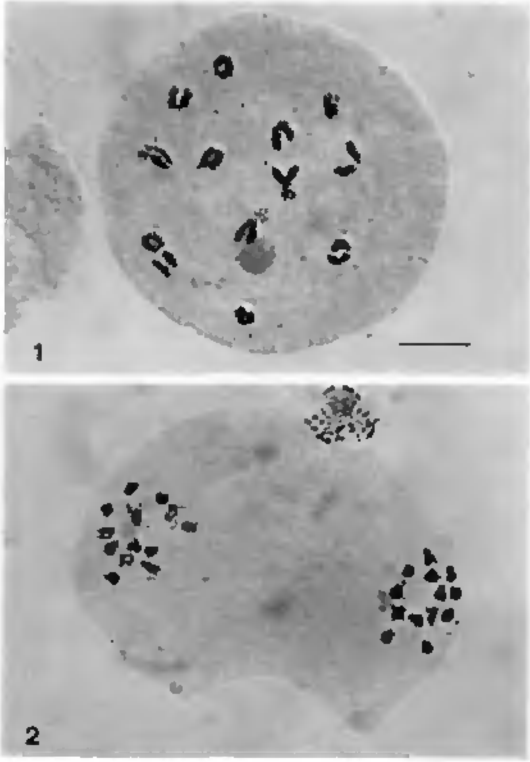
** New chromosome number for the species.

Results

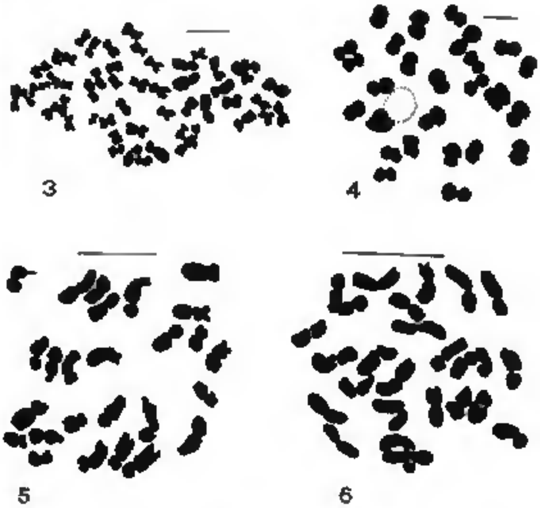
The present investigation documents chromosome numbers of 11 taxa representing 5 genera of *Gentianaceae* from China. The results are shown in Table 1 and Table 2. The chromosomes of the species examined are illustrated in Figs. 1–12. Among them, observations for 7 taxa were made for the first time.

In *Gentianopsis*, *G. lutea* (Burk.) Ma, *G. paludosa* (Hook. f.) Ma var. *paludosa* and *G. paludosa* var. *ovato-deltoidea* (Burk.) Ma ex T. N. Ho, are diploid, with $2n=26$ ($n=13$) chromosomes (Figs. 2, 5–6); *G. grandis* (H. Smith) Ma is tetraploid with $2n=52$ ($n=26$) chromosomes (Fig. 4); while both diploid ($2n=26$, $n=13$) and tetraploid ($2n=52$) cytotypes were found for *G. barbata* (Froelich) Ma (Figs. 1, 3). All the numbers for these species are reported here for the first time, except that a different number, $2n=78$, has been previously reported for *G. barbata* from Russian Siberia and the Far East. The tetraploid number $2n=52$ ($n=26$) was found for the first time for the genus in the present study. The distribution pattern of the different cytotypes of the genus, according to the previous reports and the results of this study, is shown in Fig. 13.

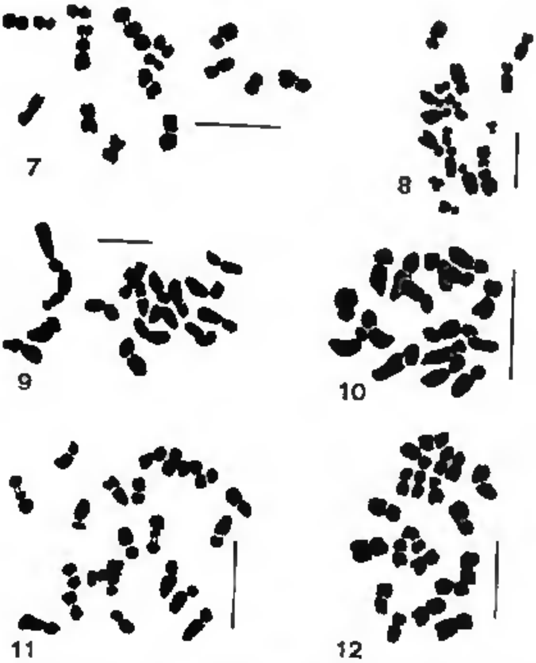
In *Comastoma*, $2n=16$ chromosomes were counted for *C. polycladum* (Diels et Gilg) T. N. Ho for the first time (Fig. 7) and a different number, $2n=18$, was recorded for *C. pulmonarium* (Fig. 8) which had previously been reported to have $2n=12$ chromosomes from Siberia. In the karyotype of the first species, two chromsomes are obviously satellited, whereas in the second no visible satellite was found, but instead two extra chromosomes. Extensive obser-varions, on different preparations, of many individuals from two populations of the second



Figs. 1–2. Microphotographs of *Gentianopsis* chromosomes. 1, *G. barbata* (G097), diakinesis, n=13 bivalents; 2, *G. lutea*, meiotic telophase I, n=13. Bar=10 μm.



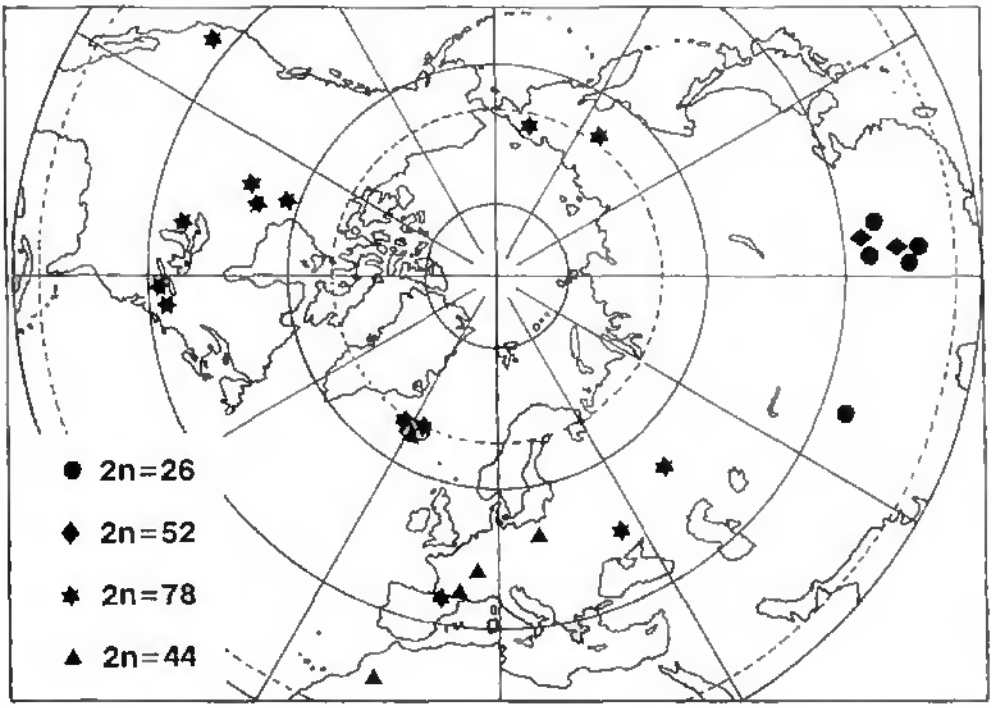
Figs. 3–6. Drawings of *Gentianopsis* chromosomes. 3, *G. barbata* (G163), mitotic metaphase, 2n=52; 4, *G. grandis*, diakinesis, n=26 bivalents; 5, *G. paludosa* var. *paludosa*, mitotic premetaphase, 2n=26; 6, *G. paludosa* var. *ovato-deltoidea*, mitotic premetaphase, 2n=26. Bars=5 μm.



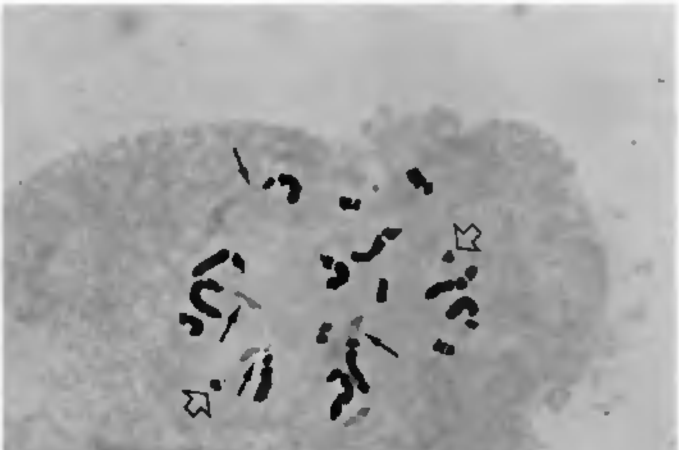
Figs. 7–12. Chromosomes drawings. 7, *Comastoma polycladum*, mitotic metaphase, 2n=16; 8, *C. pulmonarium*, mitotic metaphase, 2n=18; 9, *Lomatogonium rotatum*, mitotic premetaphase, 2n=16; 10, *L. macranthum*, mitotic premetaphase, 2n=16; 11, *Halenia elliptica*, mitotic metaphase, 2n=22; 12, *Swertia diluta*, mitotic metaphase, 2n=20. Bars=5 μm.

species indicated that this difference was by no means a technical artefact due to squashing. However, this species showed very distinct telomeric heteropycnosis which was not found in the former (Fig. 14). The chromosomes of the genus were rather small-sized.

Both *Lomatogonium rotatum* and *L. macranthum* (Diels et Gilg) Fern. were found to have



Figs. 13. Geographical distribution of the known cytotypes of *Gentianopsis*, according to the data in Table 1. Indefinite counts and counts without localities are not shown. Diploid and tetraploid cytotypes occur in SW and W China as well as in the adjacent Himalayan area.



Figs. 14. Microphotograph of mitotic premetaphase chromosomes of *Comastoma pulmonarium* showing the two smallest chromosomes (empty arrows) and the distinct telomeric heteropycnosis (solid arrows). Bar = 10 μm.

2n=16 chromosomes with similar morphology (Figs. 9, 10). The chromosome number of the latter species is reported here for the first time.

Swertia diluta (Turcz.) Benth. et Hook. f. was counted as 2n=20 here for the first time (Fig. 12) and *Halenia elliptica* D. Don as 2n=22 (Fig. 11) which confirmed the previous reports for it.

Discussion

1. The basic chromosome number of the genus *Gentianopsis* and its systematic implications

Löve and Löve (1975) and Löve (1953) had suggested the basic number x=11 for the genus *Gentianopsis*, based on their observations of *G. ciliata* and *G. detonsa*. Meanwhile, they suggested that taxa such as *G. crinita* (Froel.) Ma and *G. procera* (Th. Holm) Ma with 2n=78 chromosomes should perhaps be placed in a different genus with a basic number of x=13. With more chromosome data available now, we are able to draw the conclusion that the basic number of the genus is x=13 rather than x=11, since most of the species observed, including the type species of the genus *G. barbata*, are diploids, tetraploids or hexaploids of x=13 (Table 1). However, the exceptions of 2n=44 found in *G. ciliata* do indicate a separate position of this species and seem rather stable. The only different report of 2n=78 chromosomes from Central Pyrenees (Löve and Löve 1986) is doubtful and needs to be confirmed. Correlated with its distinct chromosome number, some morphological characters such as the shorter pedicels (as long as the internodes of its stem) and the winged and reticulated seedcoat are distinctly different from that of the other members of the genus which possess pedicels much longer than the stem internodes, and seeds unwinged and sculptured with finger-like projections on their seedcoats (Yuan (b) submitted). Nevertheless, as a whole, this species fits the circumscriptions of the genus *Gentianopsis* well (Iltis 1965). For *G. detonsa*, the report of 2n=44 from Iceland (Löve 1953) needs to be confirmed because the other reports were either hexaploid 2n=78 in the same area (Löve 1983, Löve and Löve 1986), or diploid with n=13 from Kashmir (Vasudevan 1975).

Ma (1951) and Iltis (1965) have elucidated the generic characters of *Gentianopsis* well. But some authors still consider it as a section of the genus *Gentianella* (e.g. Pritchard and Tutin 1972). The chromosome data however, suggests a separate generic position. The chromosome numbers available for *Gentianella* (excluding *Comastoma* and *Gentianopsis*) are tetraploid (2n=36) or occasionally diploid (2n=18) with the basic number x=9. Whereas, as elucidated above, the basic number of *Gentianopsis* is x=13 which is by no means similar to that of *Gentianella* but is the same as that of some sections of the genus *Gentiana* (e.g. sect. *Cruciata* and sect. *Pneumonanthe*). The seedcoat sculptures also indicate its closer relationship to *Gentiana* than to *Gentianella* (Yuan (b) submitted), although some floral characters (e.g. the position of glands and the unplaited corolla lobes) indicate a closer relationship with *Gentianella*.

2. The origin and diversification of the genus *Gentianopsis*

From the distribution map of the cytotypes of *Gentianopsis* (Fig. 13), one can conclude that except the European *G. ciliata*, all the examined species from Eastern Europe, Far Eastern Russia and North America to Iceland are uniformly hexaploid with 2n=78 chromosomes. Diploid cytotypes (2n=26) were found only in southwestern and western parts of China and the adjacent Himalayan area. Tetraploid cytotypes (2n=52) revealed by the present study, restricted to Southwestern and Western China. Therefore, we suggest that the unglaciated mountainous regions of Southwestern and Western China and the adjacent Himalayan area may represent the most important centre of origin and primary diversification of the genus, where at least three different diploid species and two tetraploid species exist. Polyploidization may be the main course of adaptive radiation from the centre towards the higher latitude area. The tetraploid (inter- and intraspecific) cytotypes may be formed by primary polyploidization around the centre. The hexaploid (inter- and intraspecific) cytotypes may have arisen from secondary polyploidization or hybridization. The distinct European species *G. ciliata* may be a specialized form derived from a 2n=52 ancestor either by down dysploidy or hybridization. This still needs to be confirmed by a more detailed study.

3. The karyological aspects of the other genera.

The existing chromosome data suggest that *Comastoma* is a polybasic taxon. *C. tenellum* was always found to possess 2n=10, *C. nanum* 2n=30; *C. falcatum* 2n=28 or 30; *C. polycladum* 2n=16 and *C. pulmonarium* 2n=12 from Siberia (Krogulevich 1978) but 2n=18 in the present study. The relationships among those numbers are still far from clear. However, the appearance of the smallest pair of chromosomes in *C. pulmonarium* correlated with the loss of the big satellites may give us an indication concerning the variation of the basic chromosome number (x=8, 9).

Our results revealed 2n=16 for both *Lomatogonium rotatum* and *L. macranthum*, which further confirmed the basic number of the genus to be x=8. The chromosome number and morphology suggest a close relationship between *Lomatogonium* and some species of *Comastoma*, such as *C. polycladum*.

In *Swertia* 2n=20 chromosomes has been reported from very different regions such as Japan, the Himalaya and Aftrica. Our observation on *S. diluta* indicated that this number also occurs in China. Many more observations on different species are needed before any conclusion can be drawn. Our observation also confirmed 2n=22 for *Halenia elliptica* which was congruent with the previous reports on the genus and further indicated that this genus is fairly stable karyologically.

Summary

Chromosome numbers were documented for 11 taxa representing 5 genera of Gentianaceae from China. *Gentianopsis lutea*, *G. paludosa* var. *paludosa* and var. *ovato-deltoidea* were diploid with 2n=26; *G. grandis* was tetraploid with 2n=52 (n=26); while both diploid (2n=26, n=13) and tetraploid (2n=52) cytotypes were found for *G. barbata*. The tetraploid number was found for the first time for the genus. The present results confirmed the basic number x=13 for the genus and revealed that the mountainous regions of western and southwestern China and the adjacent Himalayan area probably represent the most important origin and primary diversification centre of the genus, with regard to the distribution pattern of cytotypes. The chromosome number of *Comastoma polycladum* was 2n=16 with two satellited chromosomes is reported here for the first time. The chromosome number of *C. pulmonarium* was 2n=18, without any visible satellite which differed from previous reports. *Lomatogonium rotatum* and *L. macranthum* all had 2n=16 chromosomes. It is the first report of chromosome number for the second species. *Swertia diluta* was found to have 2n=20 chromosomes, again mentioned for the first time. The chromosome number 2n=22 was confirmed for *Halenia elliptica*.

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Karyological studies on *Gentiana* sect. *Chondrophyllae* (*Gentianaceae*) from China

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Key words: *Gentianaceae, Gentiana,* sect. *Chondrophyllae.* – Karyosystematics, cytogeography, chromosome numbers, karyotype, chromosome evolution.

Abstract: Nineteen populations of fifteen species of *Gentiana* sect. *Chondrophyllae* from China were observed cytologically. *Gentiana alsinoides, G. anisostemon, G. asterocalyx, G. exigua, G. heterostemon, G. intricata, G. praticola, G. pseudoaquatica* , *G. spathulifolia,* and *G. subintricata* all had the same chromosome number of $2n = 20$ (or $n = 10$), whereas *G. piasezkii* had $2n = 36$, *G. squarrosa* $2n = 38$, *G. prattii* $2n = 18$, *G. aristata* $2n = 14$ ($n = 7$), and *G. heleonastes* $2n = 12$. All these chromosome numbers are documented here for the first time, except for *G. squarrosa,* where it is a new number report. The basic numbers of $x = 6$, $x = 7$ and $x = 19$ are new for the section. Karyotype analyses of some species revealed that, except for a few cases, the species examined mainly had metacentric chromosomes. $2n = 20 = 2m(SAT) + 18m$ was found to be the main type of karyotype for the species with $2n = 20$. Chromosomal evolution and its mechanism in this section are also discussed.

Chondrophyllae Bunge consists of 158 species and represents the largest section of the genus *Gentiana* L. It is a subcosmopolitan section and has its highest diversity in Asia (154 species), especially in the mountain regions of SW China, where 88 species are found. One species of the section, *G. quadrifaria* Blume, extends to SE Australia through SE Asia. Three species exist in North and Central America: *G. douglasiana* Bong., endemic to West N America, *G. pumilio* Standley & Steyerm., endemic to Central America (Guatemala and Mexico) and a common species, *G. aquatica* L., in North America and northwestern Asia. Two species occur in Europe: *G. boryi* Boiss., endemic to Spain, and *G. pyrenaica* L. found in the East Pyrenees, the Carpathians, SW Bulgaria and West Asia. One species, *G. atlantica* Litard. & Maire, occurs in Africa (Morocco) (Ho & Liu 1990). Some other closely related species, including *G. prostrata* Haenke (2n=36), *G. nutans* Bunge (2n=36) and *G. sedifolia* Kunth (n=20), have recently been separated into a distinct section, *Dolichocarpa* T. N. Ho (Ho & Liu 1990), and are therefore not included in the present paper.

Recently, Löve & Löve (1975, 1978, 1986) have advocated the splitting of the genus *Gentiana* into several smaller so-called monobasic genera based on basic chromosome numbers. They suggested three new genera for some species of sect. *Chondrophyllae*, i.e., *Holubogentia* Löve & Löve (=*Holubia* Löve & Löve non Oliver,1884), *Kuepferella* Laínz and *Chondrophylla* A. Nelson, typified by the species *G. pyrenaica, G. boryi*, and *G. prostrata*, respectively. Since the real cytological mechanism influencing the variation of chromosome numbers is not clear and a greater variation of basic numbers exists in Asian species, this splitting might simply create more confusion and was therefore not followed in the present paper.

In addition, Omer (1989) established another new genus, *Qaisera* Omer, based on *G. carinata* Griseb. and including two other species. These species used to be included in sect. *Chondrophyllae* but Ho & Liu (1990) treated them as a newly named sect. *Fimbricorona* T. N. Ho (= sect. *Eurythalia* Griseb., nomen illeg.). The diagnostic character of this group is the fimbriate corolla throat. The type species is cytologically undistiguishable from many other species of sect. *Chondrophyllae* because it has a chromosome number of 2n = 20 common to many others (see below). The recognition of the new genus needs to be confirmed by more evidence. For the time being we will leave it in the original sect. *Chondrophyllae* of *Gentiana*.

At present, chromosome data are available for 23 species and 4 intraspecific taxa of the section (Table 1). Almost all the species of the section from outside Asia are known cytologically, except for the endemic Central American species *G. pumilio*. The two European species have been observed repeatedly. *Gentiana pyrenaica* was constantly found to possess 2n = 26 chromosomes (Küpfer & Favarger 1967; Favarger & Küpfer 1968; Löve & Löve 1975, 1986), while different counts of 2n=20 (Küpfer 1968, Löve & Löve 1986) and 2n = 26 (Löve & Löve 1975) were given for the Spanish endemic species, *G. boryi*. The only representative of the section on the African continent, *G. atlantica*, was once suspected to be the vicarious species of the Spanish *G. boryi* but it has a very different chromosome number of 2n = 48 (Küpfer 1980). The American species, *G. douglasiana*, was found to have n = 13, a very common number for the genus in that region (Taylor & Mulligan 1968, Pojar 1973). Among the 154 species in Asia, only 19 species have been observed cytologically. The observations were mainly made from the Russian Siberia and Far East, Japan, New Guinea and the Western Himalaya. Different numbers, such as 2n = 18, 20, 24, 26, 36, 40, 44, 48, 96–98, have been reported, although 2n = 20 (or n = 10) was found to be the commonest one. Different numbers were found between certain closely related taxa such as *G. nipponica* Maxim. var. *nipponica* (2n = 36) and var. *robusta* Hara (2n = 96–98), *G. quadrifaria* var. *quadrifaria* (n = 9) and var. *zeylanica* Kusn. (n = 10). Intraspecific polyploidy was reported for *G. carinata* from the Western Himalaya (Vasudevan 1975) and for *G. nipponica* from Japan (Shigenobu 1982a, 1984). The two Taiwanian species, *G. flavomaculata* Hayata and *G. scabrida* Hayata, have unusual numbers of 2n = 24 and 2n = 44, respectively (Chuang & al. 1963, Hsu 1968). *Gentiana aquatica* is a common species of North Asia and North America. It has 48 somatic chromosomes, the same number as the African species *G. atlantica* which is completely isolated from it geographically. Different numbers have been reported for the common Asiatic species, *G. squarrosa* Ledeb.: 2n = 20 from Japan (Shigenobu 1984) and 2n = 36 from Russian Siberia (Krasnoborov & al. 1980). Although the section appears to have its highest diversity in the mountainous

Table 1. Previous reports of chromosome data on *Gentiana* sect. *Chondrophyllae*

Taxon	Habit	Origin	Chromosome number	References
G. altaica	perennial	Altai	n = 13	Sokolovskaja & Strelkova (1938)
G. aquatica	biennial	Siberia	2n = 48	Belaeva & Siplivinsky (1977, cited in Agapova 1990)
G. argentea D. Don	biennial	W Himalaya	n = 10	Mehra & Vasudevan (1972)
		W Himalaya	n = 10	Vasudevan (1975)
G. atlantica	biennial	Morocco	2n = 48	Küpfer (1980)
G. boryi	perennial	Spain	2n = 20	Küpfer (1968)
		Spain	2n = 26	Löve & Löve (1975)
		Spain	2n = 20	Löve & Löve (1986)
G. capitata	biennial	Nepal	2n = 20	Malla & al. (1984)
		W Himalaya	n = 10	Mehra & Vasudevan (1972)
G. capitata var. *strobiliformis* Clarke	biennial	W Himalaya	n = 10	Vasudevan (1975)
G. carinata	biennial	W Himalaya	n = 20	Mehra & Gill (1968)
		Kashmir	n = 10	Vir Jee & Kachroo (1985)
		W Himalaya	n = 10, 20	Vasudevan (1975)
G. cruttwellii	perennial	New Guinea	2n = 20	Borgmann (1964)
G. djimilensis	perennial	Aragats	2n = 26	Voskaenian (1974, cited in Agapova (1990)
G. douglasiana	biennial	N America	n = 13	Taylor & Mulligan (1968)
		N America	n = 13	Pojar (1973)
G. ettinghausenii F. Muell.	?	New Guinea	2n = 20	Borgmann (1964)
G. flavomaculata	biennial	Taiwan	2n = 24	Chuang & al. (1963)
G. grandiflora Laxm.	perennial	Siberia	2n = 26	Krogulevich (1978)
G. jamesii	perennial	Hokkaido	2n = 36	Shigenobu (1982a, 1984)
G. nipponica	perennial	Fukushima	2n = 36	Shigenobu (1982a, 1984)
G. nipponica var. *robusta*	perennial	Fukushima	2n = 96, 97, 98	Shigenobu (1982a, 1984)
G. pedicellata	biennial	Nepal	2n = 18	Malla & al. (1984)
		W Himalaya	n = 9	Mehra & Vasudevan (1972)
		W Himalaya	n = 9	Vasudevan (1975)
G. pedicellata var. *wightii* Kusn.	biennial	India	2n = 20	Mallikarjuna (1985)
	biennial	India	2n = 20	Mallikarjuna & al. (1987)
G. piundensis Van Royen	?	New Guinea	2n = 20	Borgmann (1964)
G. pyrenaica	perennial	Pyrenees	2n = 26	Favarger & Küpfer (1968)
		Pyrenees	2n = 26	Küpfer & Favarger (1967)
		Spain	2n = 26	Löve & Löve (1975)

Table 1 (continued)

Taxon	Habit	Origin	Chromosome number	References
		Pyrenees	2n = 26	Löve & Löve (1986
G. quadrifaria	biennial	India	n = 9	Sharma & Sarkar (1970)
G. quadrifaria var. *zeylanica*	biennial	India	n = 10	Mallikarjuna (1985)
	biennial	India	n = 10	Mallikarjuna & al. (1987)
G. scabrida	biennial	Taiwan	2n = 44	Hsu (1968), Chuang & al. (1963)
G. squarrosa	biennial	Siberia	2n = 36	Krasnoborov & al. (1980)
		Japan	2n = 20	Shigenobu (1984)
G. thunbergii	biennial	Japan	2n = 36	Shigenobu (1982b, 1984)
G. zollingeri Fawcett	biennial	Japan	n = 10, 2n = 20	Wada (1966)
		Japan	2n = 20	Shigenobu (1984)
		Far East	2n = 20	Starodubtsev (1985)

regions of SW China, no cytological observation has ever been made from these regions before the present investigation.

From this short survey one realizes the great variation existing in chromosome numbers in this section, though 2n = 20 seems to be the commonest number. It has been found from New Guinea, Japan, India, the Himalaya and the Spanish Sierra Nevada. The question thus arises whether the distribution pattern of the number in so many different geographical regions indicates a primitive monophyletic origin or a newly formed polyphyletic origin. In other words, what is the relationship between the variation of basic number and the pattern of geographical distribution? Which number could most probably be the ancestral basic number? And what is the relationship between the ancestral number and the other derived numbers? To answer these questions, it is obviously necessary to observe as many species as possible, especially those from the diversification and origin center(s). The present paper, following our reports on sections of the genus (Yuan 1993, Yuan & Küpfer 1993), intends to contribute more karyological data towards a better understanding of the cytotaxonomy and cytogeography of the genus *Gentiana*.

Material and methods

All the species examined here are biennial except one, *G. praticola* Franchet, which is perennial. The sources of materials are listed in Table 2. The sampling sites are shown in Fig. 18. The vouchers are deposited in the Herbarium of the University of Neuchâtel, Switzerland (NEU) and the Herbarium of Lanzhou University, China (LZU). Mitotic observations were made from root tips. Seeds were germinated on wet filter papers in petri dishes. Root tips, pretreated with a saturated water solution of α-bromonaphthalene for 110 min at room temperature, were fixed with Carnoy 3 : 1. The preparation procedure

Table 2. Origins of the *Gentiana* species examined and their chromosome numbers

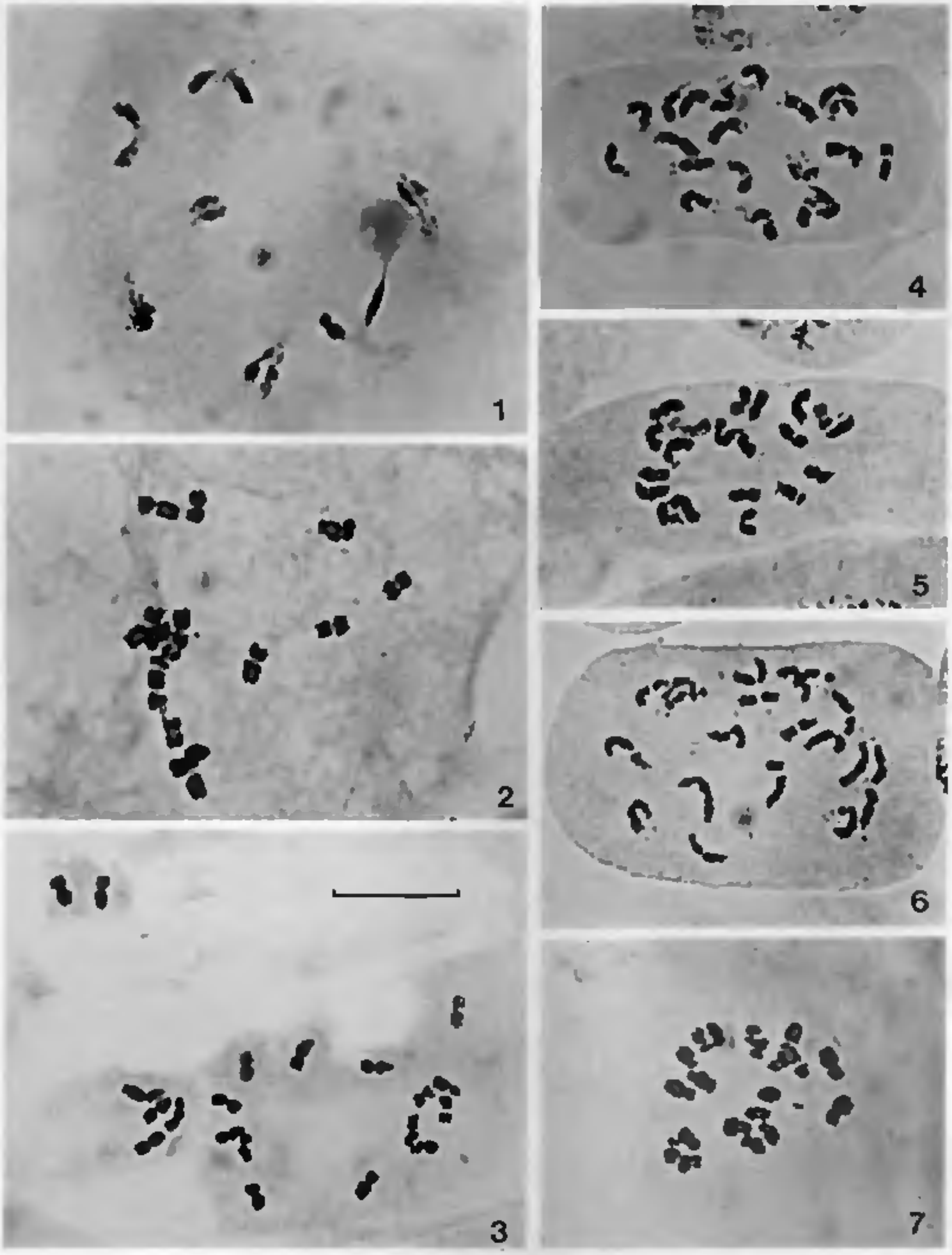
Taxon	Locality and altitude s.m.	Collection no.	Chromosome no.
G. alsinoides	Lijiang, Yunnan, 2950 m	G123	2n = 20
G. anisostemon	Kunming, Yunnan, 2200 m	G148	2n = 20
G. asterocalyx	Lijiang, Yunnan, 2900 m	G119	2n = 20
G. aristata	Luqü, Gansu, 3360 m	G014	n = 7
	Luqü, Gansu, 3450 m	G190	2n = 14
G. exigua	Lijiang, Yunnan, 2400 m	G101	2n = 20
G. heleonastes	Maqü, Gansu, 3650 m	G032	2n = 12
G. heterostemon	Dali, Yunnan, 2100 m	G133	2n = 20
G. intricata	Lijiang, Yunnan, 3200 m	G125	2n = 20
G. piasezkii	Mingxian, Gansu, 2400 m	G061	2n = 36
	Dangchang, Gansu, 2250 m	G068	2n = 36
G. praticola	Dali, Yunnan, 2200 m	G134	2n = 20
	Kunming, Yunnan, 2200 m	G149	2n = 20
G. prattii	Luqü, Gansu, 350 m	G191	2n = 18
G. pseudoaquatica	Luqü, Gansu, 3300 m	G015	n = 10
G. spathulifolia	Lanzhou, Gansu, 2400 m	G004	2n = 20
G. squarrosa	Xiahe, Gansu, 3000 m	G046	2n = 38
	Xiahe, Gansu, 2900 m	G155	2n = 38
G. subintricata	Lijiang, Yunnan, 2900 m	G121	2n = 20

was the same as in our previous report (Yuan 1993). Meiotic observations were made from pollen mother cells. Mitotic observations on *G. heleonastes* H. Sm. ex Marq. were made from ovaries. Young flower buds were fixed from the field with Carnoy 3 : 1. After washing out the fixative completely with 70% ethanol, the flower buds were stained with hydrochloric carmine (Snow 1963) for 48 h at 60 °C, and were then gently heated for about two min in 45% acetic acid. The selected anthers and ovaries were then squashed for observation.

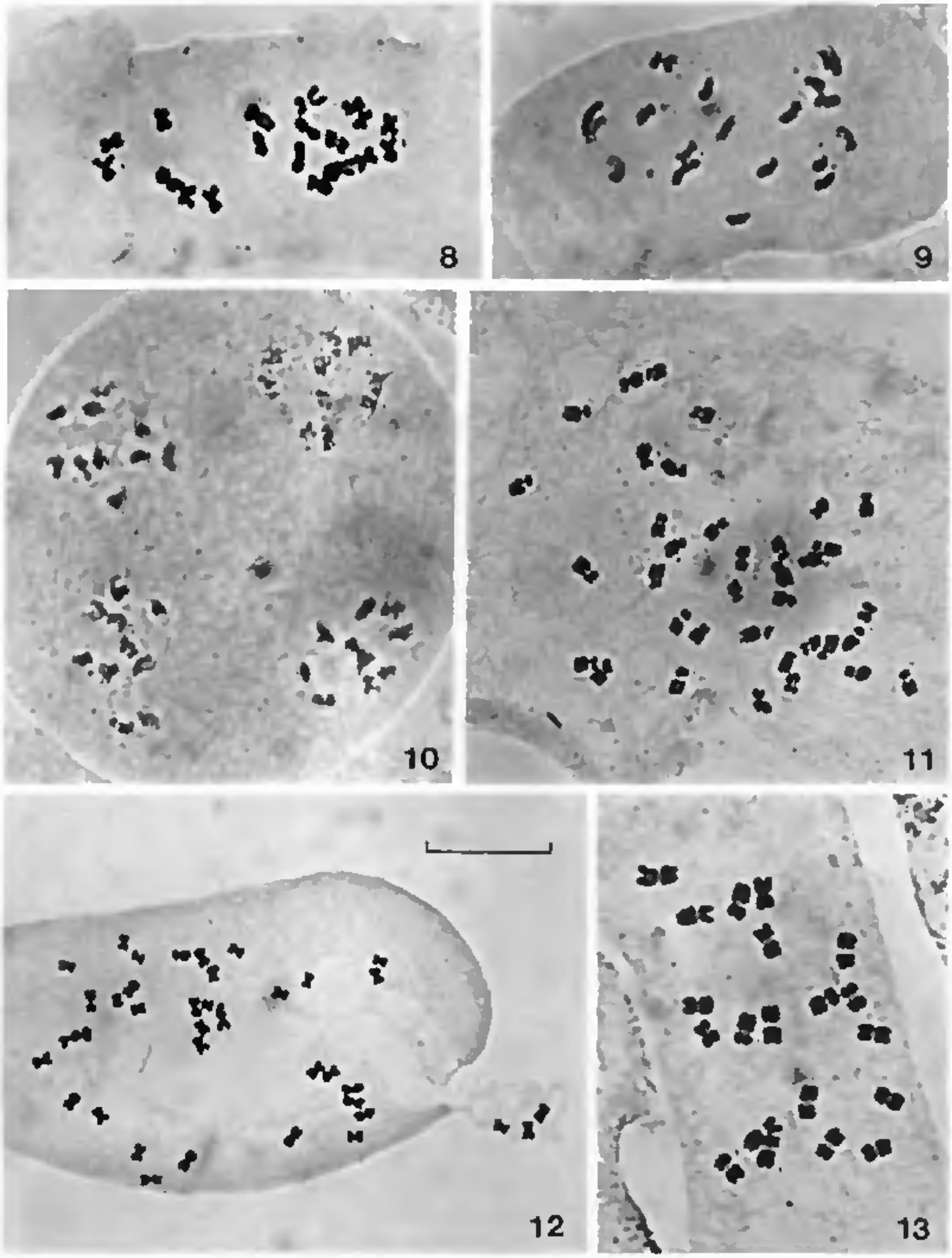
The chromosome parameters were measured from a number of cells varying from one to five. Homologous chromosomes were recognized by their morphology. The terminologies for centromere positions introduced by Levan & al. (1964), the karyotype classification of Stebbins (1971) and the karyotype asymmetry indices defined by Romero Zarco (1986) were followed.

Results

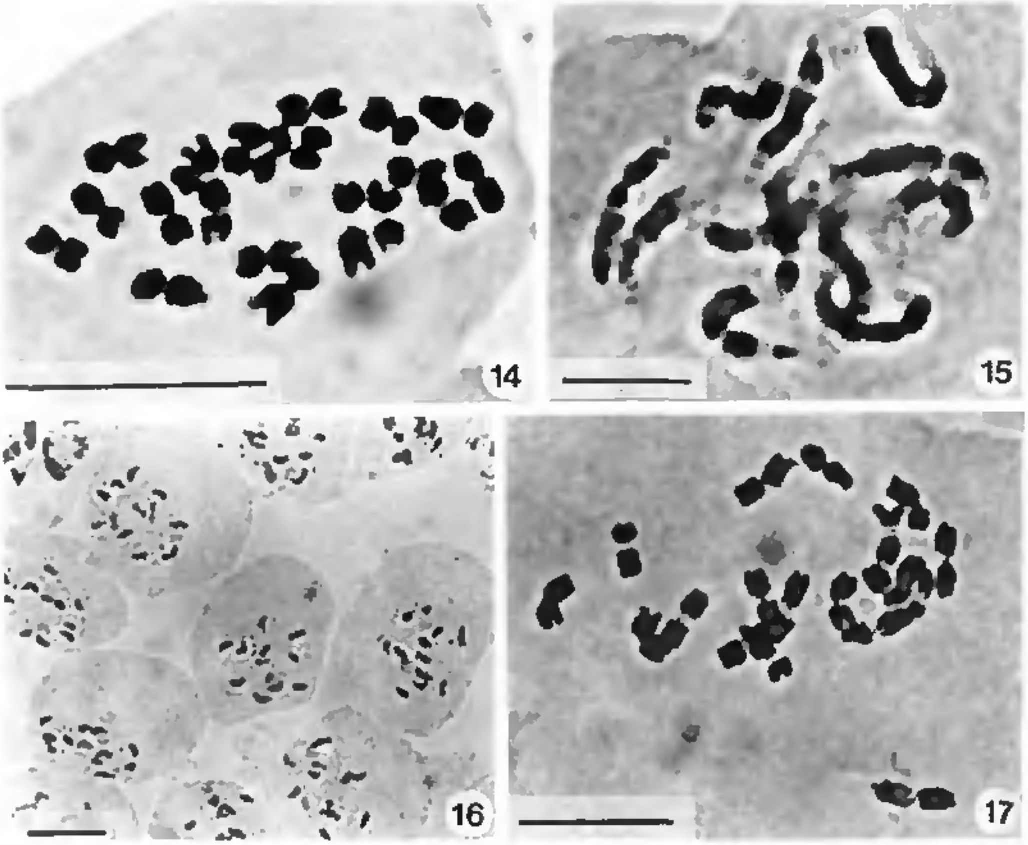
The present paper gives the first chromosome number reports for 14 species and a new number record for one species of *Gentiana* sect. *Chondrophyllae*. The chromosome numbers along with the origins of the materials examined are listed in Table 2. The chromosome morphology is illustrated in Figs. 1–17. Most of the species examined, including *G. alsinoides* Franchet, *G. anisostemon* Marq., *G. asterocalyx* Diels, *G. exigua* H. Sm., *G. heterostemon* H. Sm., *G. intricata* Marq., *G. praticola*, *G. pseudoaquatica* Kusn., *G. spathulifolia* Maxim. ex Kusn., and *G. subintricata* T. N. Ho, have the same chromosome number of 2n = 20 (or n = 10), which corresponds to the number most commonly reported for this section. *Gentiana piasezkii* Maxim. was counted as 2n = 36, which is the same as some species of the section reported from Japan (Table 1). *Gentiana prattii* Kusn. was counted



Figs. 1–7. Chromosomes of *Gentiana* sect. *Chondrophyllae*. – Fig. 1. *G. aristata*, diakinesis, n = 7 bivalents. – Fig. 2. *G. aristata*, mitotic metaphase, 2n = 14. – Fig. 3. *G. asterocalyx*, mitotic metaphase, 2n = 20. – Fig. 4. *G. anisostemon*, mitotic metaphase, 2n = 20. – Fig. 5. *G. exigua*, mitotic metaphase, 2n = 20. – Fig. 6. *G. heterostemon*, mitotic metaphase, 2n = 20. – Fig. 7. *G. intricata*, mitotic metaphase, 2n = 20. – Bar: 10 µm



Figs. 8–13. Chromosomes of *Gentiana* sect. *Chondrophyllae*.– Fig. 8. *G. praticola*, mitotic metaphase, 2n = 20. – Fig. 9. *G. subintricata*, mitotic metaphase, 2n = 20. – Fig. 10. *G. pseudoaquatica*, meitotic telophase II, n = 10. – Fig. 11. *G. piasezkii*, mitotic metaphase, 2n = 36. – Fig. 12. *G. squarrosa*, mitotic metaphase, 2n = 38. – Fig. 13. *G. spathulifolia*, mitotic metaphase, 2n = 20. – Bar: 10 µm



Figs. 14–17. Chromosomes of *Gentiana* sect. *Chondrophyllae*. – Fig. 14. *G. alsinoides*, mitotic metaphase, 2n = 20. – Figs. 15, 16. *G. heleonastes*, late prophase (2n = 12) and earlier prophase showing the 12 conspicuous chromocentres. – Fig. 17. *G. prattii* mitotic metaphase, 2n = 18. – Bars: 5 µm in Fig. 15, 10 µm in Figs. 14, 16, 17

as 2n = 18, a number previously reported only for a Himalayan species, *G. pedicellata* Wall. Nevertheless, our observations revealed that *G. aristata* Maxim. had 2n = 14 and n = 7, which was a new number in this section and previously reported only for a European species of the genus, *G. nivalis* L. of sect. *Calathianae* Froelich. We found 2n = 12 for *G. heleonastes*, a number never recorded before for this genus. In addition, we revealed a new number of 2n = 38 for *G. squarrosa*, which was previously reported as having 2n = 20 and 2n = 36, from Japan and Russian Siberia, respectively. Thus, besides further documenting numbers already reported with more species from China, our investigation has revealed three basic numbers new for this section, x = 6, 7 and 19, of which x = 6 (2n = 12) is in fact new for the genus.

Observations on the meiosis of *G. aristata* (n = 7, Fig. 1) and *G. pseudoaquatica* (n = 10, Fig. 10) indicated that both species have similar chiasma configurations with a distal crossing-over on each bivalent and regular segregations at anaphase I and II.

Gentiana heleonastes is an exception. Besides its distinct chromosome number (2n = 12), we noticed that it had rather big and obvious chromocenters in prophase

Table 3. Karyotype structure of some species of *Gentiana* sect. *Chondrophyllae*. Chromosomal size range, karyotype formula, karyotype class according to Stebbins (1971), and the intra- and interchromosomal asymmetry indices A1 and A2 according to Romero Zarco (1986) are given

Taxon	Collection no.	Size range (µm)	Karyotype formula	Class	A1	A2
G. alsinoides	G123	2.0–2.9	2n = 20 = 2m(SAT)+18m	1A	0.162	0.101
G. anisostemon	G148	2.1–3.6	2n = 20 = 2m(SAT)+18m	1A	0.154	0.168
G. asterocalyx	G119	1.6–2.2	2n = 20 = 2m(SAT)+18m	1A	0.212	0.077
G. aristata	G190	2.4–3.6	2n = 14 = 14m	1A	0.225	0.155
G. intricata	G125	1.7–2.7	2n = 20 = 4m+16 sm	3A	0.502	0.146
G. piasezkii	G068	1.9–2.8	2n = 36 = 16m+2sm(SAT)+18sm	2A	0.442	0.104
G. praticola	G134	1.7–2.7	2n = 20 = 20m	1A	0.192	0.158
G. prattii	G191	2.8–5.2	2n = 18 = 2m(SAT)+18m	1A	0.201	0.193
G. spathulifolia	G004	2.0–3.3	2n = 20 = 2m(SAT)+18m	1A	0.194	0.144
G. squarrosa	G155	1.3–2.2	2n = 38 = 2m(SAT)+36m	1A	0.192	0.178

and interphase nuclei: 12 chromocentres (prochromosomes) are constantly visible in most prophase and some interphase nuclei (Fig. 16).

Karyotype structure was analysed in some species and the results are shown in Table 3. For most species, the karyotypes are rather symmetrical and very similar to each other, with a rather high proportion of metacentric chromosomes. Most species with 2n = 20 have a pair of satellited metacentric chromosomes and 9 pairs of unsatellited metacentric chromosomes. Thus their karyotype is always 2n = 2m(SAT) + 18m. The chromosomes are small to medium. The asymmetry classification and indices of karyotypes revealed little difference among species except for *G. intricata* (and probably *G. subintricata*) which has a more asymmetrical karyotype (Table 3).

The geographical distribution of the species examined is shown in Fig. 18. It can be seen that all the samples collected from the Yunnan Province have the same number of 2n = 20, though some variation in the asymmetry of karyotypes does exist. On the contrary, the species collected from the Gansu Province show a higher diversity of chromosome numbers: each of the species we examined has a different number.

Discussion

The basic chromosome numbers and their systematic implications for the section. Since the section was found to be chromosomally polybasic, different basic numbers, such as x = 9, 10, 12, 13 or x = 5, 6, 9, 13, have been suggested. Actually, both series are not incompatible, since x = 10 and 12 may have arisen from x=5 and x = 6, respectively (Küpfer 1980). The number x = 11 should in fact be added to the series, because *G. scabrida* was found to have 2n = 44 (Chuang & al. 1963, Hsu 1968). However, some ambiguous cases exist, e. g., the species with 2n=36 can be interpreted either as tetraploid 2n = 4x = 36 (x = 9) or as hexaploid 2n = 6x = 36 (x = 6). A similar explanation can also be given for the species with

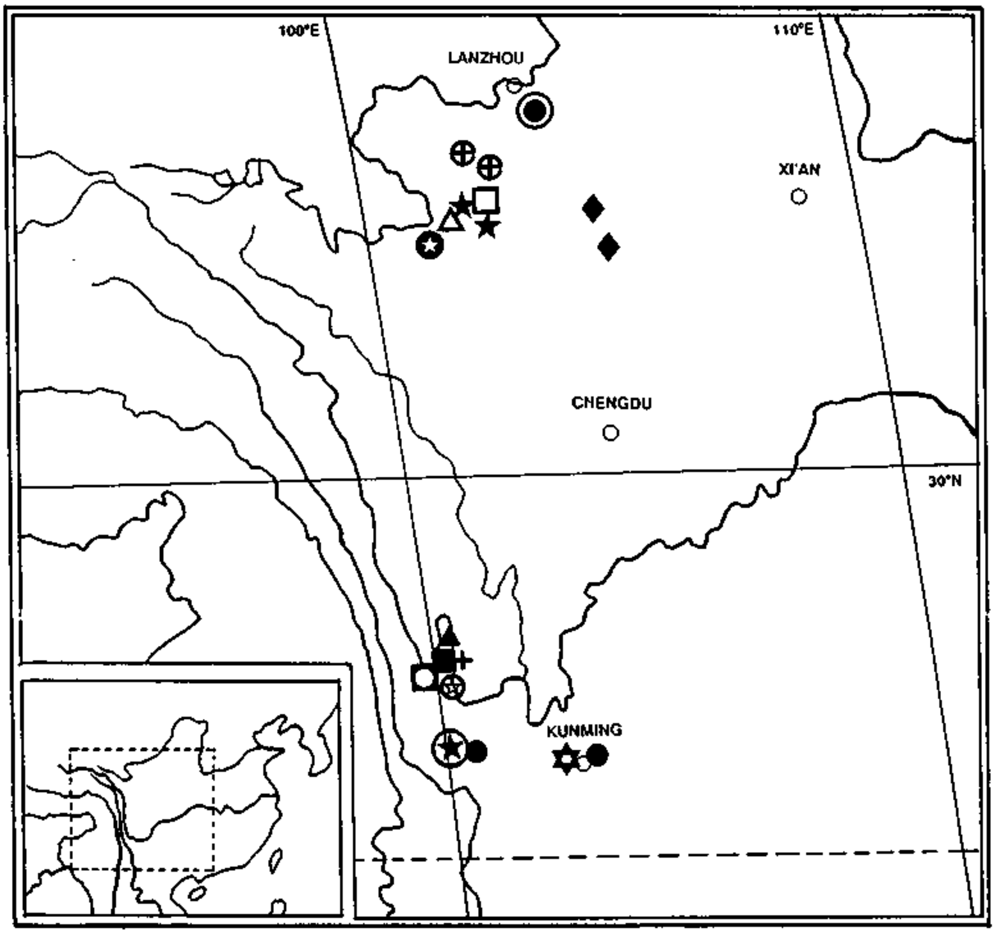


Fig. 18. The sampling sites and the chromosome numbers of the populations of *Gentiana* sect. *Chondrophyllae* examined. ■ *G. alsinoides* (2n = 20), ✡ *G. anisostemon* (2n = 20), ▲ *G. asterocalyx* (2n = 20), ★ *G. aristata* (n = 7, 2n = 14), ◘ *G. exigua* (2n = 20), ✪ *G. heleonastes* (2n = 12), ⊛ *G. heterostemon* (2n = 20), ⊗ *G. intricata* (2n = 20), ♦ *G. piasezkii* (2n = 36), ● *G. praticola* (2n = 20), □ *G. pratii* (2n = 18), △ *G. pseudoaquatica* (n = 10), ◉ *G. spathulifolia* (2n = 20), ⊕ *G. squarrosa* (2n = 38), + *G. subintricata* (2n = 20)

2n = 48 (x = 6 or 8). Referring to the species studied here, all the species with 2n = 20 or n = 10 can then be considered to be either diploid (x = 10) or tetraploid (x = 5), and fall within the commonest category of chromosome numbers for the section. Karyotype analysis of Chinese species with 2n = 20 revealed that most of them have a rather symmetrical 1A karyotype (see Table 3) and can be generalized as 2n = 20 = 2m(SAT) + 18m, except for *G. intricata* and probably *G. subintricata* which have more asymmetrical 3A karyotypes, suggesting that at the same chromosome number level, changes in chromosomal structures might play an important role in karyotype repatterning and thus in speciation of related species. Therefore *G. intricata* may have a special position in the group.

A new basic number, x = 6, was found for the section in *G. heleonastes*, at the diploid level of 2n = 12. This is also the lowest number in the genus. The newly found chromosome number and especially the very big and obvious chromocentres that exist during the mitotic prophase suggest a specific position for the species. Unfortunately, we were not able to obtain exact karyotype data for the species so as to establish a proper comparison with that of other related species. Therefore, the karyological relationships of the species and the origin of the new number still remain uncertain. However, our observation has ascertained the existence of the basic number x = 6, which had been suggested before but with no direct evidence from diploid species.

The present study revealed 2n = 14 and n = 7 for *G. aristata*. It is undoubtedly a diploid with a basic number of x = 7. This number has previously been reported only for *G. nivalis*, a geographically very isolated European species belonging to the sect. *Calathianae*, and it has been suggested that this number derived from x=5 by centric fission (MÜLLER 1982). A close relationship between *G. aristata* and *G. nivalis* is very improbable. The karyotype analysis revealed that *G. aristata* has 14 metacentric chromosomes with arm ratios ranging from 1.07 to 1.58 without any subtelocentric, telocentric, or even submetacentric chromosomes, thus reducing the possibility of direct or simple centric fission from the x = 5 type (see below for further discussion).

The chromosome number n = 9 or 2n = 18 was previously reported for the Himalayan species *G. pedicellata* (VASUDEVAN, 1975, MALLA & al. 1984). We found 2n = 18 for *G. prattii*. This species then undoubtedly has the basic number of x = 9. Its karyotype has an asymmetry similar to that of other 2n = 20 species. Therefore, it is impossible to identify any simple centric fusion. The origin of this number also remains uncertain.

The species *G. piasezkii* has 36 somatic chromosomes, which is a very common number in the perennial sect. *Ciminalis* (ADANSON) DUMORT. of Europe. But, only four species in sect. *Chondrophyllae* were reported having this number: *G. squarrosa* from Russian Siberia (see below); two perennial species, *G. jamesii* HEMSL. and *G. nipponica*, and one biennial species, *G. thunbergii* (G. DON) GRISEB., from Japan. They were considered as hexaploids of the basic number x = 6 (SHIGENOBU 1982a, b). Karyotype data of *G. piasezkii* showed a high similarity between many chromosomes and a gradual variation in size. It seems very difficult, if not impossible, to determine its basic number using only karyomorphological analysis. Thus, detailed cytogenetical analysis is needed to confirm whether it is tetraploid (x = 9) or hexaploid (x = 6). However, it is interesting to note that in the karyotype of the species many individual chromosomes have arms of equal length even if their total lengths are different. This should make us aware of the possibility of particular factors affecting the uniformity of chromosome arms and even whole chromosomes. This uniformity of structure might also benefit certain specific cytogenetical processes, such as exchange. If this were indeed the case, a translocation involving a whole arm with the breakage happening near or within the centromere might be involved.

Gentiana squarrosa is a widely distributed species in East Asia. 2n = 20 and 2n = 36 have been reported for the species, respectively, from Japan (SHIGENOBU 1984) and Russian Siberia (KRASNOBOROV & al. 1980). Our investigations on two populations of Gansu, however, constantly gave counts of 2n = 38. Thus the basic number of these populations must be x = 19, which is a new basic number for the section and obviously a secondary one. Its karyotype consists of only metacentric chromosomes, so the possibility of a simple centric fusion or fission relationship between the 2n = 36 and 2n = 38 types can be excluded. If it is possible to exclude a mis-identification of the species and the numbers can be confirmed, then this species becomes a typical example of polyploidization combined with dysploidization (see further discussion below). In this genus, the number 2n = 38 has also been reported for the European species *G. terglouensis* HACQ. of sect. *Calathianae* and has been suggested to originate from a 2n = 40 type by dysploidy changes (MÜLLER 1982).

The mechanisms of chromosome number variation in the section. The mechanisms of variation of the basic chromosome number in the genus *Gentiana* are not well understood. MÜLLER (1982) suggested a centric fission origin for n = 7 in *G. nivalis* from a supposed ancestral basic number of x = 5. But, considering the species *G. aristata* which has the same number as the above and *G. heleonastes* which has the lowest number in the genus (2n = 12), the origins of these numbers seem less simple. The karyotype of *G. aristata* consists of seven pairs of metacentric chromosomes of fairly uniform size. This does not correspond to the usual situation of centric fission which is generally associated with the presence of acrocentric or telocentric chromosomes and the reduction of chromosome length in offspring components. Furthermore, the only known diploid gentianaceous species known to be of the x = 5 type is *Comastoma tenellum* (ROTTB.) TOYOKUNI, which, although it used to belong to the genus *Gentiana*, is morphologically and karyologically (with its very small chromosomes) quite distinct from the genus. No real 2n = 10 type has yet been found in the genus *Gentiana*. Still, a symmetrical karyotype seems a common situation in the whole genus, since our primary investigation has revealed this to be the case in sect. *Cruciata*, sect. *Monopodiae* T. N. HO, sect. *Frigida* KUSN., sect. *Microsperma* T. N. HO and sect. *Stenogyne* FRANCHET (YUAN 1993, YUAN & KÜPFER 1993). As mentioned above, the uniformity of karyotype asymmetry as well as the uniformity of chromosome arm length probably either cause or else indicate particular mechanisms which are still not well understood. We suppose that polyploidization in combination with dysploidization caused by unequal reciprocal translocation may play a great part in the diversification of the basic number and the speciation of the section. As there is little intraspecific polyploidy, nothing indicates that the higher gametic numbers have arisen from simple polyploidization. Probably a lot of them are either dysploids or perhaps polybasic allopolyploids. If the cytological variability of *G. sqarrosa* is confirmed, it would represent an exemple of polyploidization (n = 10 → n = 20) followed by decreasing dysploidy (n = 20 → n = 19 → n = 18). The large series of basic chromosome numbers brings evidence for the importance of dysploidy in the karyological evolution of the genus *Gentiana* (YUAN 1993). This also applies to sect. *Chondrophyllae*. In our present state of knowledge, more speculations about the basic numbers are vain if they are founded on karyological criterion alone. Morphological and biochemical studies now in progress will throw more light on the processes of karyotype evolution in the section as well as in the genus.

Relationships between basic numbers, growth habits and classifications in the section. The present study suggests that the relationship between the basic number and growth habit of the species is more complex than some previous authors had expected. LÖVE & LÖVE (1975) and WEAVER & RÜDENBERG (1975) believed that the perennial species of the section have the basic number of x = 13, while the biennial species, which they called annual, have the basic number of x = 10. LÖVE & LÖVE (1975) went even further and gave a generic status to the perennial species with x = 13. They established a genus *Holubia*, which they later changed to *Holubogentia* (LÖVE & LÖVE 1978), based on *G. pyrenaica* (2n = 26). They included the perennial Spanish species *G. boryi* in their new genus and gave it a chromosome number of 2n = 26, unaware of an earlier chromosome number report of 2n = 20 for the species (KÜPFER 1968). A year later, LAÍNZ (1976) established another genus, *Kuepferella*, based on *G. boryi*. LÖVE & LÖVE (1986) adopted this classification. Two new genera were thus established for perennial species based on their different basic numbers. The biennial or so-called annual species were left in sect. *Chondrophyllae* or the genus *Chondrophylla* A. NELSON, as some authors suggested (WEBB 1985, LÖVE & LÖVE 1986). The present study, in connection with previous reports shown in Table 1, revealed that no constant correlation exists between growth habits and basic numbers in the section. For biennial species the basic number is not only x = 10, but may also be x = 19 as in *G. squarrosa* (2n = 38), x = 12 (or x = 6 or x = 8?) as in *G. aquatica, G. atlantica* (2n = 48), and *G. flavomaculata* (2n = 24), x = 13 as in *G. douglasiana* (2n = 26), x = 11 as in *G. scabrida* (2n = 44), x = 9 as in *G. pedicellata* (2n = 18) and perhaps *G. thunbergii* and *G. piasezkii* (2n = 36), x = 7 as in *G. aristata*, x = 6 as in *G. heleonastes*. For perennial species, the basic number is not only x = 13 as in *G. altaica* LAXM., *G. djimilensis* C. KOCH, *G. grandiflora* LAXM., and *G. pyrenaica* (2n = 26), but also x = 10 as in *G. boryi, G. praticola* and *G. cruttwellii* H. SM. (2n = 20), x = 9 (or x = 6?) as in *G. jamesii* and *G. nipponica* (2n = 36). Therefore, we suggest that to obtain a better understanding of the variation of basic numbers, as many species as possible should be investigated and more detailed and intensive analyses be carried out, rather than just new ranks be added based on a limited number of characters.

Neither do the karyological data fit in with the classifications of the conservative authors, who took a broader generic concept but adopted some infrageneric and intrasectional classes. In their most recent classification, HO & LIU (1990) divided the sect. *Chondrophyllae* into several series based mainly on vegetative characters. According to their system, species with different basic numbers are very often placed in the same series (e.g., *G. asterocalyx* (2n = 20), *G. aristata* (2n = 14) and *G. heleonastes* (2n = 12) were grouped together in ser. *Linearifoliae* T. N. HO; *G. boryi* (2n = 20) and *G. aquatica* (2n = 48) were subordinated to ser. *Humilis* MACQ., *G. capitata* D. DON (2n = 20) and *G. atlantica* (2n = 48) in ser. *Capitatae* T. N. HO). In another case, species with the same basic number fall within different series. *Gentiana spathulifolia, G. intricata, G. praticola, G. cruttwellii* (2n = 20) were, for example, put into different series. In other words, the gross morphology is not well correlated with the variation of the basic chromosome number, or else the correlation is not yet clearly recognizable in this section. More careful morphological comparisons as well as detailed observations on the growth habits of karyologically different taxa are thus urgently needed.

The geographical pattern of chromosomal variation in the section. Following our present results and previous data, a diversification centre of chromosome number for the section seems to be situated in SW China and the adjacent Himalayan areas. Within our limited number of samples orginating from China, a higher diversity of chromosome numbers seems to exist in the high-altitude mountain and meadow regions of SW Gansu, since all the samples collected from the northwestern mountains of Yunnan have the same number of 2n = 20, but samples collected from the southwestern high-altitude regions of Gansu showed examples of all the numbers found in the investigation, including 2n = 12, 14, 18, 20 (n = 10), 36, and 38 (see Table 2 and Fig. 18).

To some extent the presence of many Chinese species with 2n = 20 fills the geographical gap of the cytotype between New Guinea, where three species were counted as 2n = 20 (Borgmann 1964), and the Himalayas where more than five species were found to have 2n = 20 or n = 10 (see Table 1), and still further to the Spanish Sierra Nevada, where *G. boryi* (2n = 20, Küpfer 1968) grows. Whether this pattern indicates a polyphyletic or monophyletic origin of the number is still uncertain. However, in the case of *G. nivalis* and *G. aristata* (2n = 14), and of *G. terglouensis* and *G. squarrosa* (2n = 38), the identical numbers seem to have been acquired independently, since for each pair of species, the two members are completely isolated from one another morphologically, geographically and also systematically.

Gentiana aristata (2n = 14), *G. heleonastes* (2n = 12), *G. pseudoaquatica* (n = 10) are sympatrically distributed in SW Gansu. The individuals of these three species grow together but can be easily recognized. This pattern suggests that chromosome isolation may play an important role in their speciation.

In spite of the above-mentioned dicussions, however, no general conclusion on the relationships between geographical distribution and chromosome number variation can yet be drawn, because the chromosome data for the majority of the species indigenous to China is still incomplete. To understand the geographical pattern of the different basic numbers better, many more observations on species from different regions are still needed.

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Molecular phylogenetics of the subtribe *Gentianinae* *(Gentianaceae)* inferred from the sequences of internal transcribed spacers (ITS) of nuclear ribosomal DNA

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Key words: *Gentianaceae, Gentianinae, Centaurium.* – nrDNA, ITS, PCR, sequencing, phylogeny, molecular systematics.

Abstract: The internal transcribed spacer (ITS) regions of 18S-25S nuclear ribosomal DNA from representatives of 23 species of the subtribe *Gentianinae* and one outgroup species (*Centaurium capitatum)* were analyzed by polymerase chain reaction amplification and direct DNA sequencing. Within the taxa analyzed, the length of the ITS1 region varied from 221 to 233 bp, ITS2 from 226 to 234 bp. Of the aligned sequences of 497 positions, 151 sites involved gaps or nucleotide ambiguity, 133 were invariable and 213 showed divergence. In pairwise comparisons among the taxa of the subtribe *Gentianinae* and the outgroup, sequence divergence ranged from 1.3% to 34.1% in ITS1, from 0 to 28.1% in ITS2 and from 0.6% to 27.5% in combined ITS1 and ITS2. Phylogenetic trees generated from ITS sequences were highly resolutive and principally concordant with morphological classifications for the major phylogenetic divisions in the subtribe. An ancient divergence leading to two evolutionary lines was suggested in the subtribe by both DNA sequence and morphological data. One line encompasses the genera *Gentiana, Crawfurdia* and *Tripterospermum,* morphologically characterized by their glands on the base of ovary and their plicate corolla, while the other line involves all other members of the subcribe surveyed, characterized by their epipetalous glands and simple corolla without plicae. *Megacodon,* with glands on the base of ovary but without plicae on its corolla, was revealed to be more related to the latter group than to the former. *Comastoma, Gentianella* and *Gentianopsis* were shown to be well-defined monophyletic genera. *Pterygocalyx* showed much closer affinity to *Gentianopsis* than to any other genus. Some conflictions were detected in the genus *Swertia.*

The subtribe *Gentianinae* (*Gentianaceae*) comprises a major part of the tribe *Gentianieae* as well as the family *Gentianaceae*. It took its shape after the classic work of Gilg (1895), in which seven genera were recognized. Since that time, several authors have proposed classifications for this group (e.g., Gillett 1957, Toyokuni 1963, Smith 1970, Ho & Liu 1990). Several new genera have been created or split off from the original ones. An extreme example is the splitting of the collective assemblage, the genus *Gentiana* s.l. The principal classifications of the subtribe and the circumscriptions of the different genera are summarized in Table 1. The heterogeneous group of *Gentiana* s.l. with two subgenera as circumscribed by KUSNEZOV (in GILG 1895) involves a big part of the subtribe as it is recognized today. The two subgenera later recognized as distinct genera: *Gentiana* s. str. and *Gentianella*. The former almost retains the original circumscription of subg. *Eugentiana*, despite some changes of nomenclature and infrageneric classifications. Although some authors (e.g., HOLUB 1973; LÖVE & LÖVE 1975, 1976) have advocated further splitting of *Gentiana* s. str. into several so-called monobasic genera based on basic chromosome numbers, their proposals have not been widely accepted (PRINGLE 1978, HO & LIU 1990). The genus *Gentianella* for its part was further divided into four genera, namely *Comastoma* based on sect. *Comastoma*, *Gentianopsis* based on sect. *Crossopetalum*, *Megacodon* based on sect. *Megacodon* and all other sections as *Gentianella* s. str. This splitting was accepted by some authors (e.g., HO 1988, HO & LIU 1990, ILTIS 1965, PRINGLE 1978) but somehow rejected by others (e.g., TUTIN 1972, SMITH 1970). In addition, the monotypic genus *Pterygocalyx* which was included in *Crawfurdia* as a subgenus by GILG (1895), was lumped with *Gentianella* sect. *Crossopetalum* (= *Gentianopsis*) by SMITH (1967). MARQUAND (1931, 1937) included the genera *Crawfurdia* and *Tripterospermum* within the genus *Gentiana* as nominate sections. TOYOKUNI (1965) has suggested the placement of the genus *Megacodon* into *Gentiana*. In our present study, we adopt the circumscriptions of genera from HO & LIU (1990), except that we still leave *Frasera* as a distinct genus for the time being, instead of incorporating it into *Swertia*.

The phylogeny of the subtribe *Gentianinae* is still not well resolved. However, two main evolutionary lines have often been suggested: the "*Gentiana*" line comprising the genera *Gentiana* s. str. *Crawfurdia*, *Tripterospermum*, *Megacodon*, and probably *Ixanthus*, and the "*Gentianella*" line represented by all other genera of the subtribe (GILLETT 1957; TOYOKUNI 1963, 1965; HO & LIU 1990). This opinion is based on some morphological characters: The "*Gentiana*" line has glands at the base of the ovary, corollas with plicae or folds between the lobes, calyxes with a continuous membrane or rim extending completely around the interior of the tube, and corolla lobes with 3 primary vascular bundles; while the "*Gentianella*" line has so-called epipetalous glands – with glands, fovae, or spurs born on the surface of the corrola alternating with the stamens, corollas without any plicae or folds, corolla lobes with 5–9 vascular bundles and calyxes without intracalycular membrane. Within the "*Gentiana*" line, HO & LIU (1990) believe that the climbing genera *Crawfurdia* and *Tripterospermum* with twisted stems represent the primitive type and have the closest affinity with the genus *Gentiana*, while TOYOKUNI (1965) thought that the genus *Megacodon* is closely related to *Gentiana* and has to be incorporated in *Gentiana*. In the "*Gentianella*" line, the genera were suggested to be rather closely related to each other through a development series of glands to fovae, fovae accompanied by squamellae and the development of the squamellae themselves, to the final development of spurs. Thus *Halenia* was believed to be originated from a Swertia-like ancestor (GILLETT 1957).

Contribution of chromosome data towards resolving the phylogenetic problems in this subtribe is limited. As demonstrated in our previous papers (YUAN 1993,

Table 1. Selected classifications on the subtribe *Gentianinae*. The sectional classification is shown here only for the "*Gentianella*" group

Gilg (1895)	Gillett (1957)	Toyokuni (1963)	Smith (1970)	Ho & Liu (1990)
Crawfurdia				
subg. *Pterygocalyx*		*Pterygocalyx*		*Pterygocalyx*
subg. *Tripterospermum*		*Tripterospermum*	*Tripterospermum*	*Tripterospermum*
subg. *Dipterospermum*			*Crawfurdia*	*Crawfurdia*
Jaeschkea	*Jaeschkea*	*Jaeschkea*	*Jaeschkea*	*Jaeschkea*
Gentiana				
subg. *Eugentiana*	*Gentiana*	*Gentiana*	*Gentiana*	*Gentiana*
subg. *Gentianella*	*Gentianella*	*Gentianella*	*Gentianella*	*Gentianella*
sect. *Dasystephana*				
sect. *Andicola*			sect. *Andicola*	
sect. *Imaicola*				
sect. *Stylophora*				
sect. *Amerella*			sect. *Amerella*	
sect. *Antarctophila*			sect. *Antarctophila*	
sect. *Arctophila*			sect. *Arctophila*	
sect. *Crossopetalum*		*Gentianopsis*	sect. *Crossopetalum*	*Gentianopsis*
		Comastoma	sect. *Comastoma*	
sect. *Megacodon*	*Megacodon*		*Megacodon*	*Megacodon*
Ixanthus	*Ixanthus*	*Ixanthus*	*Ixanthus*	*Ixanthus*
Pleurogyne	*Lomatogonium*	*Lomatogonium*	*Lomatogonium*	*Lomatogonium*
				Lomatogonopsis
Swertia	*Swertia*	*Swertia*	*Swertia*	*Swertia*
		Frasera		
			Veratrilla	*Veratrilla*
Halenia	*Halenia*	*Halenia*	*Halenia*	*Halenia*
			Latouchea	*Lotouchea*

Yuan & Küpfer 1993 a, b) and the references listed therein, most genera of this subtribe are polybasic in nature; e.g., in *Gentiana*, a continuous series of gametic numbers from n = 6 to 24, and n = 26 and 30 has been recorded; in *Swertia*, n = 7–10, 12–14, 26, and 30 have been found. A parallel development of the dysploid series is obviously involved in the karyological evolution of this subtribe. It is very difficult, if not impossible, to evaluate phylogeny of the group only from the karyological point of view.

Although molecular systematics of plants has become flourishing recently (Doyle 1993), not much study has been performed on the family *Gentianaceae*. An attempt was made by Sytsma & Schaal (1985, 1990) on the New World tropical shrub genus *Lisianthus*, by using restriction analysis on chloroplast DNA (cpDNA) and nuclear ribosomal DNA (nrDNA). They successfully addressed phylogenetic problems among the populations of a species complex. Their DNA-based phylogeny was highly concordant with the isozyme-based one. Interestingly, they revealed a length variation of about 100 base pairs (bp) in the internal transcribed spacer (ITS) region between the 18 S and 5.8 S nuclear ribosomal genes, both within and among individuals of populations. Recently, Gielly (1994) and Gielly & Taberlet (1994 and pers. comm.) have engaged in an investigation of the phylogenetic utility of two non-coding fragments of cpDNA, the *trn*L (UAA) intron and the intergenic spacer between the *trn*L (UAA) 3′ exon and *trn*F (GAA) gene, using the European gentians of the subtribe *Gentianinae* as demonstration group. In their cladograms, the genus *Gentiana* formed a highly supported monophyletic clade. All the other genera grouped together monophyletically as the sister clade of *Gentiana*. The phylogenetic relationships among the taxa within both clades are congruent to a certain extent but not fully with morphologically defined classifications, and also conflict or remain disresolutive in some aspects, e.g., sect. *Ciminalis* of the genus *Gentiana*, a monophyletic group strongly supported by morphological and karyological characters, was split into two relatively distant clades. This problem may result from the property of uniparent inheritance of cpDNA. Furthermore, their samples included only European taxa. Some important groups from other regions are lacking. This drawback limited the possibility of getting a general conclusion of phylogeny comprising the whole subtribe. Thus analyses on both nuclear and chloroplast DNA involving more complete groups are still essentially necessary to evaluate the phylogeny of this subtribe.

The internal transcribed spacer (ITS) region of 18 S–25 S ribosomal DNA (nrDNA) has recently become of a favourite part of the nuclear genome for phylogenetic reconstruction (Baldwin 1992, 1993; Kim & Jansen 1994; Suh & al. 1993; Wojciechowski & al. 1993). This is because of its high copy numbers, rapid concerted evolution, and diverse rate of evolution within and among component subunits and spacers (Hamby & Zimmer 1992, Hillis & Dixon 1991). In addition, the relatively small size (600–700 bp usually) of ITS region and the special positions of the spacers flanked by highly conservative coding regions are very favourable for primer design and direct sequencing of polymerase chain reaction (PCR) amplified products. In flowering plants, ITS sequences have been successfully used to examine phylogenetic relationships among the genera of the tribe *Madiinae* of *Asteraceae* (Baldwin 1992, 1993), among the genera of *Winteraceae* (Suh & al. 1993), among the species of *Krigia* and its closely related genera of the tribe *Lactuceae* (*Asteraceae*) (Kim & Jansen 1994), and among a dysploid species group of *Astragalus* (*Fabaceae*) (Wojciechowski & al. 1993). All these studies have shown a high congruence between the ITS-based phylogeny and morphology-based or cpDNA-based ones, which indicated ITS to be a promising source of nuclear phylogenetic markers which are especially suitable for interspecific studies or comparisons among closely related genera.

The present study was carried out to demonstrate the molecular evolution of the ITS and the phylogeny among the closely related genera of the subtribe *Gentianinae*, with the following questions addressed particularly: (1) the utility of ITS sequences as a source of phylogenetic data in the subtribe *Gentianinae*; (2) the phylogenetic relationships among the genera of the subtribe; (3) the congruence between the ITS phylogeny and the existing classifications; (4) to find the sister group of the genus *Gentiana*, which will be employed as outgroup in our future phylogenetic studies of this genus.

Material and methods

Plant material. Sources of plant material for 23 ingroup and one outgroup species are given in Table 2. Leaves were collected directly in the field using the silica gel method (Chase & Hills 1991), except for *Gentianella biebersteinii*, *G. caucasea*, *G. umbellata*, and *Gentianopsis blepharophora*, for which leaves were taken directly from conventionally prepared herbarium specimen collected within the last two years. Fresh leaves of the outgroup, *Centaurium capitatum* (= *C. erythraea* subsp. *erythraea* var. *capitatum*) were taken from a living individual grown in the Botanical Garden of Neuchâtel. This species was selected as outgroup because it belongs to the subtribe *Erythraeinae* which is suggested to be closely related to the subtribe *Gentianinae*. All voucher specimens were prepared and deposited at the herbarium of the University of Neuchâtel (NEU).

Despite of all our efforts in material collection, some taxa, namely *Jaeschkea*, *Ixanthus*, *Veratrilla*, *Latouchea*, and *Lomatogoniopsis*, are still missing from our material list. However, these missing genera are represented by a few species only.

Total DNA extraction. Total DNAs were extracted from 100–300 mg dried leaves pulverized in liquid nitrogen or directly in 2 × CTAB buffer according to the protocol of Doyle & Doyle (1987), except the material taken from herbarium sheets mentioned above, for which a modified procedure was used: a piece of leaf (about 5 × 5 mm) was ground in 0.5 ml 2 × CTAB buffer in a 1.5 ml-microcentrifuge tube and subsequently incubated at 65 °C for 30 min. Following once chloroform extraction, the aquaceous phase containing DNA was applied to the QIAEX gel extraction kit (QIAGEN). The procedure suggested by the manufacturer was followed, with the DNA-CTAB mixture being used to replace the agarose gel designed in the original protocol of the manufacturer.

Amplification of ITS region and the primers used. A standard double-strand polymerase chain reaction (PCR) was applied to amplify the entire ITS region, using primers YP1 and YP4. Two internal primers, YP2 and YP3 were also used in subsequent sequencing. The primers YP1, YP2, YP3, and YP4 described here are equivalent with the primers ITS5, ITS2, ITS3, and ITS4 designed by White & al. (1990), respectively, except a minor modification in YP1. The primer sequences are: YP1 = GGAAGTAGAAGTCGTAACAAGG, YP2 = GCTGCGTTCTTCATCGATGC, YP3 = GCATCGATGAAGAACGCAGC, YP4 = TCCTCCGCTTATTGATATGC (see Baldwin 1993: fig. 1, for the location of each primer). All these primers were purchased from Microsynth (Switzerland).

PCRs were carried out in 50 µl reaction mixture containing 34.75 µl sterile double-distilled water, 5 µl of 10 × Taq polymerase reaction buffer, 2 µl equalmolar 5 mM dNTP, 2, 5 µl each of 10 µM primer YP1 and YP4, 0.25 µl of 5 Unit/µl Taq DNA polymerase, and about 3–12 ng (3 µl of 1–4 ng/µl) genomic DNA. For multiple sample amplifications, a master mix containing all the components except the genomic DNA was premixed and aliquoted. The aliquots were heated till 70 °C and then genomic DNAs were added. The reaction mixtures were sealed with about 15 µl mineral oil to prevent evaporation. The thermal cycling was performed on a Perkin-Elmer thermal cycler with the following conditions: 1 cycle of 2 min at 94 °C linked to 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min with 4 sec extension for each cycle at 72 °C; and then 5 min at 72 °C to complete primer extension.

Cleaning of the PCR products. After the cycling was completed, 5 µl reaction products were run on a 0.8% agarose gel to check the quality of amplification. To clean the ITS fragments, 40 µl PCR products were run on a 0.8% agarose gel with bigger wells which were prepared by taping a regular comb to get bigger tooth. The gel was prepared and run in 1 × TAE buffer. The agarose blocks containing ITS fragments were excised from the gel with a scalpel under UV light, and then applied to the QIAEX gel extraction kit according to the manufacturer's protocol (QIAGEN). ITS fragments were finally recovered in 40 µl TE buffer, which is sufficient to run many times sequencing.

Table 2. The species, the origin of plant material, their representative numbers and the abbrevations of generic names used in the following tables and illustrations. Voucher: *K* Ph. Küpfer; *Y* Y.-M. Yuan; *Z* L. Zeltner. * *C. capitatum* Willd. was treated as *C. erythrea* Rafn. subsp. *erythreae* var. *capitatum* (Willd.) Melderis

No.	Taxon	Voucher	Locality
	Gentiana		
1	*G. algida* Pall.	Y91–S1	Trail Ridge, Rocky Mt Natl. Park, USA
2	*G. aristata* Maxim.	Y92–328	Maqü, Gansu, China
3	*G. boryi* Boiss.	Z93–S1	Hoya del Moro, Sierra Nevada, Spain
4	*G. lutea* L.	Y91–S5	La Tourne, Neuchâtel, Switzerland
5	*G. verna* L.	Y93–12	Grand Chavalard, Valais, Switzerland
	Comastoma		
6	*Cm. cyananthiflorum* (Franch. ex Hemsl.) Holub	Y92–230	Mt Yulong, Yunnan, China
7	*Cm. pulmonarium* (Turcz.) Toyokuni	Y92–279	Ruoergai, Sichuan, China
	Crawfurdia		
8	*Cw. tibetica* Franch.	Y93–121	Mt Gongga, Sichuan, China
	Frasera		
9	*F. speciosa Dougl.* ex Hook.	Y91–S2	Towhee Trail, Boulder, Colorado, USA
	Gentianella		
10	*Gl. biebersteinii* (Bunge) Holub	K91–G1	Terek Valley, Mt Caucasus, Georgia
11	*Gl. caucasea* (Loddiges ex Sims) Holub	K91–G2	Kazbek, Mt Caucasus, Georgia
12	*Gl. campestris* (L.) Börner	K93–G1	Col du Pt. St Bernard, Italy
13	*Gl. umbellata* (Bieb.) Holub	K91–G3	Djvari Pass, Mt Caucasus, Georgia
	Gentianopsis		
14	*Gs. blepharophora* (Bordz) A. I. Galushko	K91–G4	Djvari Pass, Mt Caucasus, Georgia
15	*Gs. grandis* (H. Smith) Ma	Y92–222	Lijiang, Yunnan, China
16	*Gs. paludosa* (Hook f.) Ma	Y92–314	Maqü, Gansu, China
	Halenia		
17	*H. elliptica* D. Don	Y93–53	Shiqü, Sichuan, China
	Lomatogonium		
18	*L. macranthum* (Ciels ex Gilg) Fern	Y93–91	Ganzi, Sichuan, China
	Megacodon		
19	*M. stylophorus* (C. B. Clarke) H. Smith	Y93–182	Mt Baima, Yunnan, China
	Pterygocalyx		
20	*P. volubilis* Maxim.	Y93–120	Mt Gongga, Sichuan, China
	Swertia		
21	*S. franchetiana* H. Smith	Y93–129	Xining, Qinghai, China
22	*S. tetraptera* Maxim.	Y92–315	Maqü, Gansu, China
	Tripterospermum		
23	*T. cordatum* (Marq.) H. Smith	Y92–267	Mt Hualong, Shaanxi, China
	Centaurium		
24	* *C. capitatum* Willd.	Z93–A	Högby, Öland, Sweden (cultivated in the Botanical Garden of Neuchâtel)

Sequencing of ITS region. Purified double-strand DNA (dsDNA) were directly sequenced from both strands by the standard dideoxy chain termination technique. We initiated our sequencing with the Sequenase kit (version 2.0, U.S. Biochemical), but shifted to cycling sequencing later on, using the *fmol* DNA sequencing system (Promega). The protocols recommended by the manufacturer were followed, using the primers YP1, YP2, YP3, and YP4 end-labelled with [gama-^{32}P]-dATP (Amersham). The following program was performed on a thermal cycler (Perkin-Elmer): 1 cycle of 2 min at 94 °C, followed by 35 cycles of 45 sec at 94 °C, 45 sec at 55 °C, and 1 min at 72 °C, and finally cooled to 4 °C. This system gives good resolution for ITS sequences.

DNA sequence samples were separated on 6% acrylamide – 8 M urea gels. The gels were fixed in 20% ethanol – 10% acetic acid for 10 min, transferred onto Whatman 3 MM paper, vacuum dried at about 80 °C for 2 h, and then exposed to Kodak XAR X-ray films for 10–14 h.

Sequence alignment. The sequence boundaries among the two spacers and the three coding regions (18 S, 5.8 S, and 25 S genes) of nrDNA were determined by comparison with published sequences from *Daucus carota* and *Vicia faba* (Yokota & al. 1989). The combined sequences of ITS1 and ITS2 were aligned using the program Clustal (Higgins & al. 1992), with fixed gap penalty and floating gap penalty at 10 and DNA transitions unweighted. The generated alignment was slightly adjusted manually to minimize gap number. The inclusion of the outgroup did not introduce any difficulty for sequence alignment.

Numbers and proportions of nucleotide site divergence were calculated for all possible pairwise comparisons of ITS1, ITS2 and the combined ITS1 and ITS2 sequence data. Only the sites without gap and nucleotide ambiguity were included in the comparisons. The formular $P = N_d/(N_d + N_i) \times 100\%$ was used for calculation, where P is the percentage of site divergence, N_d is the number of divergent nucleotide sites and N_i is the number of identical nucleotide sites between the two sequences compared.

Phylogenetic analysis. The potentially informative site, i.e., those with each of at least two nucleotide states shared by two or more sequences, were used for phylogenetic reconstruction. In our basic analysis, gaps were treated as missing data and character state changes were weighted equally. Phylogenetic trees were reconstructed using Fitch parsimony, i.e., assuming unordered character states as implemented in PAUP 3.0s (Swofford 1991). In order to explore the possible impact of gap coding and character state weighting on phylogenetic tree construction, we implemented the following analyses in addition to the basic analysis: (1) the informative insertion/deletion sites (i.e., shared by at least two sequences) were excluded; (2) the informative insertion/deletion sites were excluded and were coded as unweighted binary characters (0 for gap, 1 for insertion) regardless of their size; (3) gaps were simply coded as a new state; and (4) gaps were coded as missing and transversions were weighted 2 or 5 times over transitions, respectively, using the stepmatrix method. In view of the high number of taxa included in this study, heuristic search strategies were employed for the phylogenetic searches. Three different regimes of addition sequences and branch-swapping were performed, in order to be sure the most parsimonious trees have been found: (1) SIMPLE addition sequence and TBR (tree bisection-reconnection) swapping; (2) CLOSEST addition sequences and TBR; and (3) 100 replicates of RANDOM addition sequence and TBR swapping. Bootstrap values were calculated from 100 replicates of heuristic searches with SIMPLE addition sequence.

Results

ITS sequences and their divergence. For most taxa, approximately 640 bp of the ITS1-5.85S-ITS2 regions were sequenced. However, we included only the ITS1 and ITS2 regions in this paper, since sequence data for the 5.8 S gene was incom-

Table 3. Aligned DNA sequences of ITS1 and ITS2 of 23 representatives of subtribe *Gentinaninae* and one outgroup *Centaurium capitatum*. See Table 2 for species names

```
Taxon                                        Nucleotide sites

    >ITS1
                                                                                                       1
             1111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990
    1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
        **          * *      *          *    *   ***                         *                       ***

1   TCGAT-TCCTGCGAASRA--GGACCCGAGAACATGTCT-AACGCAC--GGGCGTTCGGGACGAGGGAAACCAC-GGACCGATGCCCCGAGCACGGCG---
2   ....--......C--TT.AG.A..A.C.-...TG..T.TT.---..-C...T-..T...-.C.A.......C.-..G..A..T......-.CTTAM.GGW
3   ....A-......T..GC.GA................-.T.T.....--......-................G.A..............C...T.A..---
4   ....A-.........GC.GA.....-..........-.T..A....--....T................GG..-.......................---
5   ....A-.........WC.GA.........G......T.T..AC...--..............G..........-..................T....---
6   ....AG.........GC.GCG...............-.T...A.T.G-.KA...C..................-.A....GC.T.............---
7   ....--..-......GC.GA.....T..........-.T...A.TNG-..-...CT.................-......GC.T.....T.......---
8   .....-.GG......GC.GA................-.T...T...--....A..T.....T..........T-....T.............A....---
9   ....A-.........SR.-CG....T..........-.T...CAH---......C..A..A.G..........-......GC..T............TGT
10  ....A-.........S-.GA................-AT...AA..--......CT...G...........C.-......GC............T..---
11  ....A-.........GC.GA.........W......-AT...AA..--......CT...G...........C.-......GC....T.......T..---
12  ....A-.........GC.GA..............T.-.T...AA..A-......CT...............C.-......GC............T..---
13  ....A-.........S-.GA................-.T...AA..--......C.......T........C.-A.....GC...............GGA
14  ....A-.........S-.GA............T...-AT....T..--......C.A.....T.........T-.C..T.G.......G........---
15  ....AG.........GT.GA.....-.T....T...-AT.T..K.---......C.......C..........-......GC.G.............---
16  ....A-.........GC.GA............T...-AT.T-....--......C.......C......GG..-......G................---
17  ....A-.........S-.GAT...............-.T....A..--......C...A.AA..........T-......GC......T..G.....---
18  ....A-.........CC.GA................-.T..N....--.......................TT-......GCA........G.....---
19  ....A-.........S-.GA................-.T.......--......C....C..G..........-......GC.T.......G.....C--
20  ....A-.........AC.GA............T...-AT.......--......C..T....T..........A......GA..............T---
21  ....A-.....GCG.GC.GA................-CT...AA..--..T.A.C..................-......GC...............---
22  ....A-......A..GC.GA................-.T...AA..--......C...A.A............-......GCA....AT...T.A..---
23  .....-.Y.-.....GC.GA................-.N...TT..--....A..T....T.TA.........-...................A...---
24  ....A-.........GC.GA.......T........T-TTCTTTG.AC....A.C--...KCT..........-..G...G..-....C........---

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    0000000001111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990
    1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
          ****         *                     *  ***                              *  *                *

1   TCGACC-ACCGGTCGCTCG-TCGTGCAAA--CAACCAACCCCGGGCGCAGAAACGCG-CCAAGGAAAACG--AAAAAAGG-ATG-GCCTGCCTCCCGTAG
2   R-.C..--.......R.W.-......C..AC............SS--..A...AC.CC...........CTT........A..TTCYY.C..C.....C.
3   ......-.........C..-.........--................T.A....A..-............--T.......-..T-.T.....C.....C.
4   K.-...----..C.....T-C........--.............---..T.......-............--........-...-.............C.
5   ......-............-.......C.--...............A........T.-............--........-...-..........TT.CA
6   ......-.TA..C......-.......C.--...A.............GT...A...-...........A--.G...-..G...-.........TT.AC.
7   ......-.TA..C......-.......C.--...A.....NN.C.-..GT...A...-...........A--.G...-..G...-.........NT.AC.
8   A..G..-.......A.AA.-......T..--..T.A.............TT..-.T.-............--.....-..-...-..K.....A..A-CA
9   A.C...----.....T...-.......C.--...A....A.........T...A...-...........A--........--.T-....T...TTT.-CA
10  ....A.C.TA...T.....-C......C.--...A..............T...A...-...........A--.G......G...-..T......T..AC.
11  ......-.TA...T.....-C......C.--...A..............T...A...-...........A--.G......G...-..T......T..AC.
12  ......-.TA...T.....-.......C.--...A..............T...A...-...........A--.G......G...-..T......T..AC.
13  ......-.TA...T..C..-.......C.--A..A......-.......T...A...C...........A--.G...-..G...-..T......T..AC.
14  .....A-.TTT..T.....-.......C.--T..A..............T...A...-...........A--TG...-..G...-..T......T..ACA
15  ......-.TT......C..-.......C.--T..A.............GT...A...-...........A--.G...-..G..T-.........T..AT.
16  .GC...-.T.......C..-.......C.--T..A........--....T...A...-...........A--.G...-..G..T-CGG......T..AT.
17  ......AG.......TG..-..A....C.--...A............T.....A...-...........A--.G...-..G...-.........TT.AT.
18  ......--TA.........T....K..C.--...A..............T...A...-...........A--.G......-...-.....H...T..AC.
19  -.....-............-.........--...A..................A...-............--..G.....G...-..T......T..AC.
20  .T....AT.....T..AA.-A......C.--T..A..............T...A...-...........A--TG...-..T...-..T......T.AACA
21  ......-TTA.........-.......C.--...A..............T...AAGCG...........A--.G......-...-..T......T..ACA
22  ......AG.......TG..T..-....C.--...A..............T...AAGCG-..........A--.G...-..G.A.--........TT.AC.
23  A..G..-..........N.-......T..--....A....---......TT..A...-...........A--........-...-.......NNNNA-CA
24  ...G..--TT.....T...-.....TT.----.C..T.AA..C.....T.C..A...-...........C--.G...T..ACA.-C..GCG...T..AC.
```

Table 3 (continued)

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Taxon                                      Nucleotide sites

                                                          »ITS2
        2222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222223
        0000000001111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990
        1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
             *            *              *       *                        **         *        *        **

1       TGCCGATACGCGGTGTGCGACGGGAGGTACACG-GGCGCCTAAAGAAAC-AATCGCGTCGCCCCCCCA-ACACCGTG-GATGATATCATGCCGGT--CGT
2       .....TA.T..C..AC..-........AC....-TA.............-...GT...........A--........-.....A.AAT..T....--T..
3       ..T..-..T......C..-........AT....-.AT............-..................-........-.....A.CAT...AT..--T..
4       C....-..S-.....C.T-G.T.....AT....-.......G.......-G..T............---.....CGTG.....A...........--...
5       C....-...........A.G..-....AG...AG...A.......G...-................A--......CTG.....AGC.........--...
6       CTT..-.T.....A....-........GCA..A-...A...G.......-...............AA--..C.....TGT.A.CTC-G.A.G...GA.T.
7       CTTN.-.TN....A....-........GCA..A-...A...G.......-...T..............-..CT....TGT.A.CTN-G.A.G...GAAT.
8       .T...-...........TTTT..T...AT..T.-...A...........-....CT.........----.A...CGTCG.C..CG..G...T..A--T..
9       A.G.CT....G.--..T.-.AAA.G..GC-..A--.TA.-..T.C....-................A.-..C.-...TGT.A.GTCAG.A.G..GGA.A.
10      CT...-.C.....A....-...A....GCA..A-...A...-.......-..................-..C.T...TGT.A.CTC-G.A.G...GA.A.
11      CT...-.C.....A....-...A....GCA..A-...A...G.......-..................-..C.T...TGT.A.CTC-G.A.G...GA.A.
12      CT...-.C.....A....-...A....GCA..A-...A...G.......-..................-..C.....TGT.A.CTC-G.A.G...GA.A.
13      CT...-.C.....A....-........GCA..A-.......G.......-..................-..C.T...TGT.A.CTC-C.A.G...GA...
14      .A...-.......A....-........GCA..A-.......-..T....-..................-..CT....CGT...CTC-G.A.G.C-GA.A.
15      C....-.......A....-........G.A.AA-.......G..T....-..................-..CT....CGT...CTC-G.A.G...GA.A.
16      CTG.CG.......A....-........G.A.AA-.......G..T....-..................-..CT....CGT...CTC-G.A.G...GA.A.
17      CC...-.......A....-........GCAG.A-...A...G....T.-T..............T.A--C.C.A...TGT.A.CC.-TCA.G...CAT..
18      CT...-.T.....A...T---A.T...GCA...-...A...........-...............A.--..C.....TGT.A.CTC-G.A.G...TA.T.
19      C....-.......A....-........GCA..AT.......-.......-................A--..CT....CGT.A.CTC-G.A.G...GG...
20      C....-.G.....A....-.T.A....GCA..A-A......G..C....-..................-..CT....CAT...CT.-G.ATG...GA.A.
21      CT...-.T.....A...A-........GC...A-...A...........-......CC.-.......G-..C.....TGT.A.CTC-G.AGG...GAT..
22      C....-.......A....-...A....GCA..A-...A...G.......-................A--..C.....TGT.A.GTC-GCATG...GA...
23      .-...-.......C-..T-.A.AT...AT....-..A...N........-...T............A--..-..TATG..C.GC.A.G..TT.AC--A..
24      CC...-............-T..A....AT.G..-T......GTT.T...C................A.C..C.....CACC.TATG-G.A.A...GGT.G


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             **                                                                            *

1       CGGAGGGGCGGATATTGGCTTCCCGTG-----CTTCGGTGCGGCTGGCCTAAAC-GCAAGTCCCTTGCGA-CGGACACGACGA-CAAGTGGTGGTTGATT
2       ..............A............GTCGTT.CG.C...............T-...............-............-................
3       ..TC......................A-----.....................T-..............G-............-................
4       ...........................-----.....................T-.G............G-....G.......-................
5       AT.........................-----..C..................T-...............-............-................
6       GATG..-.....A.C............-----...G.TC..............T-.TG....T..C....-.....G......-................
7       GATG..-.....A.C......NNN...-----...G.CC..............T-.TG............-.....G......-................
8       .A........................A-----...........T.........T-.......T..CT...-T..CG.......-................
9       GA.G.--.....A.C............-----.CCGTTC..............T-.TG............-.....G......-................
10      GA.G..-.....A.C............-----..-G.CC..............TT..G............-.....G......-...............A
11      GA.G..-.....A.C............-----...G.CC..............T-..G............-.....G......-...............A
12      GA.G..-.....A.C............-----.....CC..............T-..G............-.....G......-...............A
13      GA.G..-.....A.C............-----.....CC..............T-.TT.........A..-.....G......-...............A
14      GA.G.--.....AGA............-----TC.A.C...............T-..G..-.........-T....G......-................
15      GA.G..-......GA............-----TC.ATTC..............T-.TG............-.....GT.....-................
16      GA.G.--......GA............-----TC.A.TC..............T-.TG............-.....GT.....-................
17      GA.G..-A....A.C............-----.....TC..............T-ATG............-.....G......-................
18      TA.G..--.K..G..............-----.....CGCA............T-..G............A.....G......A................
19      GA.G..-.....A.A............-----...G.CC..............T-..GC...........-.....G......-................
20      GA.G...CA...AGA............-----.C.A.CC..............T-..G........A...-.....G......-................
21      GA.G..-.....A.C............-----...G.CC..............T-..GG...........-.....G......-................
22      GA.G......T.A.C............-----...T.TC..............T-ATG............-............A................
23      T-.................NNN--...-----..AN.................T-.......T.......-............-................
24      .ATG..-.....G.C....C.......-----.CCGT.C..............T-..G...A-..C....-.....G....A.-................
```

Table 3 (continued)

Taxon	Nucleotide sites
	4444444444444444444444444444444444444444444444444444444444444444444444444444444444444444444444444
	0000000001111111111222222222233333333334444444444555555555566666666667777777777888888888899999999
	1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567
	* * * * ***
1	TT-TCAACTAAGGGTGCTGTCGCACGTTGCCCCGTCGGATGAGGA-GACTTCCTTGACCCTA-ACGCAAGCGTCGTCACGACGTCTGCCACGACCGC
2	ACC......C..-.......T.G......A...............-................-.T....-..K..........C.............
3	ACC......C..-T........T......A...............-........C.......-.T...T..A.........G.CG............
4	GCC..G......-..CG......G..C..................-.....A.........GT..T.G...............C.............
5	.C-...G.....-........M-G..C............C.....-.....A.........T-.T...TA...........................
6	..C.........-..........G..AC....-....A.......-....-..AC......G-.T...C.........T.....T...T........
7	..C.........-..CG......G.-AC....-....A.......-....-..AC......G-.T...C.....A...T.....T...T........
8	GCC.........-.........TG......A.T....A.C..A.G-....C.G.A......G-.T...T.........T..T.C....T.....T..
9	GC-.........-.........AG..C...T.-....AT.A....-....-..CC......G-.T...T.........T..T.CA...T........
10	G.C.........-........A.G..AC....-....A..T....-....-..CC......G-.T...T..A...........CT-..T........
11	G.C.........-........A.G..AC....-....A..T....-....-..CC......G-.T...T..A...........CT...T........
12	G.CG........-..........G..AC....-....A..TT...-....-..CC......G-.T...C..A...........CT...T........
13	G.C.........-..........G..AC....-..T.A..T....-....-..CC......G-.T...C.ACG.C.T......CT...T........
14	GCC.........-......C-.TG..ACA..T-....A....AC.-....-..........G-.T...C.T.A..A..T....CT...T........
15	GCC.........-.........TT.TAC.A..-....A..T....-....-.TCC.......-.T...C.---.....TT...CTG..T........
16	ACC.........-.......T.TT.TAC.G..-....A..T..A.-....-.TCC.......-.T...C.---.....GA...CT...T........
17	.CC.....G...-..........G..AC.T.T-....AG.T....-....-..........G-.T...T............T.CT...T........
18	A.C.........-...-......G..AC....A....A.......-....-..C.......G-.T...CT..........C...T...T........
19	GCC.........-..........G..AC....-....AT......-....-..CC......G-.T...T...................T........
20	.CC.........-.......T..G..AC...T-............T....-..CC......G-.T...C.........T....CA...T........
21	G.C........A-......C..TG..AC....-....A.....A.-....-..G...T...G-.T...T..............CT...T........
22	..G......G..-..........G..AC...T-....AG.T....-....-..CC......G-.T...C..............CT...T........
23	ACC.........-..CG......G..C....N-....A.C.GA..-....NNA........G-.T...T.........T....A.............
24	ACC......CTA-.........TT-.CG.........A.G.....-....GA.CC......GT.-...C.T............CA............

The IUPAC (IUB) code was followed for nucleotide ambiguity, i. e., K=G/T, S=C/G, R=A/G, Y=C/T, M=A/C, W=A/T, H=A/C/T, N=A/C/G/T. –=gap, .=matched nucleotide to the first sequence. * represents the gap sites which were considered in gap treatments (see text)

plete for most taxa and the few complete 5.8 S sequences (164 bp) showed very low sequence divergence. The aligned sequences of ITS1 (sites 1 to 251) and ITS2 (sites 252 to 497) are given in Table 3. The length and G + C content of each sequence are given in Table 4. The length of ITS1 in the taxa surveyed ranges from 221 bp (*Gentiana lutea*) to 233 bp (*Comastoma cyananthiflorum*). The length of ITS2 varied from 226 bp (*Gentianopsis paludosa*) to 234 bp (*Gentiana aristata*). The G + C content varied from 52.8% (*Gentiana aristata*) to 63.5% (*Megacodon stylophorus*) in ITS1 and from 57.1% (*Tripterospermum cordatum*) to 65.1% (*Megacodon stylophorus*) in ITS2. Sequence alignments of ITS1 required 23 gaps of 1 to 4 bp occurring in more than two sequences and 16 gaps of 1 bp occurring in individual sequences. Alignments of ITS2 required 16 gaps of 1 to 5 bp shared by more than two sequences and 13 gaps of 1 bp occurring in individual sequences. Of the aligned 497 positions, 151 sites (96 in ITS1 and 54 in ITS2) involved gaps or nucleotide ambiguity; 133 sites (51 in ITS1 and 83 in ITS2) were identical for all the sequences surveyed; 213 sites (104 in ITS1 and 109 in ITS2) showed nucleotide divergence, i.e., possessed one or more nucleotide differences in at least one sequence.

Pairwise comparisons between all possible combinations were carried out for ITS1, ITS2 and combined ITS1 and ITS2, respectively (distance matrix not shown). Ambiguous and gap sites were excluded from these comparisons. Sequence divergence between pairs of species ranged from 1.3% (*Comastoma cyananthiflorum* vs. *C. pulmonarium* and *Gentianella biebersteinii* vs. *G. caucasea*) to 34.1% (*Gentiana aristata* vs. *Frasera speciosa*) in ITS1, from 0 (*Gentianella biebersteinii vs. G. caucasea*) to 28.1% (*Crawfurdia tibetica* vs. *Gentianopsis paludosa*) in ITS2 and 0.6% (*Gentianella biebersteinii* vs. *G. caucasea*) to 27.5% (*Gentiana aristata* vs. *Frasera speciosa*) in combined ITS1 and ITS2.

Phylogenetic analysis of ITS sequences. Using only the potentially informative data with gaps coded as missing, the same three most parsimonious trees of 631 steps with a consistency index of 0.53 and a retention index of 0.60 were found by three different heuristic searches (see Material and methods). The three

Table 4. The accession numbers of EMBL Database, length and G + C content of the nucleotide sequences of ITS1, ITS2 and ITS1 + ITS2 of *Gentianinae* and the outgroup *C. capitatum*

Taxon	ITS1			ITS2			ITS1 + ITS2	
	accession number	length (bp)	G + C (%)	accession number	length (bp)	G + C (%)	length (bp)	G + C (%)
G. algida	Z48142	230	61.3	Z48117	231	60.6	461	61.0
G. aristata	Z48100	229	52.8	Z48116	234	58.5	463	55.7
G. boryi	Z48111	230	59.1	Z48118	231	58.4	461	58.8
G. lutea	Z48122	221	61.1	Z48119	231	62.8	452	61.9
G. verna	Z48133	232	60.3	Z48120	229	60.7	461	60.5
Cm. cyananthiflorum	Z48143	232	61.4	Z48125	229	60.3	462	60.8
Cm. pulmonarium	Z48144	227	61.7	Z48121	229	60.7	456	61.0
Cw. tibetica	Z48145	228	53.5	Z48123	229	57.2	457	55.4
F. speciosa	Z48146	222	53.4	Z48124	228	60.5	451	57.0
Gl. biebersteinii	Z48147	230	59.1	Z48126	229	61.6	459	60.3
Gl. caucasea	Z48127	231	60.0	Z48101	230	61.7	461	60.7
Gl. campestris	Z48104	232	58.6	Z48128	230	63.5	462	61.0
Gl. umbellata	Z48102	232	60.3	Z48132	230	60.9	462	60.6
Gs. blepharophora	Z48103	228	56.1	Z48129	226	60.4	455	58.2
Gs. grandis	Z48105	229	60.3	Z48130	227	58.6	454	59.7
Gs. paludosa	Z48106	228	58.3	Z48131	226	58.0	454	58.1
H. elliptica	Z48107	230	60.0	Z48134	229	59.4	459	59.7
L. macranthum	Z48108	228	57.5	Z48135	230	58.6	460	58.0
M. stylophorus	Z48109	230	63.5	Z48137	229	65.1	459	64.3
P. volubilis	Z48110	232	56.9	Z48136	232	60.8	464	58.8
S. franchetiana	Z48112	231	59.7	Z48138	229	62.9	460	61.3
S. tetraptera	Z48115	230	57.4	Z48139	231	61.0	461	59.2
T. cordatum	Z48113	223	52.9	Z48140	226	57.1	449	55.0
T. capitatum	Z48114	230	60.4	Z481410	231	64.1	461	62.3

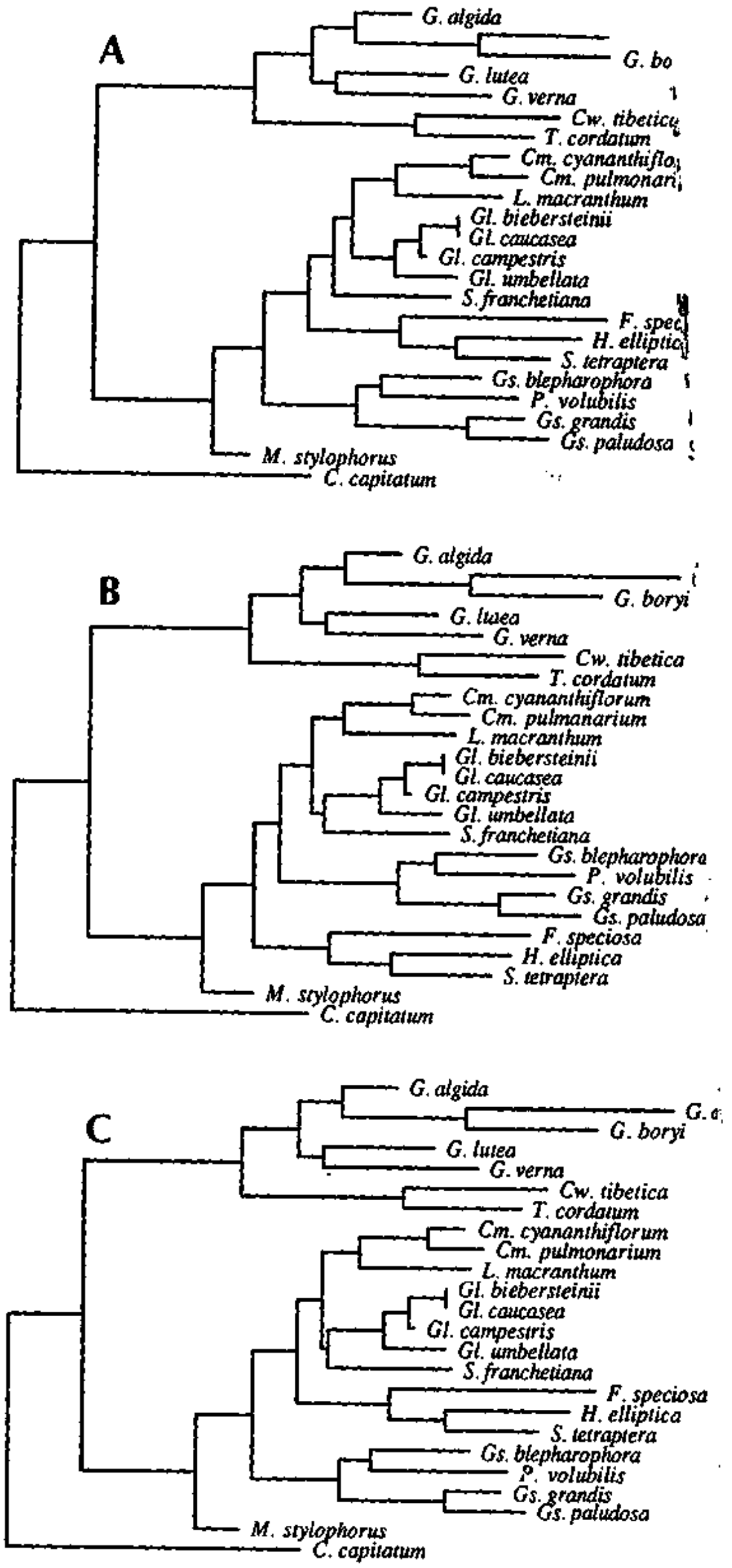

Fig. 1. A–C. The phylograms of the three equally maximum parsi enerated from potentially informative ITS sequence data where gaps were ing. The length of the branches is proportional to the number of steps co ranches. Tree length = 631; Ci = 0.53; Ri = 0.60. See Table 2 for the ab generic names

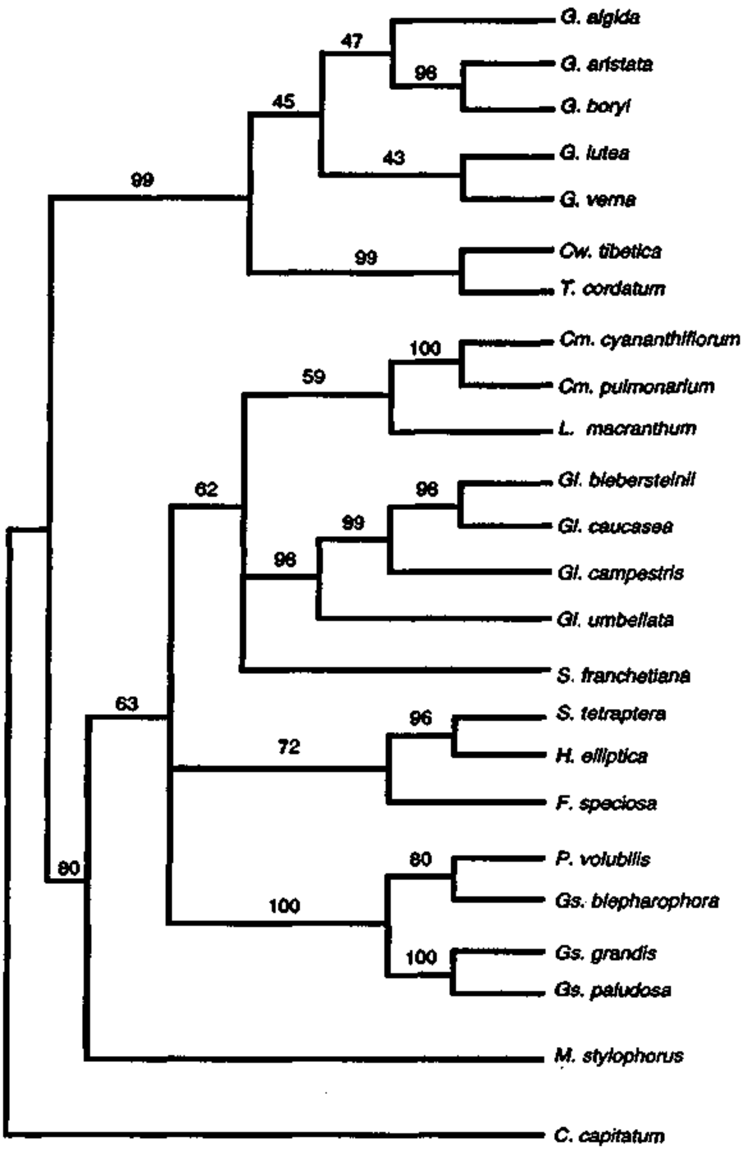

Fig. 2. The cladogram of the strict consensus of the three maximum parsimonius trees showed in Fig. 1. Numbers above the internal branches are bootstrap values of 100 replicates of heuristic searches

trees differed topologically from one another in their resolution of *Swertia franchetiana* and the clade comprising *Swertia tetraptera, Halenia elliptica* and *Frasera speciosa* (Fig. 1).

The strict consensus of the three most parsimonious ITS trees is highly resolved, and several clades are supported with rather high bootstrap values as shown in the cladogram (Fig. 2).

ITS sequences suggested an ancient divergence in the subtribe *Gentianinae*, which separated the genera *Gentiana, Crawfurdia* and *Tripterospermum* from all

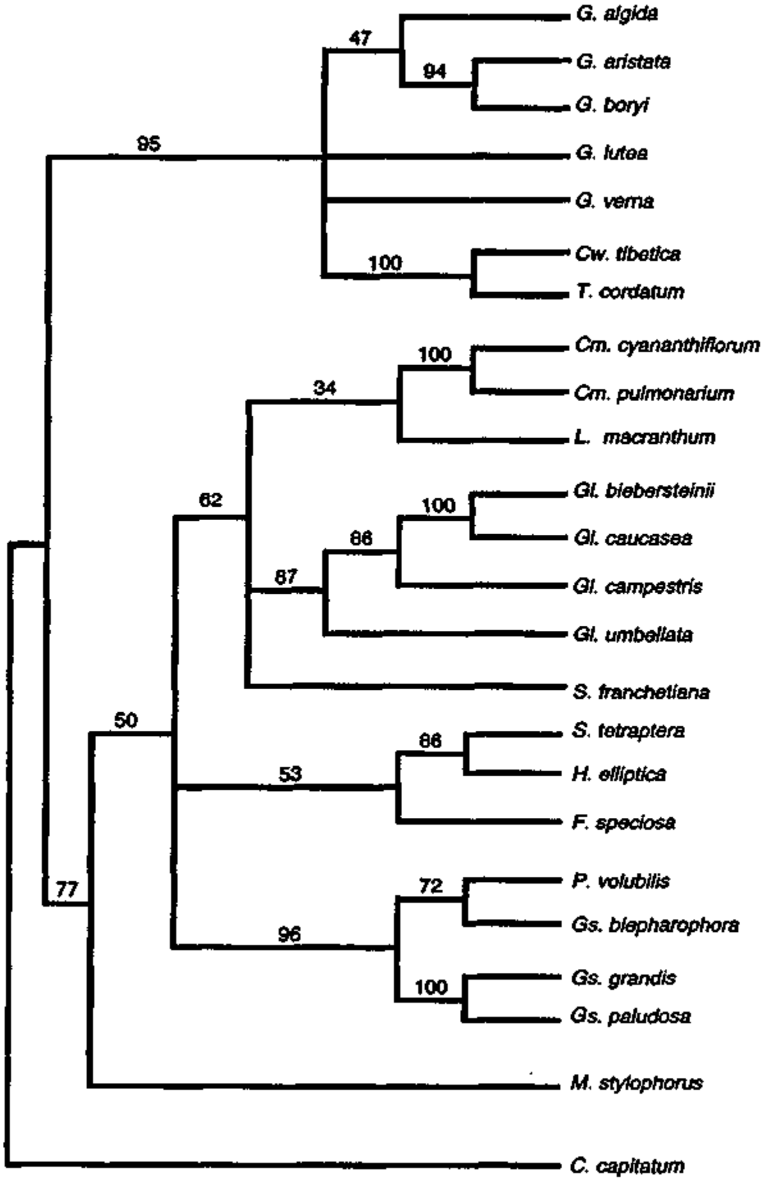

Fig. 3. The cladogram of the strict consensus of the five maximum parsimonious trees generated from the data matrix where 47 gap sites had been excluded from sequence data and were included as unweighted binary characters (0 for gap, 1 for insertion). Numbers above the internal branches are bootstrap values of 100 replicates of heuristic searches on the same data matrix. Tree length = 750; Ci = 0.53; Ri = 0.60

other members of the subtribe surveyed here, and thus two clades, the *Gentiana* and the *Gentianella* clade were formed. Both clades were supported by high bootstrap values of 99% and 90%, respectively. In the *Gentianella* clade, the genera *Comastoma, Gentianella* and *Gentianopsis* were verified as distinct groups; the genus *Lomatogonium* showed a closer relationship with *Comastoma*. The genus *Pterygocalyx* nested in the *Gentianopsis* clade, which may be a significant indication of its phylogenetic position. The genus *Megacodon* stands as sister group to all other members of the *Gentianella* clade. All these resolutions are somewhat congruent with existing morphological classifications. However, conflicts or dis-resolution exist concerning *Swertia* and its allied genera *Halenia* and *Frasera*, although a close relationship among them has been suggested.

Results under different gap treatments and character state weighting. As indicated in the method section, three additional analyses were carried out in order to explore the possible impact of gap coding on phylogenetic tree construction. In the first regime, the 47 potentially informative gap sites (indicated by * in Table 3) were excluded from phylogenetic analysis. A heuristic search generated 2 trees of 577 steps (Ci = 0.53, Ri = 0.60). The strict consensus of these two trees (not shown) is concordant with that of the basic analysis (Fig. 2) except for a higher resolution: the clade comprising *Gentianopsis* and *Pterygocalyx* standing as a sister group of the clades *Comastoma-Lomatogonium*, *Gentianella* and *Swertia-Halenia-Frasera*. *Swertia franchetiana* retained uncertain. In the second regime, the 47 gap sites were excluded and coded as unweighted binary characters which were added to the sequences data. Phylogenetic search of this regime generated 5 trees of 750 steps (Ci = 0.53, Ri = 0.59). The strict consensus (Fig. 3) of these trees is principally congruent with that of the basic analysis. However, the resolution within the *Gentiana* clade is lower. A 100 replicates of bootstrap analysis on this data matrix gave out a similar result of the basic analysis. The collapsing of the resolution involved only those clades supported by low bootstrap values, comparing to the trees of the basic analysis shown in Fig. 2. In the third regime, gaps were simply coded as a new state (fifth nucleotide). This regime generated 5 trees of 787 steps (Ci = 0.55, Ri = 0.58; trees not shown). Despite of the difference of tree length, the consensus of these trees is exactly the same with that generated from the second regime.

Two rounds of character state weighted analyses were also made to explore the relative value of transversions versus transitions. The sequence data where gaps were coded as missing generated 5 trees of 1000 steps when transversions were weighted 2 times over transitions. The strict consensus of these trees is shown in Fig. 4. The same data matrix generated a single tree of 1935 steps (Fig. 5) when the weighting factor was set at 5 : 1. The topology of these character state weighted trees is principally concordant with that of the unweighted trees. The discrepancy embodied the different resolution on *Swertia franchetiana*, *Lomatogonium macranthum*, the clade comprising *Frasera speciosa*, *Halenia elliptica* and *Swertia tetraptera* and the arrangement within the *Gentiana* clade. This discrepancy is interestingly restricted to those clades supported by lower bootstrap values (Figs. 2, 3).

Discussion

ITS sequence comparison and evolution. The difference in the length between ITS1 and ITS2 was similar in all the samples surveyed. In most species, it ranged from 1 to 3 bp. The biggest difference is shown by *Gentiana lutea* where ITS2 was 10 bp longer than ITS1. Such small difference in length between ITS1 and ITS2 has also been observed in *Solanaceae*, but in some other plant group, e.g., *Compositae*, ITS1 was much longer than ITS2 (Kim & Jansen 1994).

Among the taxa studied, the length variations of DNA sequences ranged from

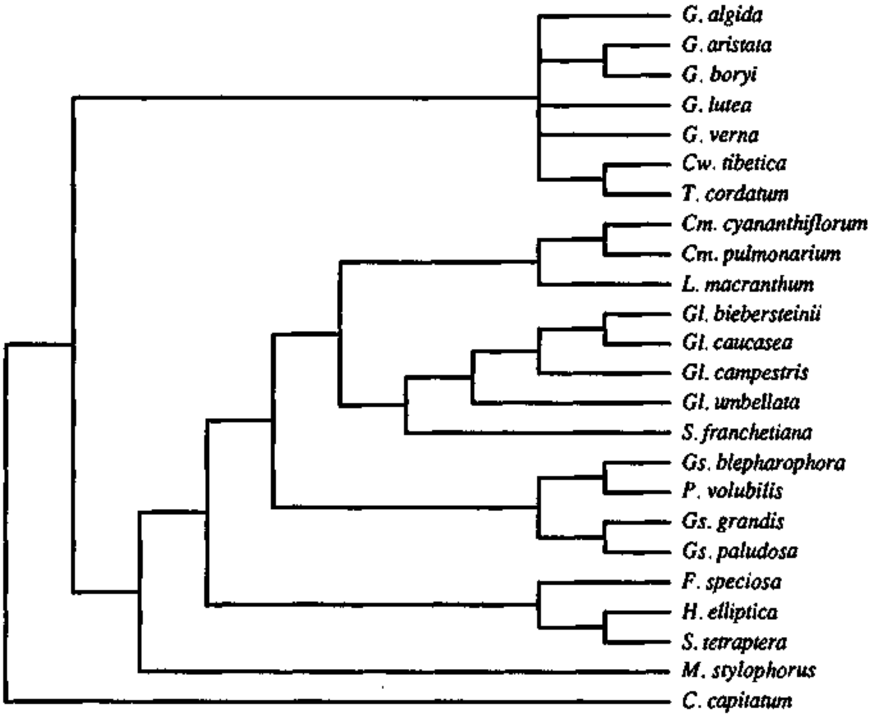

Fig. 4. The cladogram of the strict consensus of the five maximum parsimonious trees generated from character state weighted analysis where transversions were weighted two times over transitions. Tree length = 1000. Note that the resolution in the *Gentianella* clade is higher but in the *Gentiana* clade is lower

0 to 12 bp in ITS1 and from 0 to 8 pb in ITS2. These variations were primarily due to short insertion/deletion mutations. However, the present results are very different from those reported for *Lisianthus* of the same family *Gentianaceae*. A length variation of 100 bp in ITS1 has been reported among individuals within a population of *L. skinneri* (Sytsma & Schaal 1990). The reason and phylogenetic significance of such a difference are not clear yet. For the samples we investigated, no such big length variation was found, neither in the PCR nor in the sequencing step.

Point mutations were the primary source of the evolution of ITS sequences in species surveyed, which can be seen from the rather high levels of sequence divergence among and within genera (1.3%–34.1% in ITS1; 0–28.1% in ITS2). Such relatively high divergence increased the phylogenetic resolutions of the ITS sequences in the subtribe *Gentianinae*.

Phylogenetic resolution of ITS sequences in subtribe *Gentianinae*. (1) The subdivisions of the subtribe and the position of *Megacodon*. As mentioned in the Introduction, it has been constantly suggested that the subtribe *Gentianinae* contained two main subdivisions, the *Gentiana* and the *Gentianella* line (Gillett 1957, Toyokuni 1963, Ho & Liu 1990). This was based on morphological considerations. The former included the genera *Gentiana, Crawfurdia, Tripterospermum, Megacodon*, and probably *Ixanthus*, the latter all other members of the subtribe. The principal morphological criteria are the position of

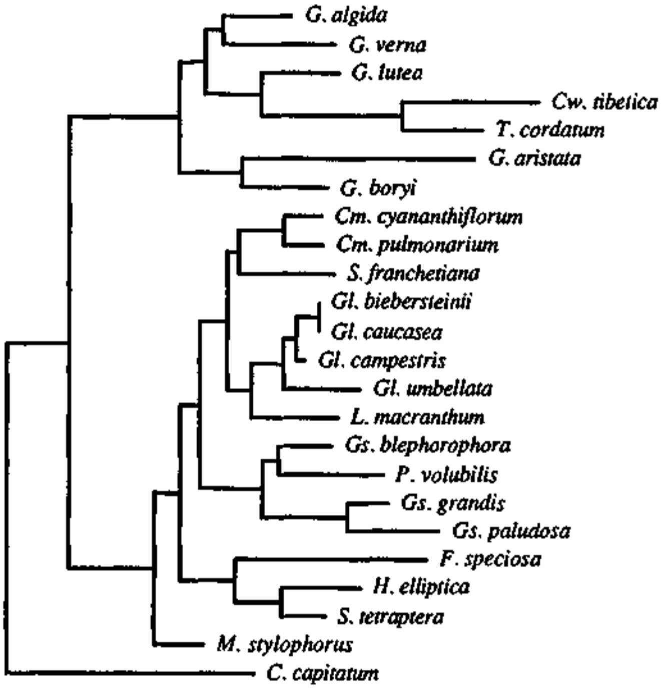

Fig. 5. The phylogram of the single maximum parsimonious tree generated from character state weighted analysis where transversions were weighted 5 times over transitions. The length of branches is proportional to the steps constituting the branches. Tree length = 1935. Note the resolution in the *Gentiana* clade and on the species *Swertia franchetiana* and *Lomatogonium* were different from those shown in Figs. 1–4

glands and persistence of plicate or folds on corolla. The ITS phylogeny of the genera, as far as it has been investigated here, supports such a subdivision. An ancient divergence of ITS sequences was suggested, which leads to the formation of two clades: the "*Gentiana*" clade encompassing the genera *Gentiana, Crawfurdia* and *Tripterospermum* and the "*Gentianella*" clade including all other members investigated (Figs. 1, 2). However, the ITS data suggest that the genus *Megacodon* belongs to the "*Gentiantianella*" line as a sister group of all the other members of this clade, rather than to the "*Gentiana*" line as suggested by morphological classifications (Toyokuni 1963, Ho & Liu 1990): However, if the morphological characters are used for the discrimination of the subdivisions, *Megacodon* really shows its intermediate and ambiguous position, because it has the "*Gentiana*" type of glands located on the base of ovary, but does not have any plicae or folds on its corolla which suggests a closer relationship with "*Gentianella*" line. The ITS data clarifies the position of *Megacodon* as being in the "*Gentianella*" line. Thus the inclusion of *Megacodon* in the "*Gentiana*" line (Ho & Liu 1990) or even within *Gentiana* (Toyokuni 1965) might be incorrect. The congruence of ITS data with such morphological characters as the position of glands and persistence of corolla plicae or folds indicates that those characters are phylogenetically truly informative in subtribe *Gentianinae*, although no explicit phylogenetic treatment of morphological data has yet been attempted on this group.

(2) The recognition and relationships of the genera *Comastoma* and *Gentianopsis*. There is a controversy among morphological classifications on the recognition of the genera *Comastoma* and *Gentianopsis* that used to be included in *Gentianella* as distinct sections (see Table1). ITS sequence data, however, recognize them as distinct genera, since, as shown in the strict consensus trees (Figs. 2–4), the species of each of these groups clustered together, respectively, as distinct monophyletic clades which were highly supported and parallel to *Gentianella* clade, except that *Pterygocalyx* nested within *Gentianopsis* clade, indicating their affinity (see discussion below). The two rather distant species of *Comastoma*, one annual and another perennial, clustered together as a monophyletic group and showed close affinity with *Lomatogonium*. *Gentianopsis*, however, was less related to both *Comastoma* and *Gentianella*.

(3) The phylogenetic position of *Pterygocalyx*. The systematic position of the monotypic genus *Pterygocalyx* is also in dispute. It was once treated as a subgenus of *Crawfurdia* (GILG 1895) because of its climbing habit. SMITH (1967) transferred it to *Gentianella* sect. *Crossopetalum* (= *Gentianopsis*), while others treated it as a distinct genus. The ITS data strongly support its close relationship with *Gentianopsis*, since it nested within *Gentianopsis* clade, supported by a rather high bootstrap value. However, we hesitated to transfer the species to *Gentianopsis* nomenclatural, since it differs markedly from *Gentianopsis* in having a twisted climbing stem and winged seeds, despite its high similarity of floral morphology with *Gentianopsis*.

(4) The sister groups of Gentiana. The genera *Crawfurdia* and *Tripterospermum* had been transferred to *Gentiana* as nominate sections by MARQUAND (1931), since he thought they overlapped with *Gentiana* sect. *Stenogyne*, but SMITH (1965) maintained them as genera distinct from *Gentiana* and thought they had no connection with *Gentiana* sect. *Stenogyne*. However, HO & LIU (1990) treated them as distinct genera but suggested a certain affinity between *Gentiana* and them. Our analysis on ITS sequences precisely indicated that *Crawfurdia* and *Tripterospermum* together formed a monophyletic clade standing as the sister group of *Gentiana* (Figs. 1–3).

The phylogenetic utility and limitation of ITS sequences. The significant congruence between ITS phylogeny and the morphological classifications described above suggested that ITS region can provide useful information for addressing phylogenetic questions among closely related species and genera. However, some conflictions or disresolutions have also been encountered, e.g., for the genus *Swertia*. Although *S. tetraptera*, *Halenia elliptica* and *Frasera speciosa* formed a monophyletic clade which was congruent with morphological suggestions, *S. franchetiana*, however, stood as sister group of *Gentianella-Comastoma* clade (Fig. 1 A) or nested in the *Gentianella* clade (Fig. 1 B, C). Gap coding and character state weighting raised another problem, since they may also slightly influence the resolution and topology of the trees (Figs. 3–5). Furthermore, there is a relatively high level of homoplasy in ITS data of the subtribe *Gentianinae*. This can be seen from the low consistency index. The relatively small size of ITS region may also limit its application in phylogenetic studies on taxonomic levels higher than genera. Therefore, ITS investigation reinforced with other data sets, such as cpDNA or other nuclear DNA fragments and morphological data, will give a better resolution or more reliable conclusions than ITS alone. Such an investigation is now under way in our laboratory to address phylogenetic problems in the subtribe *Gentianinae*.

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INFRAGENERIC PHYLOGENY OF THE GENUS *GENTIANA* (GENTIANACEAE) INFERRED FROM NUCLEOTIDE SEQUENCES OF THE INTERNAL TRANSCRIBED SPACERS (ITS) OF NUCLEAR RIBOSOMAL DNA[1]

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The internal transcribed spacers (ITSs) of nuclear ribosomal DNA have been sequenced for 20 species of *Gentiana*. By incorporating previously released sequence data of eight species, phylogenetic analyses using Fitch parsimony and character-state weighted parsimony were carried out. The length of ITS1 in the taxa surveyed ranged from 223 to 238 bp and ITS2 from 216 to 234 bp. Sequence divergence between pairs of species ranged from 5.0% to 48.9% in ITS1, from 1.1% to 45.3% in ITS2, and from 3.2% to 46.1% in combined data of ITS1 and ITS2. The ITS phylogeny was generally congruent with morphological classifications except that *G. asclepiadea* was revealed to be closely related to section *Gentiana* instead of section *Pneumonanthe* and section *Stenogyne* was shown to be a paraphyletic group of the genus *Gentiana* that would be better excluded from the genus. A divergence among the three European endemic sections and the remaining sections of the genus other than section *Stenogyne* was revealed. Thus the European species of the genus together do not form a monophyletic group. A close relationship between the sections *Chondrophyllae* s. l. (including section *Dolichocarpa*), *Cruciata* and *Pneumonanthe* was suggested. The section *Frigidae* s. l. (including sections *Monopodiae*, *Isomeria*, *Microsperma*, and *Phyllocalyx*) contained two well-supported clades: section *Frigidae* s. str. and all others together. The monophyly of the typically dysploid group section *Chondrophyllae* s. l. was confirmed. Optimization of chromosome numbers on the ITS phylogeny suggested that $2n = 26$ is a plesiomorphic state for the clade comprising sections *Frigidae* s. l., *Cruciata*, *Pneumonanthe*, and *Chondrophyllae* s. l., and probably $2n = 20$ is a plesiomorphic state for the dysploid group, section *Chondrophyllae* s. l.

Key words: *Gentiana*; Gentianaceae; internal transcribed spacers; nuclear ribosomal DNA.

Gentiana, comprising 361 species, is the largest genus in the family Gentianaceae. It is found mostly in the temperate and alpine regions of the world: widely throughout Asia (312 spp.), less commonly in Europe (29 spp.) and North and Central America (35 spp.), and sparsely in South America (three spp.), Africa (two spp. in Morocco only), and eastern Australia (one sp.). Two centers of diversity can be recognized: the principal one in the mountains of SW China and adjacent NE Burma, between 25°–34°N and 91°–105°E, where 190 species, including 98 endemics, occur; the second in the Alps and Pyrenees with 27 species, including 17 endemics (Ho and Liu, 1990). *Gentiana* is also a typical alpine plant, with most species growing at altitudes >1 000 m, *G. urnula* being an extreme at 4000–6000 m in the Himalayas.

Taxonomists disagree about the circumscription of the genus *Gentiana*. The name, in its traditional broadest sense, covers most genera of the subtribe *Gentianinae*, including *Gentianella, Gentianopsis, Comastoma, Crawfurdia, Tripterospermum, Pterygocalyx*, and *Megacodon*, which are now treated as distinct genera by most authors; but in its strict sense, this name covers only the five species usually treated as its nominate section. This narrowest circumscription was advocated by Löve and Löve (1972, 1975, 1976, 1986), Holub (1973), Laínz (1976), Weber (1985), Omer (1989), Omer and Qaiser (1992), and Omer, Ali, and Qaiser (1988). At least 12 new genera (*Calathiana, Chondrophylla, Ciminalis, Dasystephana, Favargera, Gentianodes, Holubogentia, Kuepferella, Pneumonanthe, Mehraea, Qaisera*, and *Tretorhiza*) have been created by these authors. These segregates, however, have not been widely accepted. Most modern authors, e.g., Pringle (1967, 1977, 1978, 1979), Ho (1985), Ho and Liu (1990), have accepted a circumscription of the genus, principally based on subgenus *Eugentiana* of Kusnezow (1891), excluding *Gentianella* and the other six genera mentioned above but still including all the new "genera" split by Holub, Löve and Löve, Omer, and others within the genus *Gentiana*. This circumscription was widely accepted in such standard references as *Flora Europaea* (Tutin, 1972) and *Flora Reipublicae Popularis Sinicae* (Ho, 1988), and is therefore followed here. In this circumscription, the infrageneric rank of section has been applied to the major subdivisions. In their worldwide studies, Pringle (1978) recognized ten sections, but Ho and Liu (1990), followed here (Table 1), recognized 15.

Concerning the infrageneric phylogeny of the genus

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TABLE 1. Selected classifications of the genus *Gentiana*.[a]

Kusnezow (1895)		Smith (1970)		Pringle (1978)		Ho and Liu (1990)		Splitters
Subgenus	Section	Genus	Section	Genus	Section	Genus	Section	Genus
Eugentiana	1. *Coelantha*	*Gentiana*	1. *Coelantha*	*Gentiana*	1. *Gentiana*	*Gentiana*	5. *Gentiana*	*Gentiana*
	2. *Pneumonanthe*		2. *Pneumonanthe*		2. *Pneumonanthe*		6. *Pneumonanthe*	*Pneumonanthe*
								Dasystephana
	3. *Othophora*		3. *Othophara*		10. *Othophora*		1. *Othophora*	*Gentianodes* subg. *Othophora*
	4. *Stenogyne*		4. *Stenogyne*		4. *Stenogyne*		12. *Stenogyne*	*Tripterospermum* subg. *Stenagyne*
							7. *Phyllocalyx*	*Mehraea*
	5. *Frigida*		5. *Frigida*		5. *Frigidae*		4. *Frigidae*	*Favargera*
					3. *Kudoa*		3. *Monopodiae*	*Gentianodes*
	7. *Isomeria*						10. *Isomeria*	included in *Gentianodes*
	6. *Aperta*						11. *Microsperma*	included in *Gentianodes*
	8. *Chondrophylla*		6. *Aperta*		6. *Cruciata*		2. *Cruciata*	*Tretorhiza*
			7. *Chondrophylla*		7. *Chondrophyllae*		14. *Chondrophylla*	*Chondrophylla*
							13. *Dolichocarpa*	*Halubogentia*
							15. *Fimbricorona*	*Qaisera*
	9. *Thylacites*							*Kuepferella*
	10. *Cyclostigma*		8. *Thylacites*		8. *Ciminalis*		9. *Ciminalis*	*Ciminalis*
			9. *Cyclostigma*		9. *Calathianae*		8. *Calathianae*	*Calathiana*

[a] The numbers leading each section refer to the sequence of sectional arrangement in the original literature.

Gentiana, section *Gentiana* (=*Coelanthe*) was suggested be the sister clade to the remainder of the genus, and section *Pneumonanthe* was suggested to be sister to the other sections such as *Calathianae, Cruciata, Frigidae,* and *Ciminalis* (Müller, 1982). Carbonnier, Massias, and Molho (1977), based on their chemical analyses from limited European taxa, have drawn a similar conclusion: section *Gentiana* formed a branch near the base of their intuitive evolutionary scheme; section *Pneumonanthe* was at the intermediate position; section *Ciminalis* and section *Calathianae* grouped together at a higher level. However, such suggestions have limited significance for the whole genus, since the phytochemical analyses did not take into account many Asian sections. Ho and Liu (1990) believed that section *Stenogyne*, endemic to SW China and the adjacent area, could represent the most primitive type of the genus, through which the genus *Gentiana* showed affinity with the genera *Crawfurdia* and *Tripterospermum*, which they believed to be close to the ancestral type of the genus *Gentiana*.

The geographical distribution pattern of the sections may give some hints about the origin and phylogeny of the genus. As mentioned above, two centers of diversity exist. Sections *Gentiana, Ciminalis,* and *Calathianae* are highly concentrated in the European center, whereas sections *Stenogyne, Phyllocalyx, Othophora, Monopodiae, Microsperma,* and *Fimbricorona* are restricted to the Asian center, and some sections such as *Isomeria, Cruciata, Chondrophyllae, Dolichocarpa,* and *Frigidae* are highly concentrated in the Asian center but have a wide distribution throughout Eurasia to North America. Sections *Chondrophyllae* and *Dolichocarpa* are found only in Australia and South America. In the continent of Africa, only two species are found: *G. atlantica* (section *Chondrophyllae*) and *G. sierrae* (section *Calathianae*). In the New World, although sections *Calathianae, Chondrophyllae, Dolichocarpa, Frigidae,* and *Pneumonanthe* are found, only the section *Pneumonanthe* is well diversified. Based on the geographical pattern, we suggest that there are probably parallel diversifications between the two centers of diversity: sections *Gentiana, Calathianae,* and *Ciminalis* diversified from the European one, and all other sections diversified from the Asian one except *Pneumonanthe*, whose origin is unclear.

Karyological characters, principally basic chromosome numbers, have been the critical characters used by some authors to split this genus. We have recently reviewed the relevant karyological studies (Küpfer and Yuan, 1992; Yuan, 1993; Yuan and Küpfer, 1993a, b). Of a total of 361 species 158 have been observed cytologically so far. The existing chromosome data show an extensive range of numerical variation : $2n$ = 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 52, 60, and 96. The sections *Gentiana* ($2n$ = 40), *Ciminalis* ($2n$ = 36), *Cruciata* ($2n$ = 26 or 52), *Pneumonanthe* ($2n$ = 26 with one exceptional species, *G. asclepiadea* $2n$ = 44), *Frigidae* ($2n$ = 24 with one exceptional species *G. froelichii* $2n$ = 42), and *Monopodiae* ($2n$ = 24 or 48) are relatively stable; the section *Stenogyne* has higher secondary basic chromosome numbers of x = 17, 21, 23 ($2n$ = 34, 42, and 46), while the sections *Chondrophyllae, Dolichocarpa,* and *Calathianae* show the highest variation and a typical dysploidy series. Thus dysploidization, in conjunction with polyploidization, may play an important role in the karyological evolution of the genus, especially in the three sections just mentioned. However, we are still confronted with many karyological problems, for example, the polarity of dysploidization, karyological relationships among different cytotypes, and the origin of some common cytotypes that were geographically isolated (e.g., $2n = 48$ of *G. atlantica* in northern Africa and *G. aquatica* in central Asia). In short, one has to know to what extent the basic chromosome numbers of the genus *Gentiana* reflect its phylogeny before evaluating the phylogenetic significance of karyological data. Such a task is obviously beyond the resolution of conventional karyological study.

Few studies on DNA have been conducted on the family Gentianaceae. Phylogenies reconstructed by Sytsma and Schaal (1985, 1990) on the New World tropical genus *Lisianthius* using restriction analysis of chloroplast DNA (cpDNA) and nuclear ribosomal DNA (nrDNA) were highly congruent with relationships inferred from isozyme variations. Gielly (1994) and Gielly and Taberlet (1994) have investigated the phylogenetic utility of two noncoding regions of cpDNA, the *trn*L (UAA) intron and the intergenic spacer between *trn*L (UAA) 3′ exon and the *trn*F (GAA) gene, using the European gentians of subtribe *Gentianinae*. In the resulting cladograms, the genus *Gentiana* formed a highly supported monophyletic clade; the remaining genera grouped together as the sister clade of *Gentiana*. The phylogenetic relationships among the taxa within both clades were somewhat but not totally congruent with morphologically defined classifications. Examples of conflict included the splitting of section *Ciminalis* of the genus *Gentiana*, a group strongly supported as monophyletic by morphological and karyological characters, into two relatively distant clades, and the placement of *G. frigida* (section *Frigidae*) as sister to the remainder of *Gentiana*. Recently, the internal transcribed spacer (ITS) region of nrDNA has widely been used to infer phylogenetic relationships in different taxa (Baldwin, 1992, 1993; Wojciechowski et al., 1993; Suh et al., 1993; Sang et al., 1994; Kim and Jansen, 1994; Hsiao et al., 1995a, b; Jansen and Bain, 1995; Wendel, Schnabel, and Seelanan, 1995). Our recent studies (Yuan and Küpfer, 1995) on ITS sequences of nrDNA of the subtribe *Gentianinae*, which included five species of the genus *Gentiana*, indicated that ITS sequences were informative in addressing phylogenetic relationships among the genera of the subtribe. The genera *Crawfurdia* and *Tripterospermum* were revealed to be the most closely related groups to the genus *Gentiana*, and the five species of *Gentiana* formed a highly supported monophyletic clade. Since then we have concentrated our investigations on the largest genus of the subtribe, *Gentiana*, to study further the molecular evolution of the ITS regions and its significance for resolving the infrageneric phylogeny of the genus *Gentiana*. The following issues in particular are addressed in this paper: (1) the utility of ITS sequences as a source of phylogenetic data in inferring infrageneric phylogeny in *Gentiana*; (2) the phylogenetic relationships among the sections of the genus; (3) the congruence between the ITS phylogeny and the existing classifications; (4) evaluation of the karyological conclusions.

MATERIALS AND METHODS

Plant species and materials—NrDNA ITS sequences of four *Gentiana* species and the outgroups were directly taken from our previous study (Yuan and Küpfer, 1995). Sequences were obtained from a single individual, or, in a few cases when the individual was too small, 2–3 pooled individuals of the same population from each of a further 20 species representing 12 sections. The reported chromosome number of each species and the locality and altitude of each plant investigated are given in Table 2. Leaves were collected directly from the field using the silica gel method (Chase and Hills, 1991). All voucher specimens were prepared and deposited in the herbarium of the University of Neuchâtel (NEU). *Crawfurdia tibetica* and *Tripterospermum cordatum* were initially designated as outgroups, based on our previous study (Yuan and Küpfer, 1995). However, our subsequent phylogenetic analysis revealed that a section of our ingroup, section *Stenogyne* of the genus *Gentiana*, nested within the outgroup clade. Thus we considered *C. tibetica* and *T. cordatum* as ingroup taxa and included two more distant taxa as outgroups, *Gentianopsis grandis* and *Megacodon stylophorus*, which belong to the sister clade of *Gentiana–Crawfurdia* clade in the ITS phylogenetic tree of the subtribe *Gentianinae* (see Yuan and Küpfer, 1995).

Collection of materials has presented of the biggest challenge in the current investigation. Despite persistent attempts, three relatively small sections, *Fimbricorona* (four spp.), *Phyllocalyx* (one sp.), and *Othophora* (12 spp.) remain unavailable to us.

Total DNA extraction—Total DNA was extracted from 100 to 300 mg of dried leaves pulverized in liquid nitrogen or directly in hot 2x CTAB buffer according to the protocol of Doyle and Doyle (1987).

Amplification of ITS region and the primers used—A standard double-strand polymerase chain reaction (PCR) was used to amplify the entire ITS region, using primers YP1 and YP4. Two internal primers, YP2 and YP3, were also used in subsequent sequencing. The primers YP1, YP2, YP3, and YP4 are identical to ITS5, ITS2, ITS3, and ITS4, respectively, of White et al. (1990) except for a minor modification in YP1 (see also Baldwin [1993] for the locations of the primers). The sequences of the primers are: YP1 = 5′-GGAAGTAGAAGTCGTAACAAGG-3′, YP2 = 5′-GCTGCGTTCTTCATCGATGC-3′, YP3 = 5′-GCATCGATGAAGAACGCAGC-3′, YP4 = 5′-TCCTCCGCTTATTGATATGC-3′. All of these primers were purchased from Microsynth (Windisch, Switzerland).

PCRs were carried out in 50-μL reaction mixtures containing 34.75 μL of sterile double-distilled water, 5 μL of 10× Taq polymerase reaction buffer (Appligene, Illkirch, France), 2 μL equimolar of 5 mmol/L dNTP, 2.5 μL each of 10 μM primer YP1 and YP4, 0.25 μL of 5 Unit/μL Taq DNA polymerase (Appligene, Illkirch, France), and ≈3–12 ng (3 μL of 1–4 ng/μL) of genomic DNA. For multiple-sample amplifications, a master mix containing all the components except the genomic DNA was prepared and aliquoted. The aliquots were heated to 70°C and then the genomic DNA was added last. The reaction mixtures were sealed with ≈15 μL mineral oil to prevent evaporation. PCR was performed on a Perkin-Elmer thermal cycler under the following conditions: one cycle of 2 min at 94°C linked to 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min with 4 extension for each cycle at 72°C. Samples were held for a final 5 min at 72°C to complete primer extension.

Purification of PCR products—Five microlitres of each PCR reaction was run on a 0.8% agarose gel to check the quality of amplification. To purify the ITS fragments, 40 μL of each PCR reaction was loaded on a 0.8% TAE agarose gel. The agarose blocks containing ITS fragments were excised from the gel with a scalpel under UV light, and applied to the QIAEX gel extraction kit according to the manufacturer's protocol (QIAGEN AG, Basel, Switzerland). ITS fragments were re-

TABLE 2. The taxa, chromosome numbers, origins, and altitude of plant materials studied. Voucher: Y = Y.-M. Yuan; Z = L. Zeltner. (sect. Chondrophyllae s. l. = sect. Chondrophyllae + sect. Dolichocarpa; sect. Frigidae s. l. = sect. Frigidae + sect. Isomeria + sect. Microsperma + sect. Monopodiae)

No.	Taxon	$2n$	Voucher	Origin of material
	Gentiana			
	sect. *Calathianae*			
1	*G. bavarica* L.	30	Y93-11	Grand Chavalard, Valais, Switzerland; 2 200 m
2	*G. verna* L.	28	Y93-05	Grand Chavalard, Valais, Switzerland; 2 100 m
	sect. *Chondrophyllae*			
3[a]	*G. aristata* Maxim.	14	Y92-328	Maqü, Gansu, China; 3 500 m
4[a]	*G. boryi* Boiss.	20	Z93-S1	Hoya del Moro, Sierra Nevada, Spain; 2 300 m
5	*G. pyrenaica* L.	26	Y93-14	Rila Mt., Borovetz, Bulgaria; 2 600 m
	sect. *Ciminalis*			
6	*G. alpina* Vill.	36	Y93-09	Grand Chavalard, Valais, Switzerland; 2 200 m
7	*G. clusii* Perr. et Song.	36	Y93-13	Grand Chavalard, Valais, Switzerland; 2 100 m
	sect. *Cruciata*			
8	*G. macrophylla* Pall.	26	Y92-271	Dangchang, Gansu, China; 2 400 m
9	*G. straminea* Maxim.	52	Y92-313	Maqü, Gansu, China; 3 500 m
	sect. *Dolichocarpa*			
10	*G. crenulato-truncata* T. N. Ho	18	Y92-310	Maqü, Gansu, China; 4 200 m
11	*G. haynaldii* Kanitz	20	Y92-201	Zhongdian, Yunnan, China; 3 400 m
	sect. *Frigidae*			
12[a]	*G. algida* Pall.	24	Y91-S1	Trail Ridge, Rocky Mt. Natl. Park, USA; 3 100 m
13	*G. frigida* Haenke	24	Y93-17	Rila Mt., Borovetz, Bulgaria; 2 800 m
	sect. *Gentiana*			
14[a]	*G. lutea* L.	40	Y91-S5	La Tourne, Neuchâtel, Switzerland; 1 200 m
15	*G. punctata* L.	40	Y93-15	Rila Mt., Borovetz, Bulgaria; 2 600 m
	sect. *Isomeria*			
16	*G. depressa* D. Don	?	Y92-118	Zhangmu, Tibet, China; 2 800 m
17	*G. urnula* H. Smith	26	Y92-71	Karu Pass, Langkazi, Tibet, China; 5 200 m
	sect. *Microsperma*			
18	*G. delavayi* Franch.	26	Y92-229	Lijiang, Yunnan, China; 2 900 m
	sect. *Monopodiae*			
19	*G. callistantha* Diels et Gilg	26	Y92-298	Luqü, Gansu, China; 3 500 m
	sect. *Pneumonanthe*			
20	*G. affinis* Griseb.	26	Y91-S1	Towhee Trail, Boulder, Colorado, USA; 2 300 m
21	*G. asclepiadea* L.	44	Y93-20	Izgorjaloto Gjune Reserve, Bulgaria; ≈400 m
22	*G. parryi* Engelm.	26	Y91-S4	South Park, Colorado, USA
	sect. *Stenogyne*			
23	*G. gentilis* Franch.	42	Y92-256	Western Hill, Kunming, China; 2 100 m
24	*G. rhodantha* Franch. ex Hemsl.	46	Y93-124	Yingjing, Sichuan, China; 1 100 m
	Crawfurdia			
25[a]	*C. tibetica* Franch.	?	Y93-121	Mt. Gongga, Sichuan, China; 2 700 m
	Gentianopsis			
26[a]	*Gs. grandis* (H. Smith)Ma	52	Y92-222	Lijiang, Yunnan, China; 2 900 m
	Megacodon			
27[a]	*M. stylophorus* (C. B. Clarke)H. Smith	?	Y93-182	Mt. Baima, Deqin, Yunnan, China; 4 100 m
	Tripterospermum			
28[a]	*T. cordatum* (Marq.)H. Smith	?	Y92-267	Mt. Huanlong, Shaanxi, China; 2 700 m

[a] Sequences taken from our previous study (Yuan and Küpfer, 1995).

covered in 40 μL TE buffer, which is sufficient to run several sequencing reactions.

DNA Sequencing—Both strands of purified double-strand DNA (dsDNA) were directly sequenced by standard dideoxy chain termination techniques, using the cycling method of the *fmol* DNA sequencing system (Promega, Madison, WI). The protocols recommended by the manufacturer were followed, using the primers YP1, YP2, YP3, and YP4 5'end-labeled with [gamma-^{32}P]-ATP (Amersham Rahn, Zürich, Switzerland). The following program was performed on a thermal cycler (Perkin Elmer, Foster City, CA): one cycle of 2 min at 94°C, followed by 30 cycles of 45 at 94°C, 45 at 55°C and 1 min at 72°C, and finally cooled to 4°C. This system gave good resolution for these ITS sequences.

DNA sequencing samples were separated on 6% acrylamide–8M urea gels. After electrophoresis, the gels were fixed in 20% ethanol–10%

acetic acid for 10 min, transferred onto Whatman 3 MM paper, vacuum dried at ≈ 80°C for 2 h, and then exposed to Kodak XAR X-ray films for 10–14 h.

Sequence alignment—The sequence boundaries between the two spacers and the three coding regions (18S, 5.8S, and 25S genes) of nrDNA were determined by comparison with published sequences from *Daucus carota* and *Vicia faba* (Yokota et al., 1989) and with our previous data (Yuan and Küpfer, 1995). The combined sequences ITS1 and ITS2 were aligned using the program Clustal (Higgins, Bleasby, and Fuchs, 1992), with fixed gap penalty and floating gap penalty at 10 and DNA transitions unweighted. The generated alignment was slightly adjusted manually to minimize the number of gaps. The inclusion of the outgroups did not introduce any additional alignment ambiguity.

Phylogenetic analysis—Potentially informative sites, those with each of at least two character states shared by two or more sequences, were used for phylogeny construction. The 13 sites from position 13 to 25 were excluded from most phylogenetic analyses because of their alignment ambiguity, although the inclusion of these sites does not affect the topology of the resulting trees. For our basic analysis, phylogenetic trees were reconstructed from the data set where gaps were coded as missing, using unweighted Fitch parsimony, i.e., assuming unordered character states as implemented in PAUP 3.1.1 (Swofford, 1993). To test for the effect on phylogeny reconstruction of the expected higher rate of transition mutations over transversions, we tested a range of character-state weighting schemes. Analyses were conducted using the stepmatrix function of PAUP to weight transversions 2:1, 5:1, or 10:1 over transitions. Three methods were used to treat characters for which alignments suggested that one or more taxa had experienced deletions or insertions: (1) the 51 potentially informative gap sites, as indicated by * in Fig. 1, were excluded from analysis; (2) the 51 potentially informative gap sites were converted into 36 multistate characters where both substitutions in the insertions and the deletions were taken into account, e.g., the three sites 156-158 were considered as a single character unit and six different character states (---, A--, AA-, AC-, ACG and ACT) were rescored for that character; (3) the gaps were simply coded as a new state (gapmode = newstate).

Unweighted analyses were also conducted using only ITS1 or ITS2, with gaps coded as missing, to examine the relative informativeness of the two spacers.

In view of the number of taxa included in this study, heuristic search strategies were conducted. Four different heuristic searches using different taxon addition sequences, in combination with TBR (tree bisection–reconnection) branch-swapping methods, were performed for the basic analysis, to maximize the probability of identifying the most parsimonious trees: (1) SIMPLE addition sequence; (2) CLOSEST addition sequences; (3) ASIS addition sequences; and (4) 100 replicates of RANDOM addition sequence. For all the other analyses, only the CLOSEST and TBR options were performed. Bootstrap values were calculated from 500 replicates of heuristic searches with SIMPLE addition sequences for the basic analysis where gaps were coded as missing.

Chromosome numbers were optimized on the consensus ITS tree generated from the basic analysis, using MacClade 3.0 (Maddison and Maddison, 1992).

RESULTS

Variation in repeat unit—No clear evidence of multiple repeat types was found. Each PCR product was resolved, in most cases, as a single band on 0.8% agarose gels. In a few cases, a weak smaller band was also resolved in addition to the regular band. The weak band generated the same sequence as the regular band in two examples examined, suggesting that they represent single-stranded ITS DNAs. From the sequencing step, however, nucleotide polymorphism was often found for the site 18 (C or G) and site 19 (A or G) (Fig. 1), in addition to some occasional polymorphic or ambiguous sites occurring in individual sequences at low frequency.

ITS sequences and their divergence—The aligned sequences of ITS1 (sites 1 to 255) and ITS2 (sites 256 to 505) are shown in Fig. 1. The sequences were deposited in the EMBL Nucleotide Database. The length, G + C content, and accession number of EMBL Nucleotide Database of each sequence are given in Table 3. The length of ITS1 in the taxa surveyed varied from 219 bp (*G. crenulato-truncata*) to 237 bp (*G. verna*). The length of ITS2 varied from 216 bp (*G. straminea*) to 234 bp (*G. aristata*). The multiple alignment of the sequences required 255 sites for ITS1 and 250 for ITS2. The G + C content varied from 52.8% (*G. aristata*) to 63.5% (*M. stylophorus*) in ITS1 and from 56.4% (*G. straminea*) to 65.1% (*M. stylophorus*) in ITS2. Of the 505 aligned positions, 155 sites (96 in ITS1 and 59 in ITS2) involved gaps; 27 sites (17 in ITS1 and 10 in ITS2) involved nucleotide polymorphism or ambiguity in individual sequences; 129 sites (50 in ITS1 and 79 in ITS2) were identical for all the sequences surveyed; 194 sites (92 in ITS1 and 102 in ITS2) showed nucleotide divergence, i.e., possessed one or more nucleotide differences between sequences.

Pairwise sequence similarities were calculated for all possible combinations for ITS1, ITS2, and the combined data of ITS1 and ITS2, using the data distance matrix option in PAUP. Within the genus *Gentiana* (including section *Stenogyne*), the mean sequence divergence between pairs of species ranged from 5.0% (*G. algida* vs. *G. frigida*) to 48.9% (*G. aristata* vs. *G. verna*) in ITS1; from 1.1% (*G. algida* vs. *G. frigida*) to 45.3% (*G. bavarica* vs. *G. gentilis*) in ITS2 and from 3.2% (*G. algida* vs. *G. frigida*) to 46.1% (*G. aristata* vs. *G. gentilis*) in the combined data of ITS1 and ITS2 (distance matrix not shown).

Phylogenetic analysis of ITS sequences—Basic phylogenetic analyses were performed on the data matrix where gaps were coded as missing data. Only the potentially informative sequence data were analyzed using Fitch parsimony. The autapomorphic and constant sites were ignored from analyses, since they do not contribute any resolution on internal branches of the phylogenetic trees. Four rounds of heuristic searches using different regimes of stepwise addition of sequences generated the same set of six equally most parsimonious trees of 535 steps, with a consistency index (CI) of 0.578 and a retention index (RI) of 0.606. The strict consensus of the six most parsimonious ITS trees was highly resolved, and several clades were supported with relatively high bootstrap values (Fig. 2).

The phylogeny of the ITS sequences is largely congruent with morphological classifications, with the morphologically best defined sections supported. There is a major dichotomy between the three European endemic sections (*Calathianae*, *Ciminalis*, and *Gentiana*) and the remaining sections (other than section *Stenogyne*). Two clades, one involving section *Chondrophyllae* s. l. (including section *Dolichocarpa*) and the another involving

```
        »ITS1*   *                    *             **            *
1       TCGAA-TGGTGC--GAAAG-AGAC-GACCCGAGGACA-TGTTTGAAA-CCACGGGCGTTCGGGA-CGGGGGAAACCTC-GGACCGATGCCCTAAGCATGG (100)
2       TCGAA-TCCTGC--GAAAC-AGAC-GACCCGAGGACA-TGTTTTAAATCCACGGGCGTTCGGGA-CGGGGGAAACCAC-GGACCGATGCCCCGAGCATGC
3       TCGA-TTCCTGCCTTAAGCAACACCAAACTG--------GTTTTTAAC---CGGG-TTTTGGG--CCAAGGAAACCCC-GGGCCAATTCCCCGACCTTAM
4       TCGAA-TCCTGC--TAAGC-AGAC-GACCCGAGAACA-TGTTT--ATCGCACGGGCGT-CGGGA-CGAGGGAAACCGCAGGACCGATGCCCCGCGCATGA
5       TCGAA-TCCTGC--TAAGC-AGAC-GACCCGAGAACA-TGTTT--ATCGCACGGGCGT-CGGGA-CGGGGGAAACCTT-GGACCGATGCCCCGAGCACGA
6       TCGAA-TCCTGC--GAASR---AC-GACCCGAGGACA-TGTTT--AAAGCACGGGCGTTCGGGA-CGAGGGAAACCAC-GGACCAGTGCCCCGAGCACG-
7       TCGAA-TCCTGC--GAAGA--GACGGACCCGAGGACA-TGTTT--AAAGCACGGGCGTTCGGGA-CGAGGGAAACCAC-GGACCGATGCCCCGAGCACGG
8       TCGAT-TCCTGC--TAAGC-AGAC-GACCCGAGAACA-TGTTT--AACGAATGGGCGTTCGGGA-CGAGGGAAACCAC-GGCCCGATGCCCCGAGCATGG
9       TCGAT-TCC-GC--TAAGC-AGAC-GACCCGAGAACA-TGTTGT-AACGAACGGGCGTTCGGGA-CGAGGGAAACCAC-GGCCCGATGCCCCGAGCATGG
10      TCGAA-TCCTGC--TAAGC-AGAC-GACCCGAGAACA-TGTTT--ATCTCACGGGCGT-CGGGA-YGAGGGAAACCRC-GGACCGATGCCCCGAGCAGGA
11      TCGAA-TCCTGC--TAAGC-AGAC-GACCCGAGAACA-TGTTT--ACCGCACGGGCAT-CGGGA-CGGGGGAGACCAC-GTACCGATGCCCCGAGCACGG
12      TCGAT-TCCTGC--GAASR---AC-GACCCGAGAACA-TGTCT--AACGCACGGGCGTTCGGGA-CGAGGGAAACCAC-GGACCGATGCCCCGAGCACGG
13      TCGAT-TCCTGC--GAAGC-AGAC-GACCCGAGAACA-TGTCT--AACGCACGGGCGTTCGGGA-CGAGGGAAACCAC-GGACCGATGCCCCGAGCACGG
14      TCGAA-TCCTGC--GAAGC-AGAC-GACC-GAGAACA-TGTTT--AAAGCACGGGCTTTCGGGA-CGAGGGAAAGGAC-GGACCGATGCCCCGAGCACGG
15      TCGAATTCCTGC--GGAACGAGACCGRCCCGAGGACAATGTTT--AAACCACGGGCGTTCGGGA-CGAGGGAAACCAC-GGACCCATGCCCCGAGCACGG
16      TCGAT-TCCTGC--GAAGC-AGAC-GACCCGAGAACA-TGTTT--AACGCACGGGCATTCGGGA-CGAGGGAAACCAC-GGACCGATGCCCCGAGCATGG
17      TCGAT-TCCTGC--RAAGAGAG-CGGACCCGAGAACA-TGTTT--AACGCACGGGCATTCGGGA-CCAGGGAGACCAC-GGACCTGTGCCCCGAGCATGG
18      TCGAT-TCCTGC--GAAS--AGAC-GACCTGAGAACA-TGTTT--AACGCACGGGCATTTAGGA-CCAGGGAAACCAC-GGACAAATGCCCCGAGCATGG
19      TCGAT-TCCTGC--GAMR----AC-GACCCGAGAATA-TGTTT--AATGCACGGGCATTCGGGA-CGAGGGAAACCAC-GGACCGATGGCCCGAGCATGG
20      TCGAA-TCCGGC--TAAGC-AGAC-GACCCGAGAACA-TGTTT--AACACACGGGCGTTCGGGAACCAGGGAAACCAC-GGACCGATGGCCCGAGCATAG
21      TCGAA-TCCTGC--GAASR---AC-GACCCGAGGACA-TGTTT--AAAGCACGGGCGTTCGGGA-CGAGGGAAACCAC-GGACCGATGCCCCGAGCACGG
22      TCGAT-TCCTGC--TAAGC-AGAC-GACCCGAGAACA-TGTTT--AACACACGGGCATTCGGGA-CGAGGGAAACCAC-GGACCGATGCCCCGAGCATTT
23      TCGAT-TCCTGC--GAAGC-AAAC-GACTCGAGAACA-TGTTC--AACTCACGGGCATTCGGGA-CGAGGGAAAGGAC-GTACCGATGCTCCGAGCAAGG
24      TCGAT-ACCTGC--GAASR---AC-GACCCGAGAACA-TGTTC--AACTCACGGGCGTTCGGGA-CGAGGGAAACCAC-GTACCCATGCCCCGAGCAAGG
25      TCGAT-TGGTGC--GAAGC-AGAC-GACCCGAGAACA-TGTTT--AACTCACGGGCATTTGGGA-CGTGGGAAACCAT-GGACTCATGCCCCGAGCAAGG
26      TCGAAGTCCTGC--GAAGT-AGAC-GACC-GTGAACT-TGTAT--ATCGKA-GGGCGTCCGGGA-CGCGGGAAACCAC-GGACCGGCGGCCCGAGGACGG
27      TCGAA-TCCTGC--GAAS--AGAC-GACCCGAGAACA-TGTTT--AACGCACGGGCGTCCGGGC-CGGGGGAAACCAC-GGACCGGCGTCCCGAGCGCGG
28      TCGAT-TYC-GC--GAAGC-AGAC-GACCCGAGAACA-TGTTN--AACTTACGGGCATTTGGGA-TGTAGGAAACCAC-GGACCGATGCCCCGAGCAAGG

           *             ***     *                   ***    *  ***                **             *   *
1       CGTCGACCACCGGTCGG---TCGTC-GTGCACACAACCAACCCCGGGCGCAG-AAAC-GCGCCAAGGAAAACCA---AAAAGGATGGCC-TGCCTCCTTT (200)
2       CGTCGACCACCGGTCGC---TCGTTCGTGCACACAACCAACCCCGGGCACAG-AAAC-GTGCCAAGGAAAACGA-AAAAATGCATGGC--TGGTTCTTCT
3       GGGWRGCCCCGGTCGRTWGTCGTG--CCCAAACAACCAACCCCGSSCAAA--AAAC-CCCCCAAGGAAAACCTTAAAAAAGGAATTTYYTCCCCCCCGT
4       CGTCGACCACCGGTCGC---CCGTC-GTGCAAACAACCAACCCCGGGCGTAA-AAAC-ACGCCAAGGAAAACGT--AAAAAGGATTGTC-TGCCCCCCGT
5       CGTCGACCACCGGTCGG---CCGTC-GTGCAAACAACCAACCCCGGGCGCAG-AAAC-GCGCCAAGGAAAACGT-AAASAAGGATTGTC-TGCCCCCGGT
6       CGTCGACCACCGGTCGC---TCGTC-GTGCAAACAACCAACCCCGGGCGCAG-AAAC-GCGCCAAGGAATACGA--AAAAAGGATGGCC-TGCCTCCCGT
7       CGTCGACCACCGGTCGC--GTCGTCGTCTCAAACAACCAACCCCGGGCGCAG-AAACTGTGCCAAGGAATACGA--AAAAAGGATGGCC-TGCCTCCCGT
8       CGTGGACCACCGGTCGC---TCGTC-GTGCAAACAACCAACCCCG--CGCAG-AAAC-GCGCCAAGGAAAACGA--AAAAAGGATGGCC-TGCCTCCCGT
9       CGT-GACCACCGGTCGCTCGTCGTGTATG-AAACAACCAACCCCG---GCAG-AAAC-GCGCCAAGGAAAACCA--AAAAAGGATGGCC-YG--TCCCGG
10      CGTCGACCACCGGTCGC---TCGTC-GTGCAAACA-CCAACCCCGGGGGAAGGAAAC-G-GC-AAGGAAACCCT--AAAAAGGTTTCCC-TTCCCCCCGT
11      CGTCCACCACCGGTCGA---TCGTG-GTGCAAACAACCAACCCCGGGCGCAG-AAAC-GCGCCAAGGAAAACGT-AAAAWGGATTTGTC-TGCCCCCCGT
12      CGTCGACCACCGGTCGC---TCGTC-GTGCAAACAACCAACCCCGGGCGCAG-AAAC-GCGCCAAGGAAAACGA--AAAAAGGATGGCC-TGCCTCCCGT
13      CGTGTACCACCGGTCGC---TCGTC-GTGCAAACAACCAACCCCGGGCGCAG-AAAC-GCGCCAAGGAAAACGA--AAAAAGGATGGCC-TGCCTCCCGT
14      CG----KCACCGGCCGC---TC-TCCGTGCAAACAACCAACCCCG---GCAT-AAAC-GCGCCAAGGAAAACGA--AAAAAGGATGGCC-TGCCTCCCGT
15      CGTCGGCCAACGGCCGC---TTGTCCSTGCAAACAACCAACCCCGGGCGCATTAAAC-GCGCCAAGGAAAACGA--AAAAAGGATTGCC-TGCCCTCCGT
16      CGTGCACCACCGGTCGT---TCGTC-GTGCAAACAACAACCAACCYYGGCAG-AAAC-GCGCCAAGGAAAACTA-AAAAAAGGATTGCC-TGCCTCCCGT
17      CGTCGACCACCGGTCGT---TCGTC-GTGCAAACAACCAACCCCGGGCGCAT-AAACGACGCCAAGGAAAACGA-AAAAAAGGATTGCC-TTCCTCC-GT
18      CGTCGACCACCGGTCGT---TCGTC-GTGAAAAAAACC-ACCCCGGGCGCAG-AAAC-GCGCCAAGGAAAACCA--AAAAAGGATTGCC-TGCCTCCCGT
19      CGTCGACCACCGGTCGT---TCGTC-GTGCAAACAACCAACCCCGGGCGCAT-AAAC-GCGCCAAGGAAAACGA--AAAAAGGACTGCC-TGCCTCCCGT
20      CGTCGACCACCGGTGCC---TCGTC-GTGCAAACAACCAACCCCGGGCGCAG-AAAC-GCGCCAAGGAAAACGA--AAAAAGGATGGCC-TGC-TCCCGT
21      CGTCGGCCACCGGTCGC---TCG-CCGTGCAAACAACCAACCCCGGGCGCAT-AAAC-GCGCCAAGGAAAACCA--AAGAAGGATGGCC-TGCCTCCCGT
22      CGTCGACCACCGGTCGC---TCGTC-GTGCAAACAACCAAACCCGGGCGCAG-AAAC-GCGCCAAGGAAAACGA--AAAAAGGATGGCC-TGCCTCCCGT
23      CGY-GGCCATCGGTCGC---TCGTC-GTGCTAACAACAAACCCCGGGCGTGG-AAA--GCGCCAAGGAAAATGA--AAAAAGGATGGCC-TGCTTGCCGG
24      CGGCGGCCACCGGTCGT---CCGTC-GTGCTAACAACAAACCCCGGGCGTCGAAAA--GCGCCAAGGAAAATTT--AAGAAGGATTGGTCTGCTTGCTGA
25      CCACGGCCACCGGTCAC---AAGTC-GTGCTAACATCAAACCCCGGGCGCATTAA---GTGCCAAGGAAAACG---AAAAAGGATGGCK-TGCCTACC-A
26      CGTCGACCATTGGTCGC---CCGTC-GTGCACATAAACAACCCCGGGCGCGT-AAAA-GCGCCAAGGAAAACAA--GAAAGGGATTGCC-TGCCTCTCGA
27      CGCCGACCACCGGTCGC---TCGTC-GTGCAAACAAACAACCCCGGGCGCAG-AAAA-GCGCCAAGGAAAACGA-AGAAAGGGATGGCT-TGCCTCTCGA
28      CGACGGCCACCGGTCGC---TNGTC-GTGCTAACAACAAACC---GGCGCATTAAA--GCGCCAAGGAAAACAA--AAAAAGGATGGCC-TGCC---NNN

               *     **      *    *        *     **     *       »ITS2          **                     ***
1       CATGCCG-TACGC-GGTGTGAGGCGG-AGGAGCAC-GGGCG-CCTAAA-GGAACA ATCGCGTCGCCCCCCC-AACACCG-TGCATGAAGCCATG-TCGGT (300)
2       ATCGCCG-TACGCTGGTGTGAGGCGG-AGGAGCACAGGGCATCCTAAATGGAACA ATCGCGTCGCCCCCC--AACACCGCTGCATGAAGCCAGT-CCGGT
3       CGTGCCGTAATGC-CGTACGC-ACGGGAGGACCAC-GTACG-CCTAAA-GAAACA ATGTCGTCGCCCCCC--AACACCG-TGCATGAAAAATTG-TCGGT
4       CGTGTCG-TATGC-GGTGCGC-ACGGGAGGATCAC-AGATG-CCTAAA-GAAACA ATCGCGTCGCCCCCCC-AACACCG-TGCATGAAACATTG-CATGT
5       CGTGCCG-TATGC-GGTGCGC-ACGGGAGGATCAC-AGACG--CTAAA-GAAACA ATCGCGTCGCCCCCCC-AACACCG-TGCATGAAACATTG-CCGGT
6       CACCCCG-TACGC-GGTGTGC-ACAG-AAGACCAC-GG-CG-CCTAAA-GAAATA ATCGCGTCGCCCCCCCTAACACCG-TGCATGAAATCGTG--CGGT
7       CGCCCCG-TACGC--GTGTGC-ACAGAAGGACCAC-GGGCG-CCTAAA-GAAACA ATCGCGTCGCCCCCCC-AACACCG-TGCATGAAATCGTG-CCGGT
8       CGTGCCG-TACAC-GGTGTGC-ACGGGAGGATCAC-GGGAG-CCTAAA-GAAACA ATTGCGTTCGCCCGCC-AACACCG-TGTATCAAACATTG-CCGGT
9       CGTGCCG-TACGC-GGTGTGC-ACGGGAGGATCAC-GGGCG-CCTAAA-GAAACA ATCGTTTC-----CCC-AACACCG-TGTATKAAACATTG-CCGGT
10      GCTACCGATTCGT-------CGACGG--AGATAAC--GACG--CTAAA-GAAACA ATCGCGTCGCCCCCCC-AACACCG-TGCATGAAACACTG-CCGGT
11      CGTGCCG-TATTC-GGTTCGC-ACGGGAGGATCAC-GGACG--CTAA--GAAACA ATCGCGTCGCCCCCCC-AACACCG-TGTATGAAACATTGTCCGGT
12      AGTGCCCATACGC-GGTGTGCGACGGGAGGTACAC-GGGCG-CCTAAA-GAAACA ATCGCGTCGCCCCCCC-AACACCG-TGCATGATATCATG-CCGGT
13      AGTGCCG-TACCA-GGTGTGC-GCGGGAGGATCAC-GGGCG-CCTAAA-GAAACA ATCGCGTCGCCCCCCC-AACACCG-TGCATGATATCATG-CCGGT
14      CGCGCCG-TASGC-GGTGCGT-GCTGGAGGATCAC-GGGCG-CCTGAA-GAAACG ATTGCGTCGCCCCCC--ACACCCG-TGCATGAAATCATG-CCGGT
15      CGCGCCG-TACGC-GGTGCGT-CCTGGAGGATCAC-GGGCT-TCTGAA-GAAACA ATCGCGTCGCCCCCCC-ACACCCG-TGCATGAAATCATG-CCGGT
16      CGTGCCG-TACGC-GGTGTGC-ATGGGAGGATCAC-GGGCG-CCTAAA-GAAACA AT-GCGTCGCCCCCCCCAACACCG-TGCATCAAATCATC-CCGGT
17      CGTGCCG-TACGCAGGTGTGC-ATGGGAGGATCAC-GGGCG--CTAAA-GAAACA ATCGCGTCGCCCCCCC-AACACCG-TGCATGAAATCATG-CCGGT
18      CGTGCCG-TATGC-GGTGTGC-ATGGGAGGATCAC-GGGCG-CCTAAA-GAAACA ATCGCGTCGCCCCCC--AACACCG-TGCATGAAATCATA-CCGGT
19      CGTGCCG-TACGC-GGTGTGC-ATGGGAGGATCAC-GGGCG-CCTAAA-GAAACA ATCGCGTCGCCCCCCC-AACACCG-TGCATGAATTGATG-CCGGT
20      CGTGCCG-TACGC-GGTGTGC-ACGG-AGGATCAC-GGGCG-CCTAAA-GAAACA ATCGTAAGACCCCCCC-AACACCG-TGCATGAAACATTG-CCGGT
21      CGTGCCG-TACGC-GGTGCGC-GATGGAGGATCAC-GGGCG-CCTGAA-GAAACA ATCGCGTCGCCCCCCC--ACACCG-TGCATGAAATCGTG-CCGGT
22      CGTTCCG-TACGC-GGTGTGC-ACGGGACCATCAC-GGGCG-CCTAAA-GAAACA ATCGCGTCGCCCCCCC-AACACCG-TGCATGAAACATTG-CCGGT
23      CTT--CGGTACGC-GGCCTGT-TCGGTAGGATCAC-GGGCG-CCTAAA-GAAACA ATCGC-TCGCCCCTCC-AAACCCG-TGCACGACATCATG-CCGGA
24      CACGCCG-TTGGC--GCTTGT-TCGGTAGGATCAC-GGGCA-CCTAAA-GAAACA ATCGCGTCGCCCCTCC-AAC-CCG-TGCACGAAATCATG-CCGGT
25      CATTCCG-TACGC-GGTGTGTTTGGTAGGATCAT-GGGCA-CCTAAA-GAAACA ATCCTGTCGCCCCCA--AAC-CCG-TCGACGACGTCGTG-CTGGA
26      TGCGCCG-TACGC-GGAGTGC-ACGGGAGGGAAAA-AGGCG-CCTGAAT-AAACA ATCGCGTCGCCCCCC--AACCTCG-TGCGTTGACTCGT--ACGGG
27      CGCGCCG-TACGC-GGAGTGC-ACGGGAGGGCAACATGGCG-CCT-AA-GAAACA ATCGCGTCGCCCCCC--AACCTCG-TGCGTTAACTCGT--ACGGG
28      NACATCCGTACGC-GGC-TGT-AAGATAGGATCAC-GGGAG-CCNAAA-GAAACA ATTGCGTCGCCCCCC--AACCCTA-TGCACGGCAACGTG--TTGA
```

Fig. 1. The aligned sequences of ITS1 and ITS2 of *Gentiana* and its outgroups. See Table 2 for species names. The IUPAC (IUB) code was followed for nucleotide ambiguity, i.e., K = G/T, S = C/G, R = A/G, Y = C/T, M = A/C, W = A/T, N = A/C/G/T; − = gap. * = the 51 gap sites being considered in the phylogenetic analyses (see text).

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           *            *                                                  *
1     -GGTATGAGGGGC-GGATATTGGCTTCCCGTG----CTCCGA-CGCGGCTGGCCTAAACGCAAGT-CCCTTGCG-ACGGACACGACGA-CAAGTGGTGGT (400)
2     -CGTATGAGGGGC-GGATAGTGGCTTCCCGTG----CTCCGG-TGCGGCTGGCCTAAATGCAAGT-CCCTTGCG-ACGGACACGACGAACAAGTGGTGGT
3     -TGTCGGAGGGGC-GGATAATGGCTTCCCGTGGTCGTTTCGGCTGCGGCTGGCGTAAATGCAAGT-CCCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
4     -TGTCGTCGGGGC-GGATATTGGCTTCCCGTA----CTTCGG-TGCGGCTGGCCTAAATGCAAGT-CCCTTGCG-GCGGACACGACGA-CAAGTGGTGGT
5     -TGTCGGAGGGGC-GGATATTGGCTTCCCGTG----CTTCGG-TGCGGCTGGCCTAAATGAAAGT-CCCTTCCG-ACGGACACGACGA-CAAGTGGTGGT
6     -CGTCGGAGGGGCCGGATATTGGCTTCCCGTG----CTCCGG-TGCGGCTAGCCTAAATGCAAGT-CCCTTACG-ACGGACACGACGA-CAAGTGGTGGT
7     -CGTCGGAGGGGC-GGATATTGGCTTCCCGTG----CTCCGG-TGCGGCTAGCCTAAATGCAAGT-CCCTTACG-ACGGACACGACGA-CAAGTGGTGGT
8     -TGTCGGAGGGGC-GGATATTGGCTTCCTGTG----CTTCGG-TGCGGCTGGCCTAAATGCAAGT-CCCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
9     -TGTCGGAGGGGC-GGATATTGGCTTCCCGTG----CTTCGG-TGCGGCTGGCCTAAATGCAAGT-CCCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
10    --GTCGGAGGGGC-GGATATTGGCTTCCCGTG-----CCCGT-TGCGGCTGCCCTAAATGCAAGTGCCCTTWCGTACGGACACGACGA-CAAGTGGTGGT
11    TCGTCGGAGGGGC-GGAGATTGGCTTCCCGTG----CTTCGG-TGCGGCTGGCCTAAATACGAGT-CCCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
12    -CGTCGGAGGGGC-GGATATTGGCTTCCCGTG----CTTCGG-TGCGGCTGCCCTAAACGCAAGT-CCCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
13    -CGTCGGAGGGGC-GGATATTGGCTTCCCGTG----CTTCGG-TGCGGCTGGCCTAAACGCAAGTTCCCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
14    -CGTCGGAGGGGC-GGATATTGGCTTCCCGTG----CTTCGG-TGCGGCTGGCCTAAATGGAAGT-CCCTTGCG-GCGGAGACGACGA-CAAGTGGTGGT
15    -CGTCTGAGGGGC-GGATATTGGCTTCCCGTG----CTTCGG-TGCGGCTGGCCTAAATGGGAGT-CCCTTGCG-GCGGACACGACGA-CAAGTGGTGGT
16    -CGTCGGAGGGGC-GGATATTGGCTTCCCGTG----CTTCTG-TGCGGCTGGCCTAAATTTAAGT-CCCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
17    -CGTCGGAGGGTG-AGATACTGGCTTCCCGTG----CTTCGG-TACGGCTGGCCTAAATTCAAGT-CCCTTGCG-ACGGCGACGACGA-CAAGTGGTGGT
18    -CATTGGAGGGGC-GGATATTGGCTTCCCGTG----CTTCAG-TGCGGCTGGCCTAAATTCAAGT-CCCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
19    -CGTCGGAGGGGC-GGATATTGGCTTCCCGTG----CTTCGG-TGCGGCTGGCCTAAATTCAAGT-CCCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
20    -TGTCGGAGGGGC-GGATATTGGCTTCCCGTG----CTTCGG-TGCGGCTGGCCTAAATGCAAGT-CCCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
21    -CGTCGGTGGGGC-GGATATTGGCTTCCCGTG----CTTCGG-TGCGGCTGGCCTAAATGCGAGT-CCCTTGCG-GCGGACACGACGA-CAAGTGGTGGT
22    -TGTCGGAGGGGC-GGATATTGGCTTCCCGTG----CTTCGG-TGCGGCTGGCCTAAATGCAAGT-CCCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
23    -CGTCGAAGGGGC-GGATATTGGCTTCCCGTG----CTTCGG-TGTGGCTGGCCTAAATTCAAGT-CTCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
24    CAGTCGGAGGGGC-GGATATTGGCTTCCCGTG----CTTCGG-TGCGGCTGGCCTAAAT-CAAGT-CTCTTGTG-ACGGACACGACGA-CAAGTGGTGGT
25    -TGTCAGAGGGGC-GGATATTGGCTTCCCGTA----CTTCGG-TGCGGTTGGCCTAAATGCAAGT-CTCTTCTG-ATGGCGACGACGA-CAAGTGGTGGT
26    TGACATGAGGGGGCGGATGATGGCTTCCCGTG----TCTATT-CGCGGCTGGCCTAAATGTGAGT-CCCTTGCG-ACGGACGTGACGA-CAAGTGGTGGT
27    TGGCGTGAGGGGGCGGAAAATGGCTTCCCGTG----CTTGGC-CGCGGCTGGCCTAAATGCGCGT-CCCTTGCG-ACGGACGCGACGA-CAAGTGGTGGT
28    CAGT-TGAGGGGC-GGATATTGGC--NNNGTG----CTANGG-TGCGGCTGGCCTAAATGCAAGT-CTCTTGCG-ACGGACACGACGA-CAAGTGGTGGT
```

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      *    *                  * *         **   **              *              **              *
1     TGATTTACTTAGCTAAG-GTGTTGTC-GCGCGCTGCCCT-GTC-GGACGAGGAGACTTACTTGACCCTTATGCAT-ACGTCG-TCACGACGTCTGCGACG ACCGC (505)
2     TGATTT-CTCAGCTAAG-GTGGTGTC-GRGCGCTGCCCC-GTC-GGACGAGGAGACTTACTTGACCCTTATGCAT-GCGTCG-TCACGACGTCTGCCACG ACCGC
3     TGATTACCTCAACTCAG-GTGCTGTT-GGACGTTGACCC-GTC-GGATGAGGAGACTTCCTTGACCCTAATGCA--ACGKCG-TCACGACGCCTGCCACG ACCGC
4     TGATTACCTCAACTCAG-TTGCTGTC-GTACGTTGACCC-GTC-GGATGAGGAGACTTCCTCGACCCTAATGCAT-GCATCG-TCACGAGGCGTGCCACG ACCGC
5     TGATTACCTCAACTCAG-TTGCTGTC-GCACGTTGACCC-GTC-GGACGAGGAGACTTCCTCGACCCTAATGCAT-GAGTCT-TCACGACGCATGCCACG ACCGC
6     TGGTTTACTCAACTAAG-GTGCTGTC-GCGCGCTGCCCC-GTC-GGATGAGGAGACTTACTTGACCCTGATGCAT-ACGTCG-TCACGACGTCTGCGACG ACCGC
7     TGATTTCCTCAACTAAG-GTGCTGTC-GCGCGCTGCCCC-GTC-GGACGATGAGACTAACTTGACCCTGATGCAT-ACGTCG-TCACGACGTCTGCCACG ACCGC
8     TGATTGCCTCAACTAAG-GTGTTGTC-GCGCGTTTCCCC-GAC-GGATGAGGAGACTTCCTTGACCCTAATGCAACGTGTCG-TCACGACGCCTGCGACG ACCGC
9     TG-TTGCCTCAACTAAG-GTGCTGTC-GGTAGTTGCC---GTC--GATGAGGAGACTT-CTTGACCCT-ATGCAGCATGT-----ACGACGT-TGCCACG ACCGC
10    TGATTACCTCAACTCAG-GTGCTGGG-GAACGTTAAACCCGTC-GGATGAGGAGACTTCCTTGACCCTCATGCAT--CGCCG-TCACGACGCSTGCCACG ACCGC
11    TGATTACCTCAACTAAG-GTGCTGTC-GTGCGTTGACCC-GTC-GGATGAGGAGACTTCCT-GACCCTAATGCAT-GCATCG-TCACGACGCGTGCCACG ACCGC
12    TGATTTT-TCAACTAAGGGTGCTGTC-GCACGTTGCCCC-GTC-GGATGAGGAGACTTCCTTGACCCTAACGCAA-GCGTCG-TCACGACGTCTGCCACG ACCGC
13    TGATTTT-TCAACTAAG-GTGCTGTC-GCGCGTTGCCCC-GTC-GGATGAGGAGACTTCCTTGACCCTAACGCAA-GCGTCG-TCACGACGTCTGCCACG ACCGC
14    TGATTGCCTCGACTAAG-GTCGTGTC-GCGCGCTGCCCC-GTC-GGATGAGGAGACTTACTTGACCCTGATCTCGAGCGTCG-TCACGACGCCTGCCACG ACCGC
15    TGATTGCCTCGACTAAG-GTGGTGTC-GCGCGCTGCCCC-GTC-GGACGAGGAGACTTACTTGACCCTGATGCAT-GCGTCG-TCACGACGCCTGCCACG ACCGC
16    TGAATTACTCAACTAAG-GTCGTGTC-GCGCGTTGCCCC-GTC-GGATGAGGAGACTTCTTT-ACCCTAACGCAT-GTGTCG-TCACGACGTATGCCACG ACCGC
17    TGAATTACTCAACTAAG-GTGCTGTC-GCGCGTTGCCCCTGTCAGGATGAGGAGACTTCTTTGACCCTAACGCAT-GTGTCG-TCACGACGTATGCCACG ACCGC
18    TGAATTTCTCAACTAGG-GTGCTGTC-GCGCGTTGCCCC-GTC-GGATGAGGAGACTTTTTTGACCCTAACGCATGCTGTCG-TCATG-CGTATGCCACG ACCGC
19    TGAATTACTCAACTAAG-GTGCTGTC-GCGCGTTGACCC-GTC-GGATGACCAGACTTCTTTGACCCTAACGCAT--TGTCG-TCACGACGTATGCCACG ACCGC
20    TG-TTGCCTCAACTAAG-GTGCTGTACGCTCGTTGCCCC-GTC--CATTAGGAGACTTCCATGACCCTAATGCAC-GCGTTG-TCACGACGCCTGCCACG ACCGC
21    TGATTGCCTCAACTAAG-GTGCTGTC-GCGCGCTGCCCC-GTC-GGACGAGGAGACTTACTTGACCCTGATGCAC-GCGTCG-TCACGACGTCTGCCACG ACCGC
22    TGATTGCCTCAACTAAG-GTGCTGTC-GCTCGTTGACCT-GTC-GGATGAGGAGACTTCCATGACCCTAATGCAC-GCGTCG-TCACGACGCCTGCCACG ACCGC
23    TGATTGCCTCAACTAAG-GTCGTGTC---GCGCTGCCC--ATC-GAACGGAGAGACTACGTAGACCCAGATGCAA-GCATCG-TCACGATGCCTGCTACG ACCTC
24    TGATTGCCTCAACTAAG-GTGCTGTCGTCGCGCTGCCCC-ATC-GAACGGAGAGACTCTGTAGACCCTGATGCAA-GCATCGATCACGACGCTTGCTACG ACCTC
25    TGATTGCCTCAACTAAG-GTGCTGTCGT-GCGTTGCACT-GTC-GAACGAAGGGACTCCGTAGACCCTGATGCAT-GCGTCG-TCATGATGCCTGCTACG ACTGC
26    TGATTGCCTCAACTAAG-GTGCTGTCGT-TCTACGACC--GTC-GAATTAGGAGACTCTC-CGACCCTAATGCAC-GCGTCA-TTACGCTG---GCTACG ACCGC
27    TGATTGCCTCAACTAAG-GTGCTGTC-GCGCGACGCCC--GTC-GATTGAGGAGACTCCC-CGACCCTGATGCAT-GCGTCG-TCACGACGTCTGCTACG ACCGC
28    TGATTACCTCAACTAAG-GTCGTGTC-GCGCGCTGCCN--GTG-GAACGGAGAGACTNNATTGACCCTGATGCAT-GCGTCG-TCATGACGTATGCCACG ACCGC
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Fig. 1. Continued.

section *Frigidae* s. l. (including sections *Monopodiae, Isomeria, Microsperma,* and *Phyllocalyx*) agree with some classifications but conflict with others. However, the positions of section *Stenogyne* and of *G. asclepiadea* do not agree with any existing classification: *G. asclepiadea,* which has either been included in the section *Pneumonanthe* or segregated as a distinct genus (*Dasystephana*), nested as the sister group of the section *Gentiana*; section *Stenogyne,* which was suggested as a primitive section of the genus *Gentiana,* nested outside the genus as part of the *Crawfurdia-Tripterospermum* clade—the suggested sister group of the genus *Gentiana.* Both of those unusual groupings were supported by high bootstrap values (93% and 100%, respectively).

Optimization of chromosome numbers on the ITS phylogeny suggested $2n = 20$ or $2n = 26$ as the plesiomorphic condition for the clade section *Chondrophyllae* s. l., and $2n = 26$ unequivocally as the plesiomorphic condition for the clade comprising sections *Chondrophyllae* s. l., *Cruciata, Pneumonanthe* and *Frigidae* s. l. (Fig. 2).

Results under different schemes of character state weighting—Weighting transversions two times over transitions, the combined data of ITS1 and ITS2 with gaps coded as missing generated three trees of 868 steps. The strict consensus of the three trees (not shown) was identical to the strict consensus of the unweighted trees (Fig. 2) except for better resolution in the clade of section *Chondrophyllae* s. l. (*G. aristata* grouped together with *G. crenulato-truncata* as the sister clade of *G. boryi* and *G. pyrenaica*). When the weighting ratio of transversions vs. transitions was set at 10:1, six most parsimonious trees (3 081 steps) were generated. The strict consensus (not shown) was less resolved but was topologically consistent with the 2:1 strict consensus tree, except for the collapse of the basal branches differentiating the three European sections, and the positions of *C. tibetica* and *T. cordatum* relative to section *Stenogyne.* These different positions of *C. tibetica* and *T. cordatum* relative to section *Stenogyne* were also observed in the strict consensus of the three equally parsimonious trees identified at 5:1

TABLE 3. The accession number of EMBL Nucleotide Database, length, and G + C content of the nucleotide sequences of ITS1, ITS2, and ITS1 + ITS2 of *Gentiana* and its outgroups.

	ITS1			ITS2			ITS1 + ITS2	
Taxon	Accession number	Length (bp)	G + C (%)	Accession number	Length (bp)	G + C (%)	Length (bp)	G + C (%)
G. bavarica	Z48094	230	61.3	Z48075	231	59.7	461	60.5
G. verna	Z48093	237	57.8	Z48092	231	61.5	468	59.6
G. aristata	Z48100	229	52.8	Z48116	234	58.5	463	55.7
G. boryi	Z48111	230	59.1	Z48118	231	58.4	461	58.8
G. pyrenaica	Z48068	229	60.2	Z48087	231	58.9	460	59.6
G. alpina	Z48072	225	60.2	Z48073	232	60.4	457	60.2
G. clusii	Z48097	232	61.2	Z48077	231	60.6	463	60.9
G. macraphylla	Z48067	228	59.8	Z48086	232	58.6	460	59.2
G. straminea	Z48070	227	60.1	Z48091	216	56.4	443	58.9
G. crenulato-truncata	Z48098	219	58.2	Z48079	231	60.1	450	59.0
G. haynaldii	Z48065	228	59.9	Z48085	232	59.4	460	59.8
G. algida	Z48142	230	61.3	Z48117	231	60.6	461	61.0
G. frigida	Z48063	230	61.4	Z48084	231	60.7	461	61.0
G. lutea	Z48122	221	61.1	Z48119	231	62.8	452	61.9
G. punctata	Z48066	236	59.9	Z48088	231	64.1	467	62.0
G. depressa	Z48062	231	57.5	Z48081	230	57.2	461	57.7
G. urnula	Z48071	232	58.2	Z48090	233	58.0	465	58.1
G. delavayi	Z48099	228	56.1	Z48080	230	57.2	458	56.6
G. callistantha	Z48095	227	58.4	Z48078	230	58.5	457	58.4
G. affinis	Z48061	229	59.8	Z48074	230	59.1	459	59.4
G. asclepiadea	Z48083	228	62.1	Z48076	230	64.6	458	63.4
G. parryi	Z48096	230	58.5	X85370	231	60.3	461	59.4
G. gentilis	Z48064	227	57.1	Z48082	227	58.4	454	57.7
G. rhodantha	Z48069	228	56.2	Z48089	232	58.7	460	57.4
C. tibetica	Z48145	228	53.5	Z48123	229	57.2	457	55.4
Gs. grandis	Z48105	229	60.3	Z48130	227	58.6	454	59.7
M. stylophorus	Z48109	230	63.5	Z48137	229	65.1	459	64.3
T. cordatum	Z48113	223	52.9	Z48140	226	57.1	449	55.0

weighting, which was in all other respects identical to the 2:1 strict consensus tree.

Results under different gap treatments—When the 51 potentially informative gap sites (indicated by * in Fig. 1) were excluded from phylogenetic reconstruction, unweighted parsimony analysis generated eight trees of 490 steps (CI = 0.576, RI = 0.616). The strict consensus (not shown) of the eight trees had almost the same topology as that of the basic analysis (Fig. 2), except that the clade comprising *G. affinis* and *G. parryi* collapsed. When the 51 gap sites were converted into 36 multistate characters, unweighted analysis generated six trees of 638 steps (CI = 0.578, RI = 0.576). The strict consensus (not shown) was largely the same as that of the basic analysis, except for lower resolution in the section *Frigidae* s. l. clade (*G. urnula, G. callistantha,* and *G. depressa* collapsed) and a slightly different resolution in the section *Chondrophyllae* s. l. clade (*G. haynaldii* grouped with *G. crenulato-truncata* as sister of *G. boryi* and *G. pyrenaica,* and *G. aristata* nested out as sister clade of the above four species). When gaps were simply coded as a fifth character state, six trees of 687 steps (CI = 0.591, RI = 0.569) were generated whose strict consensus (not shown) was exactly the same as that generated from the second scheme where the 51 gap sites were converted into 36 new multistate characters.

The relative informativeness of ITS1 and ITS2—Two subsets of sequences, ITS1 and ITS2, were analyzed to examine the relative informativeness of the two spacers. ITS1 generated 267 trees of 282 steps (CI = 0.599, RI = 0.581) in an unweighted analysis with gaps coded as missing. The strict consensus was poorly resolved (not shown), but most sectional groups can be recognized. In contrast, ITS2 generated nine parsimonious trees of 248 steps (CI = 0.565, RI = 0.644). The strict consensus (not shown) was topologically consistent with and almost as highly resolved as the strict consensus generated from the combined data of both ITS1 and ITS2. This result indicated that even though sequence divergence appears to be higher in ITS1, ITS2 was far more informative than ITS1, concerning both the number of most parsimonious trees and the resolution of the strict consensus tree.

DISCUSSION

Infrageneric phylogeny of the genus Gentiana and existing classifications—The infrageneric phylogeny and classification of the genus *Gentiana* have been much debated. Previous phylogenetic considerations were based mainly on the European taxa. Carbonnier, Massias, and Molho (1977) and Müller (1982), based on their phytochemical and karyological studies and perhaps also on the long perennial hemicryptophyte biotype, suggested that section *Gentiana* represents the primitive and ancestral type of the genus, that section *Pneumonanthe* was derived from it, and in turn the other sections such as *Cruciata, Frigidae, Calathianae,* and *Ciminalis* originated from section *Pneumonanthe.* The ITS phylogenetic hypothesis, however, is not consistent with such suggestions, because, as can be seen from Fig. 2, all of the European species of *Gentiana* together do not form a monophyletic group. The ITS phylogeny suggests an an-

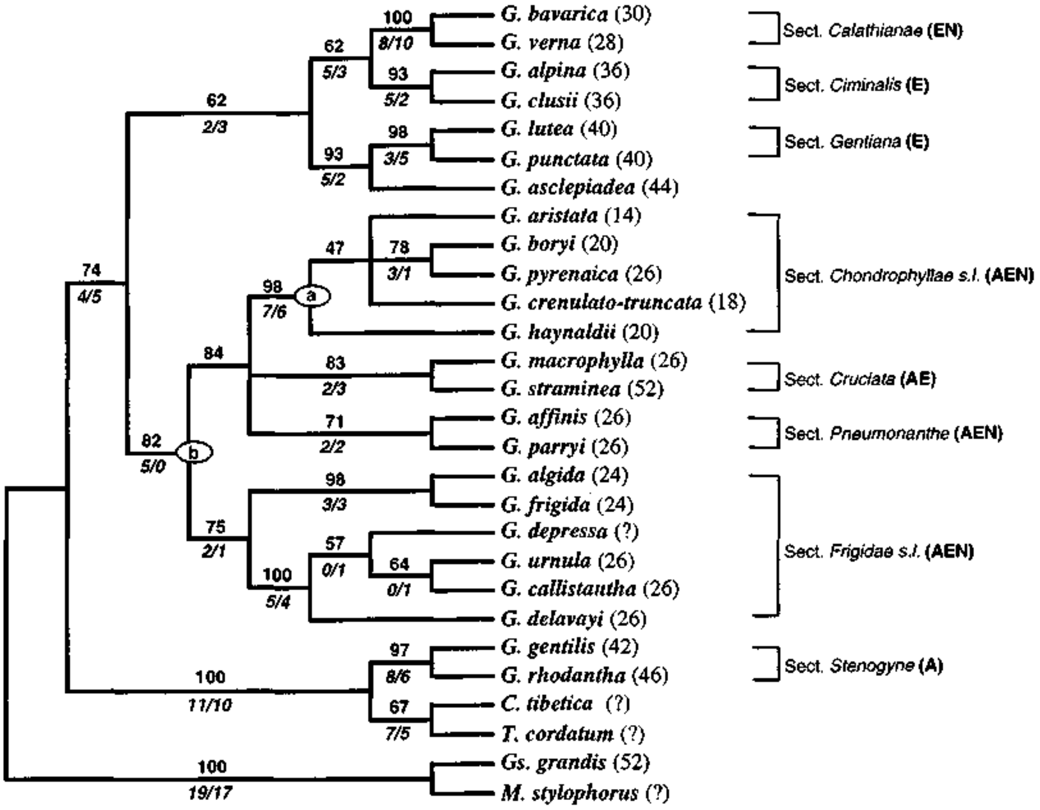

Fig. 2. The strict consensus tree of the six equally parsimonious trees generated from four rounds (SIMPLE, CLOSEST, ASIS, and 100 replicates RANDOM) of heuristic searches on the combined sequence data of ITS1 and ITS2 with gaps coded as missing. Tree length = 535, CI = 0.578, RI = 0.606. The sectional classification is also shown. The numbers above the internal branches represent the bootstrap estimates from 500 replicates of heuristic searches. The numbers under the internal branches are the numbers of character state changes (transition/transversion) forming the corresponded consensus branches. The "a" and "b" in the circles represent the optimized chromosome numbers for the corresponding nodes: "a" representing $2n$ = 20/26 and "b" representing $2n$ = 26. The numbers in the brackets following each species represent the somatic chromosome number of the relevant species (? means chromosome numbers not available). The boldface letters following each section indicate the geographical distribution of the relevant sections: A = Asia, E = Europe, and N = North America.

cient divergence of the three European endemic sections *Calathianae, Ciminalis,* and *Gentiana* plus *G. asclepiadea* from the remainder of the genus. This divergence, to a certain extent, is supported by phytochemical evidence (but see below for *G. asclepiadea*): among the species investigated, only these three endemic European sections contain xanthone-O-glycosides, whereas the other sections contain mangiferin (xanthone-C-glycosides) (Goetz, Hostettmann, and Jacot-Guillarmod, 1976a, b; Carbonnier, Massias, and Molho, 1977; Massias, Carbonnier, and Molho, 1982; Mészàros, 1994). In the ITS phylogeny, section *Gentiana* is not basal, and although it is sister to sections *Ciminalis,* and *Calathianae,* it is only distantly related to sections *Cruciata* and *Frigidae* s. l.

Section *Frigidae* s. l., corresponding to the sectional circumscription of Smith (1970), was divided into sections *Frigidae* s. str., *Monopodiae, Isomeria, Microsperma,* and *Phyllocalyx* by Ho and Liu (1990). Except for the section *Phyllocalyx,* which is missing from our samples, our ITS phylogeny supports the monophyly of this entire group and two further well-supported clades: section *Frigidae* sensu Ho and Liu (=*Frigidae* s. str.) and all the others together, which also seems to be relevant to chromosome number differentiation (Fig. 2). However, the relationships and phylogenetic position of *G. frigida* inferred from ITS sequences here is significantly different from that suggested by cpDNA sequences (Gielly, 1994; L. Gielly and P. Taberlet, Laboratoire de Biologie des Populations d'Altitude, Université Joseph Fourier, France, personal communication). In the cpDNA tree of European *Gentiana, G. frigida* was sister to all other members of the genus. This placement, which also is contradictory to morphological conclusions, could be due to relatively poor sampling of their ingroup. As demonstrated above, the European *Gentiana* species together do not form a monophyletic group, and the closest relatives of *G. frigida* are found outside Europe and therefore were not included in their analysis, which made the species *G. frigida* somewhat distinct from others. Nevertheless, we should not disregard such incongruence between cpDNA phylogeny and nuclear DNA phylogeny, because it may tell us something of biological interest, such as hybridization or lineage sorting.

A closer relationship among sections *Pneumonanthe, Cruciata,* and *Chondrophyllae* s. l. than previously hypothesized has been suggested by ITS sequences. Section *Chondrophyllae* s. l. was shown to be a monophyletic group but further splitting of this group into two sections, *Chondrophyllae* s. str. and *Dolichocarpa,* advocated by Ho (1985), was not supported by the ITS phylogeny.

G. asclepiadea has been usually included in the section *Pneumonanthe* (Nilsson, 1967; Tutin, 1972). It is karyologically distinct from all other members of that section, with $2n = 44$ (Favarger, 1949; Skalinska, 1951; Majovsky, 1974; Gagnidze, Küpfer, and Yuan, 1992), while all the other members uniformly are $2n = 26$. It was based on its distinctive chromosome number that Löve and Löve (1976) elevated the species to a new genus, *Dasystephana.* Pringle (1977) also questioned the sectional position of the species but did not elaborate. The ITS phylogeny suggests its closer relationship with section *Gentiana,* although morphologically they are significantly different. This unexpected relationship is also suggested by cpDNA data (Gielly, 1994; L. Gielly and P. Taberlet, personal communication). Phytochemical evidence contradicts this conclusion somewhat. As mentioned above, the three European sections, including *Gentiana,* contain xanthone-O-glycosides (Carbonnier, Massias, and Molho, 1977; Mészàros, 1994), and these compounds are lacking in *G. asclepiadea,* which has only mangiferin (xanthone-*C*-glycosides) (Goetz, Hostettmann, and Jacot-Guillarmod, 1976a). Since xanthone-*C*-glycoside is apparently plesiomorphic in the entire genus, the position of the species in the ITS phylogeny, therefore, requires either a reversal or parallel gains of xanthone-O-glycoside in section *Gentiana* and sects. *Ciminalis* + *Calathianae.*

Little is known about section *Stenogyne,* although it is generally accepted as part of *Gentiana* (Smith, 1965, 1970; Pringle, 1978; Ho and Liu, 1990). It contains 14 species highly restricted to southwestern China and the adjacent area. Close relationships between the section and the genera *Crawfurdia* and *Tripterospermum* have been suggested (Smith, 1965; Löve and Löve, 1976; Ho and Liu, 1990). Smith (1965) "denied any direct connection between this somewhat mysterious group and *Crawfurdia* or *Tripterospermum,*" but still claimed that "though sharply delimited from the genera *Crawfurdia* and *Tripterospermum* section *Stenogyne* seems to have a closer affinity with them than with other *Gentiana* sections." Löve and Löve (1976) recommended the transfer of this section to the genus *Tripterospermum* tentatively as a subgenus (genus *Tripterospermum* subgen. *Stenogyne*), as they had no karyological information to make further delimination. However, Ho and Liu (1990) maintained it as a section of *Gentiana,* claiming that the section represented the primitive type of the genus *Gentiana,* through which the genus showed affinity with *Crawfurdia* and *Tripterospermum.* Its karyology supports a distinct position from *Gentiana* (Yuan and Küpfer, 1993b). If the ITS phylogeny is correct, inclusion of this section in the genus *Gentiana* makes the whole genus paraphyletic unless *Crawfurdia* and *Tripterospermum* are also included in *Gentiana.* It seems more reasonable instead to exclude this section from *Gentiana* and classify it as a distinct genus. Together with *Crawfurdia* and *Tripterospermum,* it appears to form a monophyletic sister group to *Gentiana.* However, being aware of the insufficient sampling of this section, we do not intend to make further taxonomic treatment here. Further sampling of both this section and the genera *Crawfurdia* and *Tripterospermum* may help to clarify the taxonomic position of the section.

In general, with the exceptions discussed above, the ITS phylogeny is highly congruent with most classifications (Fig. 2). It is in particular quite close to the morphologically defined sections proposed by Smith (1970). Pringle's (1978) classification was principally the same as Smith's, except for some nomenclatural changes and the recognition of the section *Kudoa,* which was considered a part of section *Frigidae* by Smith (1970) or of section *Monopodiae* by Ho and Liu (1990). Regrettably, no species of this group has been examined in our present investigation, and thus the validity of section *Kudoa* cannot yet be evaluated. The classification of Ho and Liu (1990) is convenient to use, but their further recognition of some sections, such as *Dolichocarpa, Monopodiae,* and *Microsperma,* etc., were not supported by the ITS phylogeny. Based on the ITS phylogeny, it seems advisable to keep the original circumscription of section *Chondrophyllae* s. l. and to treat section *Frigidae* s. l. as two groups: section *Frigidae* s. str. and a section to include all the other members. However, the final decision on the latter case can be made only when a more detailed analysis on this group is completed, which is currently being undertaken.

Karyological evolution in Gentiana—The monophyly of the dysploid group section *Chondrophyllae* s. l. is suggested by our ITS sequence data, including the two karyologically distinctive European species of the section, *G. boryi* and *G. pyrenaica,* which had been elevated to generic rank and treated, respectively, as *Kuepferella* and *Holubogentia* (Löve and Löve, 1975, 1976; Laìnz, 1976). As can be seen from Fig. 2, dysploidy is quite a common phenomenon in *Gentiana,* occurring in many different clades. Identical chromosome numbers are likely to have developed independently in different clades making delimination of groups solely on the basis of chromosome number inadvisable in *Gentiana.* Chromosome numbers are conservative in some clades (e.g., section *Ciminalis* and section *Pneumonanthe* excluding *G. asclepiadea*), but variable in others (e.g., section *Calathianae* and section *Chondrophyllae* s. l.).

Optimization of chromosome numbers on the ITS phylogeny suggested that $2n = 20$ or $2n = 26$ is a plesiomorphic state for the clade section *Chondrophyllae* s. l. (shown as node "a" in Fig. 2) and $2n = 26$ is a plesiomorphic state for the large clade comprising sections *Chondrophyllae* s. l., *Cruciata, Pneumonanthe,* and *Frigidae* s. l. (shown as node "b" in Fig. 2). Although the former case is equivocal, $2n = 20$ or $2n = 26$, for the ITS phylogeny, our previous karyological investigations suggested $2n = 20$ as the ancestral number for that section (Y.-M. Yuan, P. Küpfer, and L. Zeltner, unpublished data). For the latter case, it seems quite reasonable to consider $2n = 26$ as plesiomorphic for that clade, since this number is the most widely distributed one in that clade, both from the number of species and from the biogeographical point of view. If this is the case, then all other numbers of that clade, such as $2n = 24$, $2n = 20$, $2n = 18$, and $2n = 14$ are secondary derivatives of $2n = 26$. Nevertheless, such consideration is greatly dependent on the robustness of the ITS phylogeny.

In addition, the differentiation of the chromosome numbers $2n = 24$ and $2n = 26$ in the clade section *Frigidae* s. l. seems to be a good synapomorphic character and may be used for delimitation of subdivisions. However, no valid conclusion can be drawn yet on this point before an analysis involving more species of this group is carried out.

It is still impossible to evaluate the karyological relationships among the closely related dysploids of the clade section *Chondrophyllae* s. l. from the ITS phylogeny, not only because of the limited number of dysploid species analyzed but also because of the poor resolution of our ITS phylogeny in this clade.

ITS evolution and its phylogenetic utility—The relative length of ITS1 and ITS2 among the species surveyed varied slightly from species to species, with either ITS1 or ITS2 being longer. The size of both spacers fell within the range of those reported previously for other plants (Suh et al., 1993). The length variation of ITS sequences among the taxa surveyed here ranged from 0 to 13 bp in ITS1 and from 0 to 25 bp in ITS2. This variation was due primarily to short insertions/deletions of 1 to 5 bp. No case of longer length variation, such as that in *Lisianthius*, in which a length variation of 100 bp in ITS1 was reported (Sytsma and Schaal, 1990), was observed.

Nucleotide substitution was the main source of sequence divergence in the ITS. It has been shown that ITS1 is more variable than ITS2 both in length and substitution in Asteraceae (Baldwin, 1992, 1993; Kim and Jansen, 1994), Fabaceae (Wojciechowski et al., 1993) and Winteraceae (Suh et al., 1993). This was also the case in *Gentiana*, where ITS1 displayed higher divergence than ITS2 among pairwise comparisons of the sequences. However, comparison of the relative phylogenetic resolution indicated that ITS2 was more informative than ITS1 in *Gentiana*. This result is parallel to the case of *Krigia* (Kim and Jansen, 1994) where a higher level of sequence divergence and homoplasy in ITS1 than in ITS2 was observed. The reason for the differential levels of divergence between ITS1 and ITS2 is not yet clear.

The effect of character state weighting (transversion vs. transition) on the resolution of phylogenetic trees is likely to vary from case to case. Wojciechowski et al. (1993) did not find that transversion weighting had an important influence on the tree topology. In the present study, weighting of transversions by different factors resulted in slightly different tree topologies, and setting an extremely high weight for transversions (ten times transitions) led to the collapse of some clades supported by other analyses. However, the principal groups of the terminal taxa were maintained, and the collapse was restricted to internal clades supported by relatively low bootstrap values. Different gap treatments also may influence the topology of trees constructed from the same sequence data. Coding gaps as missing data seems preferable to us, since information about nucleotide substitutions in the taxa with the insertions in the regions corresponding to the gaps is retained; elimination of gap sites leads to loss of phylogenetic information. Conversion of gap sites to new characters did not significantly improve the resolution of the phylogenetic trees constructed on the same data matrix. The topologies of ITS phylogeny of *Gentiana* were, then, not sensitive to different gap codings. Therefore, in general, the principal groups of *Gentiana* suggested by our ITS phylogeny were robust against different molecular evolutionary assumptions.

Wendel, Schnabel, and Seelanan (1995) recently demonstrated that not only has there been interlocus concerted evolution among nrDNA repeats but also that this evolution was bidirectional following allopolyploid speciation in *Gossypium*. Thus inferences of phylogenetic relationships among organisms originated from hybridization or allopolyploidization can be misled, if considered only on ITS or other nrDNA data. Although the applicability and extensiveness of this phenomenon need to be confirmed by more examples, this case does indicate that considerations of other disciplines such as morphology and cytogenetics should be taken into account to elaborate the phylogenetic relationships inferred from ITS or other nrDNA data among organisms involved in hybridization and polyploidization. For the genus *Gentiana*, as far as the species examined, ITS sequences are highly homogeneous, even the polyploid species such as *G. straminea*. The process of homogenization of ITS sequences in *Gentiana*, especially in the polyploid species, is not known, since the genomic organizations of the taxa are still not clear.

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The monophyly and rapid evolution of *Gentiana* sect. *Chondrophyllae* Bunge s.l. (Gentianaceae): evidence from the nucleotide sequences of the internal transcribed spacers of nuclear ribosomal DNA

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The nucleotide sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA of 24 representative species of sect. *Chondrophyllae s.l.* have been determined and analysed phylogenetically, together with some species of other sections of the genus *Gentiana*. The ITS sequences strongly support the monophyly of the sect. *Chondrophyllae s.l.* as a whole complex including various different dysploid cytotypes. Species, such as *G. boryi* and *G. pyrenaica*, that had been split into distinct genera by some cytotaxonomists have been proven to be closely related. However, the ITS sequences do not provide sufficient information to make a robust estimation of the phylogenetic relationships among the closely related species and dysploid cytotypes of the complex, beyond recognizing their monophyly and rapid evolution.

ADDITIONAL KEY WORDS:—chromosome number – dysploidy – evolution – molecular systematics – phylogeny – polyploidy – sect. *Dolichocarpa*.

CONTENTS

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INTRODUCTION

The largest and the most widely distributed section of the genus *Gentiana* (*Gentianaceae*), sect. *Chondrophyllae* Bunge *s.l.*, comprises in its broad sense, as circumscribed by Smith (Nilsson, 1967) and Pringle (1978), about 170 mostly biennial species. There are only three species (*G. prostrata* Haenke, *G. pyrenaica* L. and *G. boryi* Boiss.) of the complex in Europe; one species (*G. atlantica* Litard. & Maire) in Africa (North Morocco); three species (*G. aquatica* L., *G. douglasiana* Bong., *G. prostrata*, in North America; three species (*G. pumilio* Standley & Steyerm., *G. prostrata* and *G. sedifolia* Kunth) in Central America; three species (*G. podocarpa* Griseb., *G. prostrata* and *G. sedifolia*) in South America; and one species (*G. quadrifaria* Blume) in Australia. All other species of the complex are found in Asia, highly concentrated in the mountain regions of southwestern China and the nearby Qinghai-Tibet Plateau and the Himalayas. While Ho (1985) and Ho & Liu (1990) treated this complex as three independent sections, sect. *Chondrophyllae* Bunge *s.s.*, sect. *Dolichocarpa* T. N. Ho and sect. *Fimbricorona* T. N. Ho, based mainly on the shape of capsule and floral appendages, Laínz (1976), and Löve & Löve (1975, 1978, 1986) have established three independent genera based only on the three European species of the complex. The segregated 'new genera' are *Chondrophylla* A. Nelson, *Holubogentia* Löve & Löve and *Kuepferella* Laínz, based on *G. prostrata* ($2n = 36$), *G. pyrenaica* ($2n = 26$) and *G. boryi* ($2n = 20$) respectively. The discriminating characters of the segregates were their different basic chromosome numbers. Omer (1989) segregated another new genus, *Qaisera* Omer, from this complex, based on *G. carinata* Griseb., which corresponds to the new section *Fimbricorona* of Ho & Liu (1990). The discriminating character was its fimbrate corolla throat. Taxonomically, a decision as to whether this complex should be treated as a single section, as three different sections, or as four or perhaps more different genera is needed as is an assessment of the monophyly of the complex and its segregates.

To evaluate the validity of the segregates based on different basic chromosome numbers, the extent of chromosome number variation among the taxa and the karyological evolution of the complex must be determined. More extensive karyological investigations on Chinese species, together with previous studies on species from other regions, revealed a wide range of chromosomal number variation among the species of the complex. Different chromosome numbers, including $2n = 12, 14, 16, 18, 20, 22, 24, 26, 30, 32, 36, 38, 40, 44, 48, 60$, and 96, have been documented for the complex (Küpfer & Yuan, 1996; Yuan, Küpfer & Zeltner, unpublished). The wide range of chromosome number variation suggested the polybasic nature and a chromosome evolution through the combination of dysploidization and polyploidization for the complex. The number $2n = 20$ was suggested as the ancestral number and the polarity of the dysploidization was thought to be predominantly descending for this complex (Yuan *et al.*, unpublished). The karyological conclusions do not favour the splitting of the complex either as different sections or as different genera. However, karyological investigations cannot supply robust estimates of monophyly and chromosome evolution because of the high homoplasy of karyological data in this complex (Yuan *et al.*, unpublished). They need to be confirmed by other lines of evidence.

DNA data, particularly DNA sequences, can contribute significantly to the cases in which morphological and karyological data are inconsistent, inconclusive, deficient or poorly analysed (Patterson, Williams & Humphries, 1993). This is particularly true for plants as the sect. *Chondrophyllae s.l.* complex where high morphological and karyological homoplasy is involved and therefore morphological and karyological conclusions conflict (Yuan *et al.*, unpublished). The internal transcribed spacers (ITS) of nuclear ribosomal DNA has been proven to be useful sources of characters for phylogenetic studies. ITS sequences can provide a valuable set of characters for addressing lower-level phylogenetic questions, and were suggested to play an especially useful role in angiosperm studies by offering independent assessment of lower-level phylogenetic hypotheses based on morphology, karyology or chloroplast DNA (cpDNA) evidence (Baldwin *et al.*, 1995). Baldwin (1992, 1993) first extensively demonstrated the utility of the ITS region for phylogenetic reconstruction in Asteraceae. Some other examples of studies using ITS sequences are: Wojciechowski *et al.* (1993) on *Astragalus* (Fabaceae); Soltis & Kuzoff (1993, 1995) on *Heuchera* and *Lomatium* (Saxifragaceae); Baum, Sytsma & Hoch (1994) on *Epilobium* (Onagraceae); Hodges & Arnold (1994) on *Aquilegia* (Ranunculaceae); Kim & Jansen (1994) on *Krigia* (Asteraceae); Campbell *et al.* (1995) on some Rosaceae; Oxelman & Lidén (1995) on some Caryophyllaceae; Wendel, Schnabel & Seelanan (1995a,b) on *Gossypium* (Malvaceae); Downie & Katz-Downie (1996) on the subfamily *Apioideae* (Apiaceae). The inferred phylogenies of ITS sequences from previous studies were principally concordant with or often better resolved than relationships inferred from cpDNA (e.g. Hodges & Arnold, 1994; Sang *et al.*, 1995), morphological data (e.g. Wojciechowski *et al.*, 1993; Campbell *et al.*, 1995), karyological and cytogenetic data (e.g. Baldwin, 1993; Wojciechowski *et al.*, 1993; Hsiao *et al.*, 1995a,b). A few cases revealed incongruence between ITS-based and cpDNA-based or morphology based phylogenies. Kim & Jansen (1994) showed in *Krigia* (Asteraceae) that ITS trees were partially congruent with morphology and cpDNA trees, but about 22.5% incongruence occurred among their data sets. They concluded that ITS sequence data may have limited utility in interspecific studies or comparisons among closely related genera, especially in the groups which exhibit high levels of sequence divergence. Soltis & Kuzoff (1995) also revealed important discordance between ITS and cpDNA trees. However, the preponderance of data, including morphology, chemistry and allozymes of the same taxa, favour the ITS trees over the cpDNA trees, and the discordance was attributed to hybridization and introgression. Among these examples, ITS phylogenies have provided useful interpretations or confirmations on karyological evolution of some dysploid species groups as in *Astragalus* (Wojciechowski *et al.*, 1993), *Epilobium* (Baum *et al.*, 1994), *Calycadenia* (Baldwin, 1993), and the *Pooideae* (Hsiao *et al.*, 1995a,b).

Our previous studies at generic level of *Gentianinae* (Yuan & Küpfer, 1995) and at sectional level of *Gentiana* (Yuan, Küpfer & Doyle, 1996) have also proven the phylogenetic utility of ITS sequences. The ITS phylogenetic trees generated by parsimony analyses were principally congruent with morphological considerations, and improved or clarified some morphological misinterpretations and conflicts. Five species of the sect. *Chondrophyllae s.l.* have been sampled. They formed a highly supported monophyletic clade (Yuan *et al.*, 1996). Optimization of chromosome numbers on the ITS phylogeny suggested that $2n = 26$ is a plesiomorphic state for a clade comprising sections *Frigidae s.l.*, *Cruciata*, *Pneumonanthe*, and *Chondrophyllae s.l.*, and $2n = 20$ or $2n = 26$ equivocally as the plesiomorphic condition for sect. *Chondrophyllae s.l.* However, this investigation sampled only a limited number of species (only five out of 170) and did not include all documented cytotypes of the complex, and thus the conclusions are not necessarily applicable over the entire complex. It is obviously necessary to do a further and more extensive study for such a big and diversified complex.

The present study is concentrated on the sect. *Chondrophyllae s.l.* complex with the following questions to be addressed in particular: (1) is the complex a monophyletic group? (2) does ITS phylogeny support the splitting of the complex into different sections (as by Ho & Liu, 1990) or different genera (as by Löve & Löve, 1975, 1978, 1986, and the others), and (3) what is the phylogenetic utility of ITS sequences for this group of morphologically similar species?

MATERIAL AND METHODS

Plant species and material

ITS sequences were obtained from single individual, or 2–3 pooled individuals in the cases of small plants of a further 19 species representing most of the documented cytotypes of the complex sect. *Chondrophyllae s.l.* The species analysed, the origins of the material and the chromosome numbers of the species are given in Table 1. The chromosome numbers shown in the table were mostly observed directly from the vouchers used for DNA extraction, except for a few cases, as indicated in the table, when the numbers were obtained from literature. Leaves were collected directly from the field using the silica gel method (Chase & Hills, 1991) or were taken from conventionally prepared and recently collected herbarium sheets. All the voucher specimens are deposited in the herbarium of the University of Neuchâtel (NEU). Regrettably, the species of the small section *Fimbricorona* (4 species) remain unavailable to us. To assess the monophyly of the sect. *Chondrophyllae s.l.* complex, previous ITS sequences of five species of the complex and ten representatives of other sections of *Gentiana* were also included. Two species of other genera, *Crawfurdia tibetica* Franch. and *Gentianella campestris* (L.) Börner, were used as outgroups.

Collection of ITS sequence data

Total DNA was extracted from about 2 g of fresh or 100 to 300 mg of dried leaves pulverized in liquid nitrogen or directly in hot 2 × CTAB buffer according to the protocol of Doyle & Doyle (1987). A standard double-strand polymerase chain reaction (PCR) was used to amplify the entire ITS region, using the primers and protocols described previously (Yuan & Küpfer, 1995; Yuan *et al.*, 1996). The amplified ITS fragments were purified by electrophoresis in a 1.6% agarose gel and subsequently using the QIAEX gel extraction kit (QIAGEN AG, Basel). Both strands of purified ITS fragments were directly sequenced using primers 5' end-labelled with digoxigenin (MWG-Biotech, Germany), the DIG-TAQ cycle sequencing kit (Boehringer Mannheim GmbH) and the GATC-1500 Direct Blotting Electrophoresis DNA Sequencer (Constantz, Germany). The sequencing bands were detected by anti-digoxigenin using DIG Nucleic Acid Detection Kit (Boehringer Mannheim GmbH).

Sequence alignment and phylogenetic analysis

The sequence boundaries between the two spacers and the three coding regions (18S, 5.8S and 25S genes) of nrDNA were determined by comparison with published sequences from *Daucus carota* and *Vicia faba* (Yokota *et al.*, 1989) and with our previous data (Yuan & Küpfer, 1995; Yuan *et al.*, 1996).

The pooled ITS1 and ITS2 sequences were aligned using the progressive multiple alignment program Clustal W (version 1.5) for Power Macintosh (Thompson,

TABLE 1. The species and the origin of materials analysed. Voucher: G, Y=Y.-M. Yuan; K=P. Küpfer; Z=L. Zeltner; A=E. Anchisi. The sequences of the species marked with '*' were previously released (Yuan & Küpfer, 1995; Yuan *et al.*, 1996). **The chromosome numbers were observed directly from the vouchers used for DNA study except for those numbers shown in bracts which were obtained from literature

Taxon	Vocher	Origin	2*n***
sect. *Chondrophyllae* Bunge *s. str.*			
G. altaica Pall.	Y95–49	Hanasi, Xingjiang, China; 3100 m	(26)
G. aristata Maxim.*	Y92–328	Maqû, Gansu, China; 3500 m	14
G. atlantica Litard. & Maire	Z93–S2	Atlas Mt., Morocco; 2200 m?	(48)
G. boryi Boiss.*	Z93–S1	Sierra Nevada, Spain; 2300 m	(20)
G. crassuloides Bureau & Franch.	Y92–265	Mt. Taibai, Shaanxi, China; 2900 m	38
G. flexicaulis H. Sm. ex Marquand	Y92–264	Mt. Taibai, Shaanxi, China; 3400 m	14
G. heleonastes H. Sm. ex Marquand	G032	Maqû, Gansu, China; 3650 m	12
G. intricata Marquand	Y92–198	Lijiang, Yunnan, China; 2700 m	20
G. pantheica Prain Burk.	Y92–248	Dali, Yunnan, China; 3200 m	20
G. piasezkii Maxim.	Y92–272	Mingxian, Gansu, China; 2900 m	36
G. pseudoaquatica Kusn.	Y92–326	Maqû, Gansu, China; 3800 m	20
G. pyrenaica L.*	Y93–14	Mt. Rila, Borovetz, Bulgaria; 2600 m	(26)
G. squarrosa Ledeb.	G046	Xiahe, Gansu, China; 3000 m	38
sect. *Dolichocarpa*			
G. crenulato-truncata (Marquand) T. N. Ho*	Y92–310	Maqû, Gansu, China; 4200 m	18
G. haynaldii Kanitz*	Y92–201	Zhongdian, Yunnan, China; 3400 m	20
G. hyalina T. N. Ho	Y93–36	Maduo, Qinghai, China; 4300 m	12
G. hyalina T. N. Ho aff.	Y92–89	Dingri, Tibet, China; 4500 m	30
G. ludlowii Marquand	Y92–35	Mt. Xiangpi, Qinghai, China; 3800 m	16
G. ludlowii Marquand	Y92–33	Heimahe, Qinghai, China; 3400 m	32
G. ludlowii Marquand aff.	Y92–99	Nyalamu, Tibet, China; 4000 m	22
G. producta T. N. Ho	Y93–79	Ganzi, Sichuan, China; 4000 m	?
G. pudica Maxim.	G178	Maqû, Gansu, China; 3700 m	20
G. sedifolia Kunth	A94–S3	Chacaltaya de Bo Paz, Bolivia; 4800 m	(40)
G. tetrasticha Marquand	Y92–128	Dangxiong, Tibet, China; 4500 m	24
other sections			
G. bavarica L.*	Y93–11	Grand Chavalard, Switzerland; 2200 m	(30)
G. clusii Perr. & Song.*	Y93–13	Grand Chavalard, Switzerland; 2100 m	(36)
G. lutea L.*	Y91–S5	La Tourne, NE, Switzerland; 1200	(40)
G. algida Pall.*	Y91–S10	Trail Ridge, Rocky Mt., USA; 3100 m	(24)
G. callistantha Diels & Gilg*	Y92–298	Luqû, Gansu, China; 3500 m	26
G. urnula H. Sm.*	Y92–71	Langkazi, Tibet, China; 5200 m	26
G. delavayi Franch.*	Y92–229	Lijiang, Yunnan, China; 2900 m	26
G. affinis Griseb.*	Y91–S1	Boulder Colorado, USA; 2300 m	(26)
G. straminea Maxim.*	Y92–313	Maqû, Gansu, China; 3500 m	52
G. rhodantha Franch. ex Hemsel.*	Y93–124	Yingjing, Sichuan, China; 1100 m	46
outgroups			
C. tibetica Franch.*	Y93–121	Mt. Gongga, Sichuan, China; 2700 m	?
Gl. campestris (L.) Börner*	K93–G1	Col du Pt. St. Bernard, Italy; 2150 m	(36)

Higgins & Gibson, 1994). Two passes were conducted to get the basic alignment, i.e. the aligned sequences of the first alignment were fed back again for a second round of alignment process, using the default parameters with opening gap penalty at 10, extension gap penalty at 5 and nucleotide transitions weighted. This alignment was then used as the basic data matrix for phylogenetic analysis. To examine the alignment ambiguity and its impact on phylogenetic analyses, the alignment processes were conducted for further eight rounds in the same way with the same alignment parameters. Each time the generated alignment was subjected to phylogenetic analysis and the aligned sequences were also loaded for a next round of alignment.

Sites from 476 to 492 (Appendix) were excluded from our basic phylogenetic analysis because of the high alignment ambiguity. Phylogenetic trees were reconstructed with gaps coded as missing, using Fitch parsimony, i.e. equal weights and unordered character states, as implemented in PAUP 3.1.1 (Swofford, 1993). Heuristic searches of 100 replicates of random addition of sequences, in combination with ACCTRAN character optimization and TBR + MULPARS branch-swapping options, were conducted to maximize the probability of identifying the most parsimonious trees and to discover multiple islands of trees (Maddison, 1991). Bootstrap values (Felsenstein, 1985) were calculated from 100 replicates of heuristic searches using Fitch parsimony.

Character-state weighted parsimonious analyses were used to test the effects of the more homoplasious character changes (transitions), and other phylogenetic inference methods including neighbour-joining and maximum likelihood were also used. No important difference in tree topologies was found, and thus the results are not shown.

RESULTS

Variation in repeat unit

No evidence of multiple repeat types was found in either PCR or sequencing. Each PCR product was always resolved as a single band on 1.6% agarose gel. Some sequences contained ambiguous sites that could represent polymorphisms, but these were present at a low frequency. With the exception of the regions around site 18, ambiguous sites were scattered throughout the sequences.

Sequence analysis

The basic alignment of the sequences of ITS1 (from site 1 through site 261) and ITS2 (site 262 through site 515) are provided in the Appendix. All the sequences were deposited in the EMBL Nucleotide Database. The length, G + C content, and accession number of EMBL Nucleotide Database of each sequence are given in Table 2 and the Appendix. The length of ITS1 of the sampled sect. *Chondrophyllae s.l.* species ranged from 215 bp (*G. heleonastes* H. Sm. ex Marquand to 233 bp (*G. flexicaulis* H. Sm. ex Marquand), and the length of ITS2 ranged from 221 bp (the accession 92-89 of *G. hyalina* T. N. Ho aff. with $2n = 30$, and *G. producta* T. N. Ho) to 235 bp (*G. flexicaulis*). The multiple alignment of the sequences of sect. *Chondrophyllae s.l.*, together with the sequences of other sections and the outgroup species, assessed 261 sites for ITS1 and 254 sites for IST2. The G + C content of the complex varied from 52.8% (*G. aristata* Maxim.) to 60.9% (*G. piasezkii* Maxim.) in ITS1 and from 55.6% (the accession 92-89 of *G. hyalina* aff. with $2n = 30$) to 60.5% (*G. pseudoaquatica* Kusn.) in ITS2.

The alignment of the sequences encountered some ambiguity. Several alignment processes have been conducted to estimate impact on the phylogenetic reconstruction. The first pass of the multiple alignment of the 36 species, using the software Clustal W for Power Macintosh (Thompson *et al.*, 1994) with its default settings (transitions weighted, gap opening penalty set at 10, and gap extension penalty set at 5), generated 504 sites for pooled ITS1 and ITS2 sequences. The generated alignment was directly loaded again for the second round of alignment using the same parameters. In the same way, ten rounds of alignments were conducted. The resulting alignment of each round was also directly used as the data matrix for a

TABLE 2. The length and G+C content of the nucleotide sequences of ITS1, ITS2 and ITS1+ITS2 of sect. *Chondrophyllae* s. l. and the other related species studied

	ITS1		ITS2		ITS1+ITS2	
Taxon	Length (bp)	G+C (%)	Length (bp)	G+C (%)	Length (bp)	G+C (%)
G. altaica	218	57.3	225	58.3	443	57.8
G. aristata	229	52.8	234	58.5	463	55.7
G. atlantica	231	57.2	232	57.8	463	57.4
G. boryi	230	59.1	231	58.4	461	58.8
G. crassuloides	225	57.7	233	60.1	458	58.9
G. flexicaulis	233	56.7	235	55.7	468	56.2
G. heleonastes	215	54.4	227	59.4	442	57.0
G. intricata	223	56.5	228	55.7	451	56.1
G. pantheica	228	57.4	229	56.8	457	57.1
G. piasezkii	220	60.9	226	59.8	446	59.9
G. psedoaquatica	227	59.9	228	60.5	455	60.2
G. pyrenaica	229	60.2	231	58.9	460	59.6
G. squarrosa	221	55.7	223	58.7	444	57.3
G. crenulato-truncata	219	58.2	231	60.1	450	59.0
G. haynaldii	228	59.9	232	59.4	460	59.8
*G. hyalina*12	226	55.7	228	57.9	454	56.8
*G. hyalina*30	224	54.9	221	55.6	445	55.3
*G. ludlowii*16	225	57.8	224	60.3	449	59.0
*G. ludlowii*32	221	57.0	228	56.1	449	56.6
*G. ludlowii*22	225	55.6	228	57.9	453	56.8
G. produncta	226	58.0	221	57.4	447	57.7
G. pudica	219	57.1	223	59.2	442	58.2
G. sedifolia	231	58.8	231	59.3	462	59.1
G. tetrasticha	222	58.6	227	58.6	449	58.6
G. bavarica	230	61.3	231	59.7	461	60.5
G. clusii	232	61.2	231	60.6	463	60.9
G. lutea	221	61.1	231	62.8	452	61.9
G. algida	230	61.3	231	60.6	461	61.0
G. callistantha	227	58.4	230	58.5	457	58.4
G. urnula	232	58.2	233	58.0	465	58.1
G. delavayi	228	56.1	230	57.2	458	56.6
G. affinis	229	59.8	230	59.1	459	59.4
G. straminea	227	60.1	216	56.4	443	58.9
G. rhodantha	228	56.2	232	58.7	460	57.4
C. tibetica	228	53.5	229	57.2	457	55.4
Gl. campestris	232	58.6	230	63.5	462	61.0

TABLE 3. A comparison of the parsimonious trees generated from different alignments of ITS sequences of sect. *Chondrophyllae s. l.* and other species studied. L: length of the shortest trees; CI: consistency index; RI: retention index

	Sequence	Number				Support for the clade**									
Matrix*	length	of trees	L	CI	RI	A	B	C	D	E	F	G	H	I	J
Basic	498	27	870	0.481	0.477	+	+	+	+	+	+	+	+	+	+
1	504	8	1091	0.459	0.454	+	–	–	+	+	–	–	+	+	+
2	515	2	975	0.467	0.460	+	+	–	+	+	–	+	+	+	+
3	531	6	957	0.469	0.458	+	+	–	+	+	+	+	+	+	+
4	538	32	904	0.477	0.467	+	+	–	–	+	–	+	+	+	+
5	547	32	887	0.484	0.469	+	+	–	+	+	–	+	+	+	+
6	547	296	867	0.490	0.473	+	–	–	–	+	–	+	+	+	+
7	552	26	858	0.479	0.463	+	+	–	+	+	–	+	+	+	+
8	552	6	856	0.481	0.470	+	+	–	+	+	+	+	+	+	+
9	556	8	837	0.484	0.474	+	+	–	–	+	+	+	+	+	+
10	553	952	834	0.492	0.478	+	+	–	–	+	–	+	+	+	+

*Basic corresponds the basic data matrix which was based on the alignment round two with sites from 476 to 492 excluded from parsimonious analyses. The numbers 1 through 10 refer to the data matrices directly from the ten successive alignments by CLUSTAL W (see text).

**The clades A, B, C, D, E, F, G, H, I, and J represent the corresponded clades shown in Fig. 1; +supported; –not supported.

heuristic search of phylogenetic trees using the software PAUP (version 3.1.1; Swofford, 1993). The consensus length of the alignments and some information of the phylogenetic trees generated are shown in Table 3. The alignment generated from the second round was selected as our basic alignment of the data matrix for further phylogenetic searches because the later rounds of the alignments tend to produce congruent estimates of trees and the resolution of these trees becomes reasonably constant, although the alignments differ slightly. The alignment of the second round generated 515 sites (Appendix). In this alignment, 217 sites involved gaps of one to eight base pairs in individual sequences. The 17 sites from 476 to 492 shown in the Appendix were omitted from further phylogenetic analyses because of alignment ambiguity. The high proportion of gaps indicates a rapid sequence evolution among the closely related species of the complex.

The pairwise sequence divergence values were calculated from the basic alignments for all possible combinations of pooled ITS1 and ITS2 sequences, using the DNADIST program of PHYLIP package (version 3.5c; Fensenstein, 1994) with Kimura's two-parameter method (Kimura, 1980). The mean sequence divergence between pairs of species of the sect. *Chondrophyllae s.l.* complex ranged from 2.62% (*G. hyalina* with $2n$ = 12 [93-36] versus *G. ludlowii* Marquand aff. with $2n$ = 22 [92-99]) to 28.3% (*G. aristata* versus *G. producta* T. N. Ho) for the combined ITS1 and ITS2 data (divergence data matrix not shown). The high sequence divergence values also indicated a rapid evolution of the ITS sequences of the complex.

Phylogenetic analyses

To estimate the impact of the slight alignment ambiguity, parsimony analyses were conducted on different data matrices generated from a series of alignments. Some information including number of the shortest trees, the length, the consistency index and retention index of the trees generated and the congruence of their resolution of some clades is given in Table 3. While most species of the sect. *Chondrophyllae s.l.* complex remained unresolved and a few species were resolved differently among the different matrices, a few clades remained constant. The matrix generated by the first pass of the alignment program produced eight trees of which the strict consensus is topologically different from all others. However, the second through the tenth gave consistent resolution for a few clades (Table 3). The other species remained unresolved or were resolved differently. The second alignment was chosen as the basic alignment used as the data matrix for further analyses.

The equally weighted parsimony analysis was conducted on the potentially informative characters of the second alignment and 17 ambiguously aligned sites (476 through 492) were excluded. Heuristic tree search strategy was employed. Alternating the starting tree led to different numbers of the most parsimonious trees being retained, suggesting the existence of multiple tree islands. Therefore, a heuristic search with 100 replicates of random addition of sequences and tree-bisection-reconnection (TBR) branch swapping was conducted to maximize the probability of identifying the tree islands. 27 equally parsimonious trees of 870 steps, belonging to four tree islands (two islands of three trees, one island of six trees and one island of 15 trees), were found. Their consistency (CI) index is 0.481 and retention index (RI) is 0.477. The strict consensus of these trees is poorly resolved for the species of sect. *Chondrophyllae s.l.* (Fig. 1). Only a few species were resolved constantly: *G. altaica* Pall. from the Mt. Altai, *G. pyrenaica* from Bulgaria and *G. boryi* from Spain form a relatively stable clade; the Chinese species *G. hyalina* with $2n = 30$, *G. producta, G. ludlowii* with $2n = 16$ and *G. pudica* Maxim. formed a well supported clade in every analysis we attempted; *G. hyalina* with $2n = 12$ always nested together with a cytotype of $2n = 22$ similar to *G. ludlowii*; and *G. crassuloides* Bureau et Franch. ($2n = 40$) often nested with *G. haynaldii* Kanitz ($2n = 20$). Many other species or cytotypes were resolved differently among the most parsimonious trees. None of the 27 equally parsimonious trees supports the monophyly of the segregates, sect. *Chondrophyllae s.s.* and sect. *Dolichocarpa*. Nevertheless, the monophyly of the sect. *Chondrophyllae s.l.* as a whole complex was strongly supported by all the 27 trees (92% bootstrap; Fig 1).

When the parsimony analysis was enforced on the monophyly of the sect. *Dolichocarpa* by topological constraint, one tree of 891 steps was generated which was 21 steps less parsimonious than the maximum parsimonious trees.

DISCUSSION

The monophyly of the sect. Chondrophyllae s.l. *complex*

As mentioned in the introduction, the sect. *Chondrophyllae s.l.* complex has been considered either as a single section, or as three different sections, or as several different genera. The ITS phylogenies do not favour these segregates. None of the ITS trees from any of the analyses supports the monophyly of the sect. *Chondrophyllae s.s.* and sect. *Dolichocarpa*. Despite of the poor resolution of the ITS gene trees on the relationships of the species and cytotypes, the monophyly of the sect. *Chondrophyllae s.l.* as a whole complex is always strongly supported regardless of methods of analyses (Fig. 1; Table 3). The sections *Cruciata* and *Pneumonanthe* were always shown to be the sister group of the complex. Particularly, *G. boryi, G. pyrenaica* and *G. altaica* grouped together as a relatively highly supported clade. Therefore, the splitting of Lainz (1976) and Löve & Löve (1975, 1978, 1986) on these species should be rejected. The different basic chromosome numbers are not indications of phylogenetic isolation of these species. The ITS phylogenies do not suggest the splitting of sect. *Dolichocarpa* of Ho (1985) either, because no monophyletic clade of either sect. *Chondrophyllae s.s.* or sect. *Dolichocarpa* was obtained. One well supported clade of the ITS tree, shown as clade 'G' in Figure 1, encompasses species of both sect. *Chondrophyllae s.s.* (*G.*

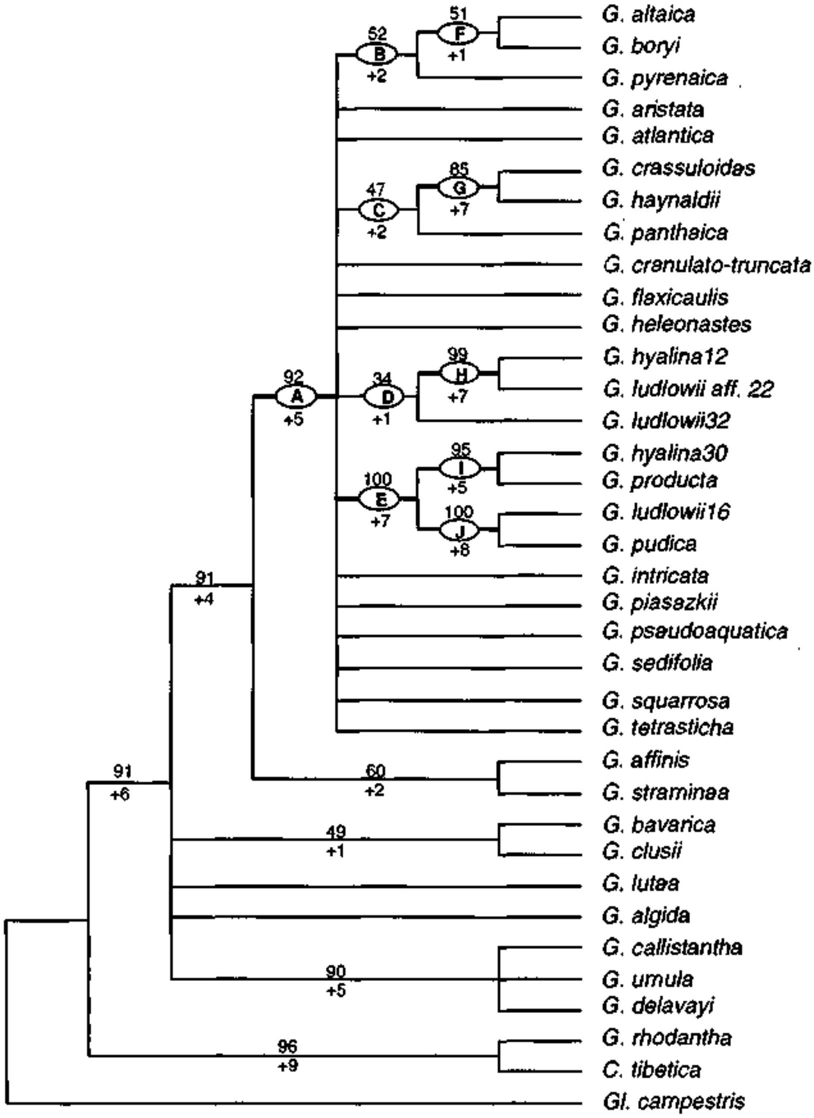

Figure 1. The strict consensus tree of the 27 equally parsimonious trees generated from PAUP heuristic searches of 100 replicates random addition sequence on the combined ITS1 and ITS2 sequences. Tree length = 870, CI = 0.481, RI = 0.477. The numbers above the internal branches are bootstrap values calculated from 100 replicates of heuristic searches. The numbers under the internal branches are the decay indices, i.e. the numbers of extra steps needed to destroy the corresponded branches. The letters A through J in the circles are the clades compared for different alignments (see text and Table 3). The thicker internal branches are supported by all analyses conducted.

crassuloides) and sect. *Dolichocarpa* (*G. haynaldii*). Regrettably, no species of sect. *Fimbricorona* or genus *Qaisera* was available for our ITS investigations.

Although ITS phylogenies cannot provide direct verification of the relationships among different dysploid cytotypes and therefore cannot verify the polarity of the dysploidization, ITS phylogenies do confirm the monophyly of the different dysploid cytotypes. Besides the clade consisting of *G. altaica* ($2n = 26$), *G. boryi* ($2n = 20$) and *G. pyrenaica* ($2n = 26$), the clade consisting of the $2n = 12$ cytotype (93-36) of *G. hyalina* and $2n = 22$ cytotype of *G. ludlowii* aff. (92-99), and the clade consisting of the cytotype $2n = 30$ of *G. hyalina*, the cytotype $2n = 16$ of *G. ludlowii*, the cytotype $2n = 20$ of *G. pudica* and *G. producta* for which the chromosome number is unknown, are also well supported examples of monophyletic dysploid complex.

It is necessary to point out here that the determination of the species names of this complex is by no means an easy task, due to the high morphological variability of the small biennial plants of the complex. Taxonomic circumscription of a certain species and the subsequent determinations based on a few morphological diagnostic characters is not guaranteed to recognize the right phylogenetic lineage and include it within a scope of definite species. A morphologically defined species may include several cytotypes belonging to more than one phylogenetic lineage. Meanwhile, different dysploid cytotypes of a specific phylogenetic lineage might be misleadingly placed into different species. Both situations, together with the existence of unrecognized cryptic species, complicated the interpretation and understanding of phylogenetic relationships among the cytotypes of the complex. The cytotype of the accession No. 92-89 ($2n = 30$) assessed as *G. hyalina* had a distinct chromosome number from the accession No. 93-36 ($2n = 12$); the cytotype of the accession No. 92-99 ($2n = 22$) assessed as *G. ludlowii* had a different number from the accession No. 92-35 ($2n = 16$) and accession No. 92-33 ($2n = 32$). If the taxonomic affiliation of the cytotypes sampled is correct, then ITS phylogenies indicated that these two species are polyphyletic. A close morphological and cytogenetic examination is needed to solve this problem. However, the nesting of different cytotypes in the same highly supported clade, such as the clade consisting of the cytotype $2n = 30$ of *G. hyalina*, the cytotype $2n = 16$ of *G. ludlowii*, the cytotype $2n = 20$ of *G. pudica* and *G. producta* of which chromosome number is unknown, does indicate that different basic chromosome numbers of this complex cannot be used as discriminating taxonomic characters.

Rapid evolution of sect. Chondrophyllae s.l. *complex*

Although the sequence divergence value among the species of the sect. *Chondrophyllae s.l.* complex ranged from 2.62% to 28.3%, the resolution of the trees was rather poor. Closer inspection of data matrix indicated a high proportion of autapomorphic mutations. Both the parsimonious trees and distance trees are characteristic in having rather short internal branches in contrast with the very long terminal branches. Such a situation was also observed in sect. *Calathianae* from chloroplast DNA sequences and was attributed to a star phylogeny, i.e. simultaneous speciation of many species (Gielly & Taberlet, 1996). This could be also the case for sect. *Chondrophyllae s.l.* Most species of the complex shared few synapomorphic characters, which suggested most of the species were derived simultaneously or within a very short period of time followed by subsequent rapid radiation. Therefore, most mutations were autapomorphic. With regard to the high sequence divergence, wide subcosmopolitan distribution, high morphological diversity, and the high variation of basic chromosome numbers, rapid evolution has probably occurred in this complex, at the molecular, chromosomal and morphological levels. Many species of this complex from the mountain regions of south-west China, centre of diversity for the complex, have very small population sizes and rather restricted distributions. Patches of species are often found on different hills of a small range, which suggested that mutations were fixed rather rapidly in different local places. However, very little is known about the reproductive and population biology of the complex. A further study of these aspects would be useful to verify any evolutionary patterns.

The rapid evolution of the complex might be attributed to the biennial and herbaceous life form of its species. Some biennial species, such as *G. aristata, G. crenulato-truncata* T. N. Ho, *G. crassuloides, G. heleonastes* etc., have accumulated more autapomorphic mutations and therefore had longer terminal branches than some other perennial species such as *G. altaica, G. pyrenaica,* and *G. piasezkii* etc. in both parsimonious and distance trees (not shown). It has been suggested that ITS sequences evolve faster in herbaceous, primarily annual groups of comparatively recent origin than in some ancient woody groups (Baldwin *et al.*, 1995). Similar correlations between plant life form and evolutionary rate have also been noted from chloroplast DNA data (Gaut *et al.*, 1992; Gaut, Muse & Clegg, 1993; Wilson, Gaut & Clegg, 1990). Generation time might be a reason for this situation in sect. *Chondrophyllae s.l.* complex, in which the biennial herbs had shorter generation time resulted in faster accumulation of mutations than perennial ones.

Limit of phylogenetic inference of ITS sequences

It is generally considered that ITS can provide valuable characters for addressing lower level phylogenetic questions (Baldwin *et al.*, 1995). In the case of the genus *Gentiana* and its allied genera, the statement remains appropriate as far as closely related genera and different sections within a genus are concerned (Yuan & Küpfer, 1995; Yuan *et al.*, 1996). In a closely related group of species such as sect. *Chondrophyllae s.l.*, ITS sequences failed to draw a robust conclusion of the species phylogeny, although the sequence divergence remained high. This is particularly obvious when a fast evolving group is examined. The weakness of the phylogenetic resolution was contributed in part by sequence alignment ambiguity, and in another part by insufficient synapomorphic mutations which were further underscored by frequent homoplasious substitutions.

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APPENDIX

The aligned nucleotide sequences of ITS1 and ITS2 of *Gentiana* sect. *Chondrophyllae s.l.* and the species of the outgroups and other sections compared. The numbers following species names are somatic chromosome numbers of the corresponded cytotypes. *C.* = *Crawfurdia, G.* = *Gentiana, G. crenulato-* = *G. crenulato-truncata, Gl.* = *Gentianella.*

```
                     >>ITS1
G. altaica           TCGAAT-CCT GCTAAGCA-- ACGACCCGAG -AACATGTT- TATCG--C-- NNNCGTTGGG
G. aristata          ....T--... ..CTTA-.-- G.A..A.C.- -...TG...- .T.AA--.-C GGGTT.....
G. atlantica         ......-... ........G- .......... -........- ....ACA.-- GGG.T.C...
G. boryi             ......-... ........G- .......... -........- .....CA.-- GGG...C...
G. crassuloides      ......-... ........G- .......... -........- ...A.CA.-- GGG...C-..
G. flexicaulis       ......T... ........G- .......... -........- .....TA.-- GGG...C...
G. heleonastes       ......-... ...G...N-- ......T... -........- .....CA.-- GGGT..C-..
G. intricata         ......-... ...G....-- ......T... -........- .....TA.-- .GGTN.C...
G. pantheica         ......-... ........G- .......... -........- .....CA.-- .GGT..C..A
G. piasezkii         ......-... ...G....G- .......... -........- .....TA.-- GGG...C...
G. pseudoaquatica    ....T.-... .....-.GA- G......... G........- ..AT.CA.-- GGG...C-..
G. pyrenaica         ......-... ........G- .......... -........- .....CA.-- GGG...C...
G. squarrosa         ......-... ........G- .......... -........- .....CA.-- .GG.T..-..
G. crenulato-        ......-... ........G- .......... -........- ....TCA.-- GGG...C...
G. haynaldii         ......-... ........G- .......... -........- ..C..CA.-- GGG.A.C...
G. hyalina12         ......-... .A.....N-- .......... -........- .....CA.-- .GG.A.....
G. hyalina30         ......-... ...G...N-- .......... -........- ....ACA.-- GGGG..C...
G. ludlowii16        ......-... ...T...N-- .......... -........- ....ACA.-- GGGG..C...
G. ludlowii32        ......-... ........G- .......... -........- .....CA.-- GGG.A.C.A.
G. ludlowii22        ......-... .......N-- CGT....... -........- .....CA.-- .GG.A.C...
G. producta          ......-... ...G....G- .......... -........- ....ACA.-- GGGG..C...
G. pudica            ......-... ..NG....-- .......... -........- ....ACA.-- .GGN.NC...
G. sedifolia         ......-... ........G- .......... -........- .....CA.-- GGG.A.C...
G. tetrasticha       ......-... ........G- .......... -........- .....CA.-- .GG...C...
G. bavarica          ......-GG. ..G..AG.G- .......... -G.......T G.AACCA.-- GGG....C..
G. clusii            ......-... ..G...AGA- CG........ -G.......- ..AA.CA.-- GGG....C..
G. lutea             ......-... ..G.....G- ......-... -........- ..AA.CA.-- GGG.T..C..
G. algida            ....T.-... ..G..NN.CG ---....... -.......C- ..A..CA.-- GGG....C..
G. callistantha      ....T.-... ..G.N---N- .......... -..T.....- ..AT.CA.-- GGG.A..C..
G. urnula            ....T.-... ..N...AGAG CG........ -........- ..A..CA.-- GGG.A..C..
G. delavayi          ....T.-... ..G..N-.G- ......T... -........- ..A..CA.-- GGG.A..TA.
G. affinis           ......-..G ........G- .......... -........- ..A.ACA.-- GGG....C..
G. straminea         ....T--T.C ........G- .......... -........G ..A..AA.-- GGG....C..
G. rhodantha         ....TA-... ..G..NN.-- -......... -........- C.A.TCA.-- GGG....C..
C. tibetica          ....T.-GG. ..G.....GA -......... -........- ..A.TCA.-- GGG.A..T..
Gl. campestris       ......-... ..G.....GA -......... -.....T..- ..A.AAA.A- GGG...CT..


G. altaica           A--CG-AGGG AAACCGCGGA CCG--ATGCC CCGA-C---- --GA-CGNCC ACCGGTCG-C
G. aristata          --C.--.A.. .....C...G ..A--..T.. ....-.CTTA AG.G-A.G.. C.......-A
G. atlantica         .--..-.... .......... ...--..... ....-GCATG AC.TCG-A.. ..A.....-.
G. boryi             .--..-.... .......A.G A.CG-..... ...C-GCATG AC.T-..A.. ........-.
G. crassuloides      GA-..-G... .....A.... ...--..... ....-GCACG GC.TCG-A.. ........-G
G. flexicaulis       .--..AG..A ..C.TC.... ...--..... ....-GCATG AC.GA..A.. ...AA...-A
G. heleonastes       GC-..-GA.A ..CAT..... ..----C.T. ....-GCACG AC.T-..A.. .......---
G. intricata         .--..-.... .....A.... ..---..... ....-GCACG GC.T-..AA. ........-T
G. pantheica         .--..-G... .....A.... ...--..... ..T.-GCACG GC.T-.CA.. ........TA
G. piasezkii         .--.A-.... .......... ...--.C... ....AGCGTG A.---.C--- ........-.
G. pseudoaquatica    GA-..-G... .....A.... ...A--.... ....-GCACG GC.T-..A.. .......T-.
G. pyrenaica         .--..-G... .....TT... ...--..... ....-GCACG AC.TCG-A.. ........-G
G. squarrosa         GA-.A-...- .......... ...TATC... .N..-GCATG A----..T.G ...ACCGT-.
G. crenulato-        .--..-.... .......... ...--..... ....-GCAGG AC.TCG-A.. ........-.
G. haynaldii         .--..-G... .G...A..T. ...--..... ....-GCACG GC.TCG-A.. ........-A
G. hyalina12         .--..-.... ........T. ...--..... ....-NCATN AC.T-NAC.A C.......-.
G. hyalina30         .--..TG... .....A.... ...--..... .-..-TGANN GC.T-N-A.. ........-.
G. ludlowii16        .--..-GN.. .....A.... ..A---.A.. T...-GCACG GC.T-..A.. ........-.
G. ludlowii32        GA-..-.T.. .....A.... ...------- -...-GGATT GC.T-..A.. ........-.
G. ludlowii22        .--..AG... ........T. ...--..... ....-NCATN AC.T-NAC.A C.......-.
G. producta          .--..-T... .....A.... ...--..... .-..-GCACG GC.TCG-A.. ........-.
G. pudica            .--..-G... .....A.... ..A---.... ....-N---- GC.T-N-A.. ......N.-.
G. sedifolia         G--..-.... .......... ...--..... ....-GCATG AC.TCGTA.. ........-A
G. tetrasticha       .--..-.... .......... ...--..... ....-.CNNA -----..A.. ........-G
G. bavarica          GA-..-G... .....T.... ...--..... .TA.-GCATG GC.TC-.A.. ........-.
G. clusii            GA-..-.... .....A.... ...--..... ....-GCACG GC.TCG-A.. ........CG
G. lutea             GA-..-.... ...GGA.... ...--..... ....-GCACG GC.GC----- .....C..C-
G. algida            GA-..-.... .....A.... ...--..... ....-GCACG GC.T-..A.. ........-.
G. callistantha      GA-..-.... .....A.... ...A--.... ....-GCATG GC.T-..A.. ........-T
G. urnula            GA-..-.... .G...A.... ..T--G.... ....-GCATG GC.T-..A.. ........-T
G. delavayi          GA-..-.... .....A.... .AAA--.... ....-GCATG GC.T-..A.. ........-T
G. affinis           GAA..-.... .....A.... ...--..... ....-GCATA GC.TCG-A.. ......GC-.
G. straminea         GA-..-.... .....A...C ...--..... ....-GCATG GC.T--.A.. ........-.
G. rhodantha         GA-..-.... .....A..T. ...--..... ....-GCAAG GC.G-..G.. ........-T
C. tibetica          GA-..-T... .....AT... .T.--..... ....-GCAAG GC..-..G.. .......A-.
Gl. campestris       GA-..-.... .....C.... ...--GC... ....-GCACG TC.T-..A.. .TA...T.-.
```

```
G. altaica          CCGTCGT-GC AAA----CAA CCAACCCACC C--GCAGAAA C-GCGCCAAG GAAAACGT--
G. aristata         TA.....-.. C..A--C... .......CG. .--CA.A... .-C.C..... ......C.TA
G. atlantica        T...T..-.. ...----... .......CGG G-C....... .-........ ......A.--
G. boryi            .......-.. ...----... .......CGG G-C.T.A... .-A....... ......-GT-
G. crassuloides     T......-.. ...----... .......CGG GGGA..AG.. --C..GGCCA .G......--
G. flexicaulis      T......-.. ...----... .A.....CGG G-C....... A-..A..... ......CGT-
G. haleonastes      ---.GA-NN. ..-----... .......CGG --GT.GC.G. --AACN.... ........--
G. intricata        T......-.. ...----... .......CGG G--CG..... .-..---... ......-GT-
G. pantheica        T......C.T ...----... .......GG- ---....... .-........ ......T.--
G. piasezkii        T......-.. G..----... .......CG- ---....... .-........ ........--
G. psaudoaquatica   T......-.. ...----... .......CG- --C....... .-........ ......TA--
G. pyranaica        .......-.. ...----... .......CGG G-C....... .-........ ........--
G. squarrosa        T......-C. ...----... .......CG- --G..GC.G. --AACG.... A.......--
G. cranulato-       T......-.. ...----..C .A.C...CGG G-G.A..G.. A--..G.... ....C...--
G. haynaldii        T......-.. ...----... .......CGG G-C....... .-........ ........--
G. hyalina12        T......-.. ...----... .......CG. ---....... .-........ ......T.--
G. hyalina30        T......-.. G..----... .......GG- ----G.A... AN-G.....N .....AANN-
G. ludlowii16       T......-.. G..----... .......CG. ---....... .-........ ......A.--
G. ludlowii32       T...A..-.T ...----... .......CG- --C....... .-........ ......T.--
G. ludlowii22       T......-.. ...----... .......CN- ---....... .-........ ......T.--
G. producta         T......-.. G..----... .......-GG G-C....... .-........ ......A.--
G. pudica           T-.C...-.N G..----... .......CG- ---....... .-........ ......A.--
G. sadifolia        T......-.. ...----... .......CGG G-C....... .-.T...... ........--
G. tetrasticha      T......-.. ...----... .......CG- ---.G..... .-........ ......T.--
G. bavarica         T......-.. .C.----... .......CGG G-C....... .-........ .......A--
G. clusii           T......CT. ...----... .......CGG G-C....... .T.T...... ...T...A--
G. lutaa            T.TC...G-. ...----... .......CGC ----..T... .-........ .......A--
G. algida           T......-.. ...----... .......CGG G-C....... .G-....... .......---
G. callistantha     T......-.. ...----... .......CGG G-C...T... .-........ .......A--
G. urnula           T......-.. ...----... .......CGG G-C...T... .GA....... .......---
G. dalavayi         T......-.A ...----A.. ...-...CGC G-C....... .-........ .......A--
G. affinis          T......-.. ...----... .......CGG G-C....... .---...... .......A--
G. straminea        T......-.T .TG-AAA... .......CGG ----...... .-........ .......A--
G. rhodantha        .......-.. T..----... .A.....CGG G-C.TC.... AG-....... .....TT.--
C. tibetica         AA.....-.. T..----..T .A.....CGG G-C...TT.. -G-T...... ......----
Gl. campastris      T......-.. .C.----... A......CGG G-C...T... AG-....... ......AA--


G. altaica          -AAATAAGGA TT-GTCTGCC CCCC-GTCGT GT-CGTA--- -TGGTGCG-C ACGGGAGGAT
G. aristata         -...A.G.A. ..-TC..C.. ....-..... .C-....A-T GCC..A..-. .........C
G. atlantica        -...A..... ..-....T.. ....C..... .C-....--T GC.....-G. .........C
G. boryi            -...A.G.AT .--....... ....-..... ..-....--T GC......-. ..........
G. crassuloides     --......TT C.--.-.... TA..C..... .C-..A.-TT TC.TATT--T CAC..GT.-.
G. flexicaulis      --..A..... ..-....... ...T-..... .C-....--T GC......-T .........C
G. heleonastas      --..A..... ..GTC-...T T...-..A.. TC-....--T AC.---T.-. GAC.......
G. intricata        -.....G.A. .--....... ....-..... .C-....--T GC.TGCTCT. .........C
G. pantheica        --..A..... ..-....... ....-..... .C-....--T GC......-. ..........
G. piasezkii        --..A..... ..-....... ....-..... .C-...---T GC......-. ..........
G. pseudoaquatica   --..A..... ..GTC..... T..--C.A.. .C-....--T GC......-. ..........
G. pyrenaica        -...C..... ..-....... ....-..... .C-....--T GC.....-G. ..........
G. squarrosa        --..AT.... ..GTC-.... T..G-TC... .C-....--T GC...CGA-. ---......-
G. crenulato-       -...-....T ..-CCT.C.- ....-..GC. AC-..-.--T TC.TC.---- ....--A...
G. haynaldii        -...A.G.AT ..-....... ....-..... .C-....--T TC...T..C- ..........
G. hyalina12        --..A..... ..-....... ..T.-..... .C-....--T GC......-. .........C
G. hyalina30        --..A..... ..-....... ....-..... .C-...T--T GC......-. .T........
G. ludlowii16       --..A..... C.-....... T...-N.... .C-....--T GC.....T-. ..........
G. ludlowii32       --..A..... ..-....... ....-..... .C-....--T -C......-. .........C
G. ludlowii22       --..A..... ..-.-..... ..T.-..... .C-....--A TC....N.-. .........C
G. producta         --..A..... ..-....... T...-..... .AAT..T--T GC...A..-- .T........
G. pudica           --..A..... ..-....... T...-..... .C-....--T GC.....TG. ..........
G. sedifolia        -...-..... C.-....... T...-..... .C-....--T GC....TCG. .........C
G. tetrasticha      --..A..... C.-....... ....-..... .C-....--A TC...T..-. ..........
G. bavarica         --..A.-... .G-.C..... T..T-T..A. .C-....--C GC....T.-A GGC......G
G. clusii           --..A..... .G-.C..... T...-....C CC-....--- CGC...T.-. ..A.A....C
G. lutaa            --..A..... .G-.C..... T...-....C .C-....--- CC......-T G.T.......
G. algida           -...A..... .G-.C..... T...-..A.. .CCGA..C-G C-....T.CG ........TA
G. callistantha     --..A..... C.-.C..... T...-..... .C-....--C GC....T.-. .T........
G. urnula           A...A..... ..-.C..T.. T...-..... .CCG-..C-G CA....T.C- .T........
G. delavayi         --..A..... ..-.C..... T...-..... .C-....--T GC....T.-. .T........
G. affinis          -...-..... .G-.C....- T...-..... .C-....--C GC....T-G. ....-.....
G. straminea        --..A..... .G-.C.N.-- T...-.G... .C-....--C GC....T.-. ..........
G. rhodantha        -..GA.G.AT .G-......T TG.T-.A.AC ..C.-GTT-G G-C.CTT.TT C-..T.....
C. tibetica         -G..A..... .G-.CN.... TA..--A.A. TCCG-..C-G C-....T.TT TT..T.....
Gl. campestris      -G..A.G... .G-.CT.... T.T.-.A..C TCCG-.CC-G C-..A.T.CA C-.A....GC
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                                               >>ITS2
G.  altaica          CACAGACGCC AAAAGAAACA -ATCGCGTCG CCCCC--AAC A-CCGTGCAT GAAACATT--
G.  aristata         ...GT..... T......... -..GT..... .....C-... .-........ ....A...--
G.  atlantica        ........-. T.......A. A......... .....CC... .A........ ....A.--TT
G.  boryi            ......T... T......... -......... .....CC... .-........ ........GC
G.  crassuloides     ...G-...-. T......... -......... .....CC... .C.T...... ........GC
G.  flexicaulis      .......... T......... -......... .....CC... .-........ ....A.A.TG
G.  heleonastes      ...G..GA.. T......... -......... .-...CC... .-........ ........--
G.  intricata        ........T. -......... -......... .....C-... .-.....T.. ........--
G.  pantheica        T..G...... T..T...... -......... .....C-... .-........ ........-G
G.  piasezkii        ...G...... T......... -..-...... .AA..C-... .-........ ....T...C-
G.  pseudoaquatica   ...G...... C......... -..GT..... .....C-... .-........ ........--
G.  pyrenaica        ........-. T......... -......... .....CC... .-........ ......--TT
G.  squarrosa        ...G...... T......... -......... ..-..C-... .-........ -.......--
G.  crenulato-       A..G-...-. T......... -......... .....CC... .-........ ......--CT
G.  haynaldii        ...G....-. T..-...... -......... .....CC.-A CA.....T.. ........GT
G.  hyalina12        .......... T......... -......... .T...CC... .-........ ......C.--
G.  hyalina30        ...G..T... T......... -......... .....C-..A .-........ ........--
G.  ludlowii16       ...G...T.. T......... -......... .....CC... .-........ ....T...--
G.  ludlowii32       ...N...... T......... -......... .....C-... .-........ ......C.--
G.  ludlowii22       .......... T......... -......... .T...CC... .-........ ......C.--
G.  producta         ...G....-. T......... -......... .....CC.-. .--....... ...-------
G.  pudica           ...G...... T......... -......... .....CC... .-........ ....T...--
G.  sedifolia        ...G....T. T.......T. -......... .....CC... .-........ ......--CT
G.  tetrasticha      ...G...... T......... -......... .....C-... .-........ ........--
G.  bavarica         ...G.G.... T...G..... -......... .....CC... .-........ ...G.CA.--
G.  clusii           ...G.G.... T.......AC A......... .....CC... .-........ ....TCG.--
G.  lutea            ...G.G.... TG.......G -..T...... .....CAC.. --........ ....TCA.--
G.  algida           ...G.G.... T......... -......... .....CC... .-........ ..T.TCA.--
G.  callistantha     ...G.G.... T......... -......... .....CC... .-........ ...TTCA.--
G.  urnula           ...G.G...- T......... -......... .....CC... .-........ ....TCA.--
G.  delavayi         ...G.G.... T......... -......... .....C-... .-........ ....TCA.--
G.  affinis          ...G.G.... T......... -....TAAGA .....CC... .-........ ......--TT
G.  straminea        ...G.G.... T......... -....TT..- ----.CC... .-.....T.. N.......--
G.  rhodantha        ...G.G.A.. T......... -......... ....TCC... --.......C ....TCA.--
C.  tibetica         ..TG.G.A.. T......... A...CT.... .....-A... --....CG.C ..CGTCG.--
Gl. campestris       A....G.A.. TG........ A......... .....CC... C-.....TG. T..CTCG.--


G.  altaica          GCCGGTTGTC GGAGGGG-CG GATATTGGCT TCCCGTG--- -CTTCGG-TG C-GGC-TGGC
G.  aristata         .T........ .......-.. ....A..... .......GTC GT.....C.. .-...-....
G.  atlantica        .......... TC.....-.. .......... .......--- -......-.. .-...-....
G.  boryi            AT--...... .TC....-.. .......... ......A--- -......-.. .-...-....
G.  crassuloides     C.A...C..T .......-.. ..G....... ......T--- -......-.. .-...-....
G.  flexicaulis      A......T.T ......A-.. .......... .......--- -......-.. TG-..A....
G.  heleonastes      ......C..A .......-.. ..C....... ......T--- -......-.. .-...-....
G.  intricata        .........T ......--.. .......... .......--- -......-.. .-...-..-.
G.  pantheica        C.A...CT.A T.....--.. ..A....... .......--- -....T.-.. .-...-.A..
G.  piasezkii        ......CT.T .......-.. ..C....... .N.....--- -......-.. .-...-....
G.  pseudoaquatica   ......C..A .......-.. ..C....... -......--- -......-.. .-...-....
G.  pyrenaica        .......... .......-.. .......... .......--- -......-.. .--.GC....
G.  squarrosa        .......... .......-.. .......... .......--- -......-.. .-...-....
G.  crenulato-       ......-... .......-.. .......... .......--- -:C-..T-.. .-...-....
G.  haynaldii        C.G.T.C... .......-.. ..G....... .......--- -......-.. .-...-....
G.  hyalina12        .T........ ......A-.. .......... .......--- -......-.. .-...-....
G.  hyalina30        .......... .-....A-.. ..C...-... .......--- -.....T-.. .-...-..-.
G.  ludlowii16       .......... .-GA...-.. ..C....... .......--- -...T..-C. .-...-....
G.  ludlowii32       .......... ......T-.. .......... .......--- -...T..-.. T-...-....
G.  ludlowii22       .T........ .-....A-.. .......... .......--- -......-.. .-...-....
G.  producta         -.AT.C...G C.....--.. ACA-.G---C .......--- -......TC. .-A.GC....
G.  pudica           ..G.TC.T.. .-GA...-.. ..C....... .......--- -......-CA .-...-....
G.  sedifolia        .......... ......A-.. .......... .......--- -......-.. .-...-....
G.  tetrasticha      .....AA... .-.....-.. .......... .......--- -.....T-.. .-...-....
G.  bavarica         .T....G..A T......-.. .......... .......--- -..C..A-C. .-...-....
G.  clusii           ......C... .......-.. .......... .......--- -..C...-.. .G-..-.A..
G.  lutea            ......C... .......-.. .......... .......--- -......-.. .-...-....
G.  algida           ......C... .......-.. .......... .......--- -......-.. .-...-....
G.  callistantha     ......C... .......-.. .......... .......--- -......-.. .-...-....
G.  urnula           ......C... ......T-GA ....C..... .......--- -......-.A .-...-....
G.  delavayi         A.....CA.T .......-.. .......... .......--- -....A.-.. .-...-....
G.  affinis          .......... .......-.. .......... .......--- -......-.. .-...-....
G.  straminea        .......... .......-.. .......... .......--- -......-.. .-...-....
G.  rhodantha        ......CAGT C.GA...G.. .......... .......--- -......-.. .G-..-....
C.  tibetica         ..T..A.... A......-.. .......... ......A--- -......-.. .G-.T-....
Gl. campestris       A.G...GACA T......G.. ..A.C..... .......--- -...G.C-C. .-...-....
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G. altaica          CTAAATGCAA -GTCCTTC-C G-GCGGACAC CACGA-CAAG TGGTGGTTGA TTACCTCAAC
G. aristata         .......... -....C.TG. .-A....... G....-.... .......... ..........
G. atlantica        ......T... GT--.C.TG. .CAA...... G....-..-. .......... ..........
G. boryi            .......... -....C.TG. .-........ G....-.... .......... ..........
G. crassuloides     ........G. GT-..C.TG. .-AT...... G....-.... .......... ..G.......
G. flexicaulis      .......... -....C.TG. .-A....... G....-.... .......... ..........
G. heleonastes      ........G. -....C.TG. .-A....... G....-.... .......... ..T.......
G. intricata        .......... -....C.TG. .-A....... G....-.... .......... .........A
G. pantheica        .......TG. -....C.TGT .-A....... G....-.... .......... .AT.....G.
G. piasezkii        ........G. -....C.TG. .-A....... T....-.... .......... ..TN......
G. pseudoaquatica   ......TTG. -....C.TGT .-A....... G....-.... .......... ..G...N...
G. pyrenaica        .......A.. -....C.TC. .-A....... G....-.... .......... ..........
G. squarrosa        .......... -....C.TG. .-A....... G....-.... .......... ..........
G. crenulato-       .......... GTG..C.TT. .TA....... G....-.... .......... ..........
G. haynaldii        ......A.G. GT-..C.TG. .-A....... G....-.... .......... ..........
G. hyalina12        .......... C....C.TG. .-A....... G....-.... .......... ..........
G. hyalina30        A......TG. -....C.TGT .-A....... G....-.... .......... ..T.T.....
G. ludlowii16       A.......G. -....C.TG. .-A....... G....-.... .........G ..T-T.....
G. ludlowii32       .......-.. -....C.TG. .-A....... G....-.... .......... ..........
G. ludlowii22       .......... -....C.TG. .-A....... G....-.... .......... ..........
G. producta         A......TG. GT-..C.TGT .-A....... G....A.... .......... ..T.T.....
G. pudica           A.......G. -....C.TG. .-A....--. G....-.... .........G ..T-T.....
G. sedifolia        .......T.. GT-..C.TG. .-A....... G....-.... .......... ..G.T.....
G. tetrasticha      .......... -..GGG.TG. .-A....... G....-.... .......... ..........
G. bavarica         .....C.... -....C.TG. .-A....... G....-.... .......... ..TA..T.G.
G. clusii           .......... -....C.TA. .-A....... G....-.... .......... ..T.......
G. lutea            .......G.. -....C.TG. .-.....G.. G....-.... .......... ..G....G..
G. algida           .....C.... -....C.TG. .-A....... G....-.... .......... -.TTT.....
G. callistantha     ......T... -....C.TG. .-A....... G....-.... .......... A.TA......
G. urnula           ......T... -....C.TG. .-A...CG.. G....-.... .......... A.TA......
G. delavayi         ......T... -....C.TG. .-A....... G....-.... .......... A.TT......
G. affinis          .......... GT-..C.TG. .-A....... G....-..-. .........- ..G.......
G. atraminea        .......... -....C.TN- .-A....... G....-..-. .........- ..G.......
G. rhodantha        ......-... -...TC.TGT .-A....... G....-.... .......... ..G.......
C. tibetica         .......... -...TC.TCT .-AT..CG.. G....-.... .......... ..G.......
Gl. campestris      ........G. -....C.TG. .-A.....G. G....-.... .......... .AGT.G....
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G. altaica          TCAGT-T--- TGT-CGCACG TTGA-CCCGT C-GGACGAGG AGACTTCCTC GACCCTA-AT
G. aristata         ....G-.G-C ...-T.G... ....-..... .-...T.... .........T .......-..
G. atlantica        .....-.G-C ...-..G... ....-..... .--.GT.... ....G.A.CT AGA..C.A..
G. boryi            .....-.G-C ...-..T... ....-..... .-...T.... .......... .......-..
G. crassuloides     .A..G-.GC- ...-..TG.. ....C..-.. .-...T.... ........CT .......-..
G. flexicaulis      ...AAA.GC- ...-..G... C...-.A... .-...T.... .........T ......-A..
G. heleonastes      .A..G-NG-- ...-...T.. C...-..... .-...T.... .....A...T .....--T.N
G. intricata        C.TCA-AG-T ...-...... ....-..... .-...TA..A .........T .....AT-..
G. pantheica        .A..G-.G-- ...-...G.. ....-..... .-...TT... ........CT .......C.C
G. piasezkii        .A..G-.G-- ...-...G.. C...-..... .-........ ........CT .....---TA
G. pseudoaquatica   .A..G-.GC- ...CGCGT.. C...-..... .-...T.... .....C...G AC..TA.CCG
G. pyrenaica        .....-.GG- ...-...... ....-..... .-........ .......... ......-A..
G. squarrosa        .....-GC-- ..N-..-... ....-..T.. .-...T.... T........T .....--T.G
G. crenulato-       ....G-.GC- ..G-..A... ..A.A..... .-...T.... .........T ......TG..
G. haynaldii        .A..G-.GC- ...-..TG.. ....C..-.. .-...T.... .......-CT ......-A..
G. hyalina12        .....-.G-C ..--TCG... ....-..... .-...TC... .........T .....---TA
G. hyalina30        .A..G-.G-- ...-...T.. .CTG-..T.. .-N..T.... ........AT .....---TC
G. ludlowii16       .A..G-.GC- ...-..TT.. .C..-....- .-.C.T.... ........AT .G...--T.G
G. ludlowii32       .....-.G-C ...-..TTT. ....-..... .-...T...C ....-...GT .NN.T..ATA
G. ludlowii22       .....-.GC- ...-..G... ....-..... .-...T.... .........T .....--T.A
G. producta         .A..G-.GC- ...-...T.. .C..C..T.. T-T..T.... ........AT .......A.C
G. pudica           .A..G-.GC- ...-..TT.. .C..-..... .-...T.... ........AT .....--T.G
G. sedifolia        ....G-.GC- ...-..G... ....-..... .-.T.T.... .........T ......-A..
G. tetrasticha      ....---TT- ...-...T.. ....-..... .-...T.... .......... .....--ATA
G. bavarica         .A..G-.GT- ...-...G.. C..C-..T.. .-........ ......A..T ......-T..
G. clusii           .A..G-.GC- ...-...G.. C..C-..... .-......T. .....AA..T ......G-..
G. lutea            .A..G-.CG- ...-...G.. C..C-..... .-...T.... ......A..T ......GT.C
G. algida           .A..GG.GC- ...CG-.... ...CC..-.. .-...T.... .........T ......-A.C
G. callistantha     .A..G-.G-C ...-...G.. ....-..... .-...T..CC .......T.T .......-.C
G. urnula           .A..G-.GC- ...CG-.G.. ...CC..T.. .A...T.... .......T.T ......-A.C
G. delavayi         .AG.G-.GC- ...-...G.. ...C-..... .-...T.... ......TT.T .......-.C
G. affinis          .A..G-.GC- ...A...T.. ...C-..... .--C.TT... ........AT ......-A..
G. straminea        .AG.--.GC- ...-..GTA. ...C-.--.. .-.-.T.... ......-..T ....---T..
G. rhodantha        .A..G-.GC- ...CGT.G.. C..CC..-A. .-.A...GA. .....CTG.A ......-G..
C. tibetica         .A..G-.GC- ...CG-TG.. ...CA.T-.. .-.A....A. G....C.G.A ......-G..
Gl. campestris      .A..G-.GC- ...CG-.G.. AC.C-..-.. .-.A.TTT.. .....C.-C. ......-G..
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					EMBL accession #		
					ITS1	ITS2	Voucher
G. *altaica*	GCATGA--TC	T-TCACGACG	AAT-GCCACG	ACCGC	Z71931	Z71932	Y95-49
G. *aristata*	...ACG--..	G-........	CC.-......	.....	Z48100	Z48116	Y92-328
G. *atlantica*	.....CGACG	--A..TC...	CC.GCG....	...A.	Z71933	Z71934	Z93-S2
G. *boryi*	.....C-A..	G-......G.	CG.-......	.....	Z48111	Z48118	Z93-S1
G. *crassuloides*	....-C-G..	G-........	CC.GC-....	.....	Z71935	Z71936	Y92-265
G. *flexicaulis*	....-C-G..	G-........	CC.T......	.....	Z71937	Z71938	Y92-264
G. *heleonastes*	-ATGAC-G..	.-........	CC.-......	.....	Z71939	Z71940	G032
G. *intricata*	AG....C-..	G-...N....	CC.-......	.....	Z71945	Z71946	Y92-198
G. *pantheica*	.ATCA--G..	G-...T....	C..-......	.....	Z71953	Z71954	Y92-248
G. *piasezkii*	A..C.T--G.	G-........	C..-......	.....	Z71955	Z71956	Y92-272
G. *pseudoaquatica*	A.T----TG.	G-........	CC.-......	.....	Z71959	Z71960	Y92-326
G. *pyrenaica*	......-G..	.-........	C..GC-....	.....	Z48068	Z48087	Y93-14
G. *squarrosa*	ATCGA---N.	G-........	CC.-......	.....	Z71965	Z71966	G046
G. *crenulato-*	....--CGC.	G-........	CG.GC-....	.....	Z48098	Z48079	Y92-310
G. *haynaldii*	.....C-G..	G-........	CG.GC-....	.....	Z48065	Z48085	Y92-201
G. *hyalina12*	--....NG..	A-........	CC.-......	.....	Z71941	Z71942	Y93-36
G. *hyalina30*	A--.ACCAC-	--.T......	CC.-......	.....	Z71943	Z71944	Y92-89
G. *ludlowii16*	-ATGAN-GC-	--G.......	CC.-......	.....	Z71947	Z71948	Y92-35
G. *ludlowii32*	...A--TG.G	C-G.....T.	C..-......	.....	Z71951	Z71952	Y92-33
G. *ludlowii22*	-TCGA-NG..	A-........	CC.-......	.....	Z71949	Z71950	Y92-99
G. *producta*	....-C-G..	--.T......	CC.GC-....	.....	Z71957	Z71958	Y93-79
G. *pudica*	-.CAN-GTC-	--A.......	CC.-......	.....	Z71961	Z71962	G178
G. *sedifolia*	.....-CG..	G-........	CC.GC-....	.....	Z71963	Z71964	A94-S3
G. *tetrasticha*	.A....CG..	G-........	CC.-......	.....	Z71967	Z71968	Y92-128
G. *bavarica*	....AC-G..	G-........	TC.-......	.....	Z48094	Z48075	Y93-11
G. *clusii*	....AC-G..	G-........	TC.-......	.....	Z48097	Z48077	Y93-13
G. *lutea*	T.GA.-CG..	G-........	CC.-......	.....	Z48122	Z48119	Y91-S5
G. *algida*	...A.C-G..	G-........	TC.-......	.....	Z48142	Z48117	Y91-S10
G. *callistantha*	....--TG..	G-........	T..-......	.....	Z48095	Z48078	Y92-298
G. *urnula*	.....-TG..	G-........	T..-......	.....	Z48071	Z48090	Y92-71
G. *delavayi*	.....CTG..	G-...T.-..	T..-......	.....	Z48099	Z48080	Y92-229
G. *affinis*	...C.C-G.T	G-........	CC.GC-....	.....	Z48061	Z48074	Y91-S1
G. *straminea*	...----GCA	.-GT......	T-.-......	.....	Z48070	Z48091	Y92-313
G. *rhodantha*	...A.C-A..	GA........	CT.-..T...	.....	Z48069	Z48089	Y93-124
C. *tibetica*	.....C-G..	G-...T..T.	CC.-..T...	..T..	Z48145	Z48123	Y93-121
Gl. *campestris*	...C.C-A..	G-........	CT.-..T...	.....	Z48104	Z48128	K83-G1

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Phylogenetic Use of Noncoding Regions in the Genus *Gentiana* L.: Chloroplast *trn*L (UAA) Intron versus Nuclear Ribosomal Internal Transcribed Spacer Sequences

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Sequence divergence was estimated within noncoding sequences of both chloroplast DNA (cpDNA) *trn*L (UAA) intron and nuclear ribosomal DNA (nrDNA) internal transcribed spacer sequences (ITS1 and ITS2) for 10 species of the genus *Gentiana* L. (Gentianaceae). Comparisons of evolutionary rates among these sequences (cpDNA versus nrDNA, ITS1 versus ITS2) were performed. It appears that sequence divergence is on average two to three times higher in ITSs than in the *trn*L intron sequences and higher in ITS1 than in ITS2. Both the cpDNA intron and ITSs of nrDNA give concordant phylogenetic trees. However, the ITS-based phylogeny displays higher bootstrap values. At the intrageneric level, at least in *Gentiana*, ITSs (especially ITS2) sequences seem to be more appropriate in the assessment of plant phylogenies. Nevertheless, the cpDNA *trn*L intron seems to be preferable at the intergeneric level. © 1996 Academic Press, Inc.

INTRODUCTION

Comparative analysis of homologuous DNA sequences represents an expanding area of plant systematics and evolution. The recent development of the PCR and direct sequencing of PCR products has resulted in an accumulation of sequence data which facilitates the study of plant phylogenies (reviewed by Olmstead and Palmer, 1994). Both chloroplast DNA (cpDNA) and nuclear ribosomal DNA (nrDNA) have been used to infer phylogenies at different taxonomic levels (reviewed by Palmer *et al.*, 1988; Hamby and Zimmer, 1992). Coding regions have been widely used at higher taxonomic levels (family, tribe, etc.), but they are less informative at lower taxonomic levels since they are highly conservative. Therefore, there is now a growing interest in the analysis of noncoding sequences at lower taxonomic levels such as genus and/or species.

Within cpDNA, the slowly evolving gene *rbc*L, which encodes the large subunit of RUBISCO, has been widely sequenced for over 1000 plant species (Olmstead and Palmer, 1994) and this has permitted the assessment of phylogenetic relationships at higher taxonomic levels (e.g., Chase *et al.*, 1993, and references therein). Although some phylogenies were addressed at inter- and intrageneric levels (see Conti *et al.*, 1993; Gadek and Quinn, 1993; Kron and Chase, 1993; Price and Palmer, 1993; Soltis *et al.*, 1993; Xiang *et al.*, 1993), *rbc*L is in some instances too conserved to resolve phylogenetic relationships at these lower taxonomic levels (e.g., Doebley *et al.*, 1990; Gaut *et al.*, 1992; Kim *et al.*, 1992; Soltis *et al.*, 1993; Xiang *et al.*, 1993). Currently the focus is on more rapidly evolving sequences, such as the genes *mat*K (Johnson and Soltis, 1994; Steele and Vilgalys, 1994) and *ndh*F (Scotland *et al.*, 1995), or some noncoding sequences, such as the intergenic spacers between *rbc*L and *atp*B (e.g., Golenberg *et al.*, 1993; Spichiger *et al.*, 1993; Ehrendorfer *et al.*, 1994; Manen *et al.*, 1994; Savolainen *et al.*, 1994) and between *trn*L (UAA) and *trn*F (GAA) (e.g., Böhle *et al.*, 1994; Gielly and Taberlet, 1994a,b; Mes and 't Hart, 1994; van Ham *et al.*, 1994) or the *trn*L (UAA) intron (e.g., Ferris *et al.*, 1993; Böhle *et al.*, 1994; Gielly and Taberlet, 1994a,b; van Ham *et al.*, 1994; Gielly and Taberlet, 1996).

When studying the nuclear genome, the internal transcribed spacer (ITS1 and ITS2) sequences of 18S–25S nrDNA are frequently used to assess plant phylogenetic relationships, due to (i) their small size and high copy number, which make them suitable for direct sequencing of PCR products, (ii) their rapid concerted evolution, and (iii) their higher amount of sequence divergence compared to that of their flanking coding regions (Hamby and Zimmer, 1991). Phylogenies have been successfully obtained at the intergeneric level (e.g., Baldwin, 1992; Suh *et al.*, 1993; Yuan and Küpfer, 1995) and even the intrageneric level (e.g., Baldwin, 1993; Ritland *et al.*, 1993; Wojciechowski *et al.*, 1993; Kim and Jansen, 1994; Nickrent *et al.*, 1994; Yuan *et al.*, 1996).

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Few comparisons of the evolutionary rates of both cpDNA and nrDNA have yet been done. Wolfe *et al.* (1987) previously showed that cpDNA evolves half as fast as nuclear DNA. In *Krigia* (Asteraceae), based on restriction fragment length polymorphisms (RFLP), it appears that nrDNA evolves 40 times faster than cpDNA. However the rapid evolution of nrDNA sequences is mostly due to variations in the intergenic spacer (IGS) sequences (Kim and Mabry, 1991). Sequencing of the ITS region of *Astragalus* (Fabaceae) (Wojciechowski *et al.*, 1993), *Dendroseris*, and *Robinsonia* (Asteraceae) (Sang *et al.*, 1994, 1995) indicated sequence divergence values 10, 38, and 65 times greater, respectively, than those estimated for cpDNA via restriction site mapping. In the Winteraceae, estimated rates of nucleotide substitutions are $3.2–5.2 \times 10^{-10}$ substitutions per site per year for ITS1 and $3.6–5.7 \times 10^{-10}$ for ITS2 and are similar to the rate of $4.2–4.9 \times 10^{-10}$ observed in *rbc*L (Suh *et al.*, 1993).

Within nrDNA, only a few estimates are available concerning the relative evolutionary rates of both spacers. In the Winteraceae, as mentioned above, it seems that ITS1 and ITS2 show the same substitution rate (Suh *et al.*, 1993). In the Asteraceae, ITS1 evolves 1.5–2 times faster than ITS2 (Baldwin, 1992; Kim and Jansen, 1994). On the other hand, in the filamentous fungus *Fusarium sambucinum*, the rate of sequence divergence within ITS2 is twofold that of ITS1 (O'Donnell, 1992).

In order to determine whether cpDNA or nrDNA is preferable for the assessment of intrageneric phylogenies, we estimate sequence divergence values within noncoding sequences of both cpDNA (*trn*L intron) and nrDNA (ITS1 and ITS2) for 10 species of the genus *Gentiana* L. (Gentianaceae) and compare evolutionary rates among these sequences (cpDNA versus nrDNA, ITS1 versus ITS2). Phylogenetic trees were also constructed for the same set of species, using *trn*L intron, ITS1, ITS2, and ITSs sequences.

TABLE 1

Species Included in the Study (Following Flora Europaea) and Accession Number in the EMBL Database of the Sequences Analyzed

Section	Species	*trn*L intron	ITS1	ITS2
Gentiana	*G. lutea* L.	X75702	Z48122	Z48119
	G. punctata L.	X77894	Z48066	Z48088
Pneumonanthe	*G. asclepiadea* L.	X77871	Z48083	Z48076
Frigida	*G. frigida* Haenke	X77883	Z48063	Z48084
Chondrophyllae	*G. pyrenaica* L.	X77895	Z48068	Z48087
	G. boryi Boiss.	X77874	Z48111	Z48118
Megalanthe	*G. clusii* Perr. & Song.	X77879	Z48097	Z48077
	G. alpina Vill.	X77868	Z48072	Z48073
Calathianae	*G. verna* L.	X75704	Z48093	Z48120
	G. bavarica L.	X75703	Z48094	Z48075

MATERIAL AND METHODS

Species Analyzed and Sequence Origin

Table 1 summarizes species analyzed in the present study (following Flora Europaea, Tutin *et al.*, 1972) as well as the accession numbers of the relevant sequences in the EMBL database. Sequences of cpDNA (Gielly and Taberlet, 1996) and nrDNA (Yuan *et al.*, 1996) were previously released independently in our two labs for the same species.

Laboratory Procedures

Total DNA was extracted from minimal amounts of fresh, silica gel-dried, or herbarium tissues, according to the protocole of Doyle and Doyle (1990). Amplification, purification, and direct sequencing of PCR products were performed according to the classical protocols (see Gielly and Taberlet, 1994b). Chloroplast *trn*L (UAA) intron was amplified with primers "c" (5′-CGAAATCGGTAGACGCTACG-3′) and "d" (5′-GGGGATAGAGGGACTTGAAC-3′) (Taberlet *et al.*, 1991); ITS1 and ITS2 were amplified with primers YP1 (5′-GGAAGTAGAAGTCGTAACAAGG-3′) and YP2 (5′-GCTGCGTTCTTCATCGATGC-3′), YP3 (5′-GCATCGATGAAGAACGCAGC-3′) and YP4 (5′-TCCTCCGCTTATTGATATGC-3′), respectively. The primers YP1, YP2, YP3, and YP4 described here are equivalent with the primers ITS5, ITS2, ITS3, and ITS4 designed by White *et al.* (1990), respectively, except for a minor modification in YP1 (Yuan and Küpfer, 1995). Single-stranded cpDNA and double-stranded nrDNA were sequenced using the standard dideoxy chain termination technique and ^{35}S-labeled dATP or [γ-^{32}P]ATP 5′ end-labeled primers (for more detailed information see Gielly and Taberlet, 1994b; Yuan and Küpfer, 1995).

Data Analysis

Sequences of *trn*L intron, ITS1, and ITS2 were aligned using the Clustal program (Higgins *et al.*, 1992), with fixed and floating gap penalties of 10 and transitions weighted, and with possible slight manual modifications to optimize the multiple alignments generated. Since no reliable genetic distance can be obtained using both substitutions and insertions/deletions (Gielly and Taberlet, 1994b), we calculated for each pair of species the percentage of mutational events in *trn*L intron, ITS1, and ITS2 by employing the formula of O'Donnell (1992): $P = [(Ts + Tv + ID)/L] \times 100$, where Ts = number of observed transitions, Tv = number of observed transversions, ID = number of observed insertions/deletions (multibase length differences are scored as 1), and L = sequence length ($Ts + Tv + ID$ + number of sites showing the same nucleotide). Such a formula, although it cannot be converted into a genetic distance, permits a comparison of the phylogenetic informations on the relevant sequences (Gielly and Taberlet, 1994b). Regressions of P_{ITS1} and P_{ITS2} over $P_{trnL\ intron}$ were performed using the REG procedure of SAS (1990) to test the null hypothesis of (i) slopes being equal to one, (ii) intercepts being equal to zero, and (iii) the equality of slopes.

Phylogenetic relationships among the 10 species of gentians were inferred via a Fitch parsimony approach, i.e., assuming unordered character states, using a PAUP 3.1.1 branch-and-bound search (Swofford, 1993). Due to the large number of insertions/deletions in these noncoding sequences, two different ways of treating information were employed: (i) gaps were treated as missing data and (ii) the different mutational events (substitutions and insertions/deletions) were coded in a matrix of multistate characters, the nucleotide stretch corresponding to one insertion/deletion (or two overlapping insertions/deletions) being considered as a single site, regardless of its length (Gielly and Taberlet, 1994b). The data sets were then submitted to a bootstrap analysis (1000 replications, branch-and-bound option) (Felsenstein, 1985).

RESULTS

Evolution of cpDNA and nrDNA

Within cpDNA, the average percentage of mutational events is 5.17%, ranging from 1.59% (*Gentiana clusii–Gentiana alpina*) to 9.25% (*Gentiana pyrenaica–Gentiana verna*). For the same set of species, the average sequence divergence for ITS1 is 15.36%, ranging from 6.49% (*G. pyrenaica–G. boryi*) to 23.75% (*G. boryi–G. verna*); the average for ITS2 is 10.15%, ranging from 3.43% (*G. clusii–G. alpina*) to 16.45% (*G. boryi–G. bavarica*). If we combine ITS1 and ITS2 sequences, the average percentage of mutational events is 12.76%, the lowest value being 6.42% for *G. clusii–G. alpina,* and the highest value being 19.66% for *G. boryi–G. verna.* Insertions/deletions represented 38.46, 23.66, and 11.69% of the divergence in the *trn*L intron, ITS1, and ITS2, respectively. In this study, length mutations observed are not attributable to variation in number of tandemly repeated sequences (multiple alignments are available upon request to the authors).

A total of 135 pairwise comparisons were made: 45 between the *trn*L intron and ITS1, 45 between the *trn*L intron and ITS2, and 45 between ITS1 and ITS2. Pairwise comparisons of nuclear ribosomal ITS1 and ITS2 over the chloroplast *trn*L (UAA) intron are plotted in Fig. 1. In all cases, divergence within ITSs sequences is higher than that within the *trn*L intron. On average, ITS1 evolves 2.97 times faster than the *trn*L intron, ranging from 1.39 (*Gentiana frigida–G. boryi*) to 7.68 times (*Gentiana lutea–G. alpina*). If we compare the *trn*L intron with ITS2, it appears that the evolution of ITS2 is, on average, 1.96 times faster than that of cpDNA, the range being from 1.24 (*G. frigida–G. pyrenaica*) to 5.79 times (*G. lutea–G. alpina*). Pooling ITS1 and ITS2 sequences showed an evolution of ITSs that is 2.47 times that of cpDNA, ranging from 1.32 (*G. frigida–G. pyrenaica*) to 6.72 times (*G. lutea–G. alpina*). In most situations, sequence divergence in ITS1 is higher than that in ITS2 (Fig. 1). In both regressions (P_{ITS1} and P_{ITS2} over $P_{trnL\ intron}$), the slopes are not significantly different from one ($P > 0.05$ in both cases, values of *t* test being 0.782 and 0.660, respectively); the intercepts are significantly different from zero ($P < 0.001$ in both cases); the two slopes are not significantly different from each other (P = 0.682) (Fig. 1).

Phylogenetic Analyses of cpDNA and nrDNA Sequences

The two different ways of treating gap information in cpDNA sequences resulted, via the branch-and-bound search of PAUP, in four (gaps treated as missing) and three (substitutions and gaps coded in a matrix) most parsimonious trees. *G. frigida* was taken as the outgroup since in a cpDNA based phylogeny it formed the most isolated clade of the European gentians (Gielly and Taberlet, 1996). Strict consensus trees with bootstrap values are presented in Fig. 2 (tree lengths and consistency indexes are reported in Table 2). The two different gap treatments gave congruent tree topologies, especially regarding the position of *Gentiana asclepiadea,* which always grouped with *G. lutea* and *Gentiana punctata,* and also regarding the internal position of *G. pyrenaica* and *G. boryi* of section *Chondrophyllae.*

For nrDNA, whatever the regions analyzed (ITS1, ITS2, or ITS1 + ITS2) or the way of treating gap information, the branch-and-bound search of PAUP always generated a single most parsimonious tree (Fig. 3; for length and consistency indexes see Table 2). In this tree, as in cpDNA trees, *G. asclepiadea* always grouped with species of section *Gentiana.* However, section *Chondrophyllae* (*G. pyrenaica* and *G. boryi*) nested outside the rest of the species. Note that we obtained the same topology for the cpDNA-based phylogenetic trees by enforcing a topological constraint (section *Chondrophyllae* nesting outside) and tree lengths were increased by only one step when treating gaps as missing data and by only two steps when including insertions/deletions (Table 2).

DISCUSSION

The Differential Level of Sequence Divergence in the Three Sequences Analyzed

The results reported here clearly show that sequence divergence is higher in ITSs sequences of nuclear ribosomal DNA than in the noncoding region of chloroplast

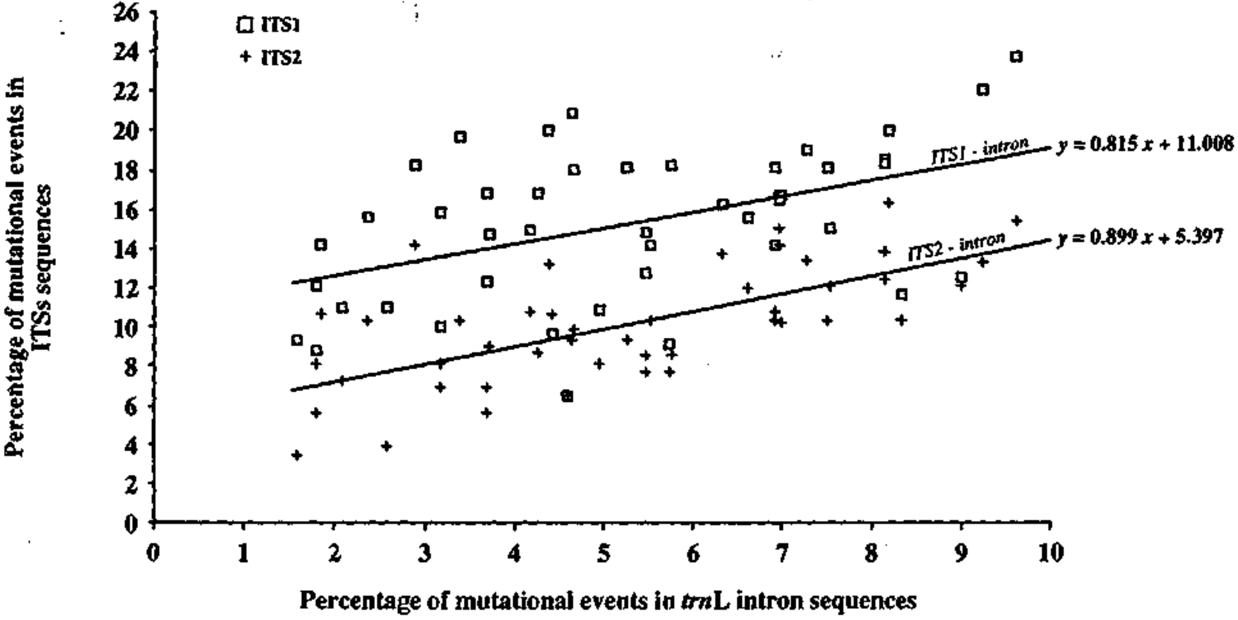

FIG. 1. Relationships among the percentages of mutational events in cpDNA and nrDNA noncoding sequences.

DNA (2–3 times on average). This is less than the rate of nrDNA observed in *Astragalus*, which is 10 times higher than that of cpDNA (Wojciechowski *et al.*, 1993), and much less than the 40-, 38- and 65-fold rates observed for *Krigia* (Kim and Mabry, 1991), *Dendroseris* (Sang *et al.*, 1994), and *Robinsonia* (Sang *et al.*, 1995), respectively. But this result is in contradiction with Suh *et al.* (1993), who estimated nucleotide substitutions rates for ITSs which appear to be similar to that of *rbc*L in the Winteraceae.

Even between the two spacers of nuclear ribosomal DNA, the sequence divergence is higher in ITS1 than in ITS2 in *Gentiana* (1.51 times on average). This result is consistent with the case of the Asteraceae, where ITS1 was found to change 1.5 to 2 times faster than ITS2 (Baldwin, 1992; Kim and Jansen, 1994), but op-

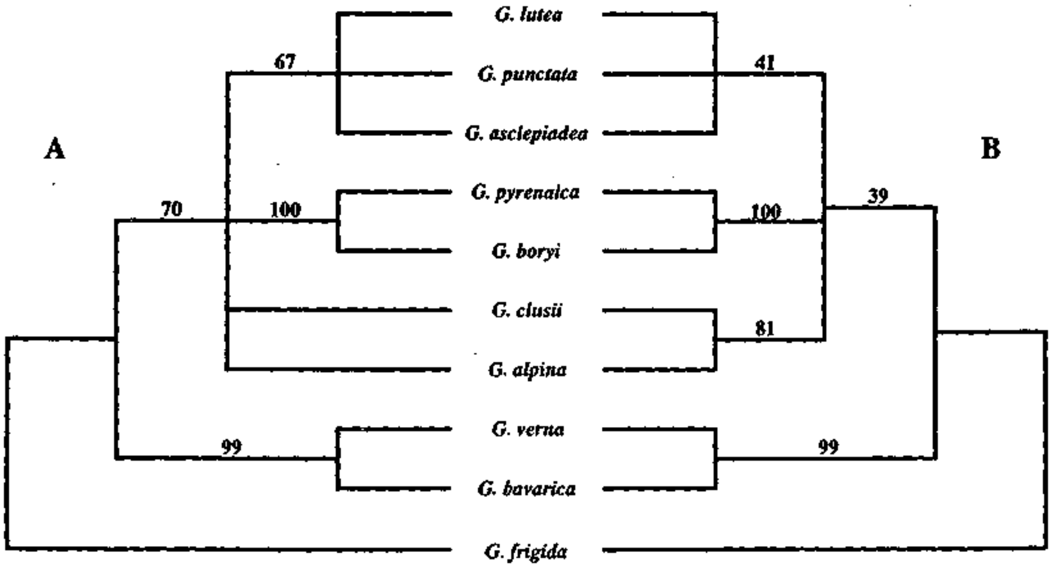

FIG. 2. Strict consensus of the trees retained by the branch-and-bound search algorithm of PAUP based on *trn*L intron sequences analyses: (A) gaps were treated as missing data (four trees produced) and (B) gaps and substitutions were coded in a multistate characters matrix (three trees produced, see text for explanations). Numbers indicate bootstrap estimates from 1000 replications. Tree lengths and consistency and retention indexes are reported in Table 2.

TABLE 2

Number, Length, Consistency Index, and Retention Index for the Trees Produced by the Branch-and-Bound Search Algorithm of PAUP

	Gaps as missing data				Gaps and substitutions coded			
Region analyzed	N	L	CI	RI	N	L	CI	RI
*trn*L intron	4	47	0.957	0.909	3	76	0.921	0.793
*trn*L intron[a]	1	48	0.938	0.864	6	78	0.897	0.724
ITS1	1	121	0.860	0.738	1	150	0.860	0.720
ITS2	1	53	0.755	0.755	1	99	0.879	0.760
ITS1 + ITS2	1	208	0.861	0.746	1	249	0.867	0.736

Note. N, number of trees retained by the branch-and-bound search algorithm of PAUP; L, tree length; CI, consistency index; RI, retention index.

[a] Search with topological constraint enforced, i.e., species of section *Chondrophyllae* nesting outside the rest of the species.

poses results for to the ascomycete *F. sambucinum*, where ITS2 evolves 2 times faster than ITS1 (O'Donnell, 1992).

Despite that ITSs display higher sequence divergences than *trn*L intron, we cannot assert that ITSs evolve significantly faster than *trn*L intron (the slopes of the regression lines presented in Fig. 1 are not significantly different from 1). The reasons for the differential level of sequence divergence of noncoding sequences among these two genomes, and between the two spacers of nrDNA, are not yet clear.

Phylogenetic Resolution of cpDNA versus nrDNA

The results indicate that both cpDNA- and nrDNA-based phylogenies are principally congruent with morphological classification. The sectional circumscriptions have been respected. The resolutions of cpDNA and nrDNA are mainly in agreement for most clades, e.g., the placement of *G. asclepiadea* which nested with species of the section *Gentiana* (Figs. 2 and 3; see also Gielly and Taberlet, 1996; Yuan *et al.*, 1996). Such congruence between phylogenies based on the two genomes has also been reported in Compositae (Baldwin, 1992) and in Fabaceae (Wojciechowski *et al.*, 1993). However, in our present study, disagreement between the cpDNA- and nrDNA-based phylogenies has also been revealed: the phylogenetic relationships of section *Chondrophyllae* with other clades are presented differently in the cpDNA (Fig. 2) versus nrDNA phylogenies (Fig. 3). Similar disagreement has been observed in *Krigia* (Kim and Jansen, 1994). However, in the present case, similar topologies for nrDNA and suboptimal cpDNA trees were obtained by enforcing a topological constraint in the cpDNA data set which increased tree lengths by only one or two steps (Swofford, 1991). For the set of species analyzed here, nrDNA seems to give higher bootstrap values than cpDNA at the intrageneric level. Referring to the two ITSs sequences, it appears that the phylogenies constructed from ITS1, ITS2, and ITS1 + ITS2 exhibit exactly the same topology (Fig. 3), although ITS2 showed lower divergence than ITS1.

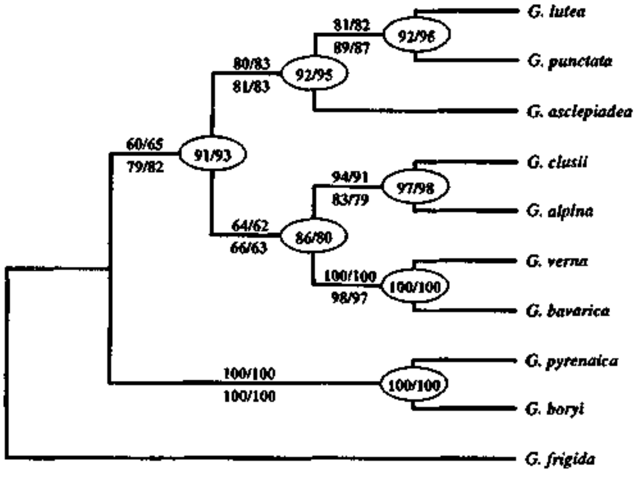

FIG. 3. Single most parsimonious tree deduced from ITSs sequences analyses (ITS1, ITS2, and ITS1 + ITS2) via the branch-and-bound search algorithm of PAUP for the two distinct ways of treating information (gaps treated as missing data/gaps and substitutions coded in a multistate character matrix, see text for explanations). Numbers on the tree indicate bootstrap estimates from 1000 replications: (i) above branches, analysis of ITS1 sequences, (ii) below branches, analysis of ITS2 sequences, and (iii) circles on nodes, analysis of combined ITS1 and ITS2 sequences. Tree lengths and consistency and retention indexes are reported in Table 2.

Phylogenetic Application of cpDNA versus nrDNA

Noncoding sequences are generally expected to reveal more information than coding regions at lower taxonomic levels. Some existing studies on both noncoding sequences of cpDNA (e.g., Golenberg *et al.*, 1993; Böhle *et al.*, 1994; Gielly and Taberlet, 1994b; Savolainen *et al.*, 1994) and internal transcribed spacer sequences of nrDNA (e.g., Baldwin, 1992, 1993; Kim and Jansen, 1994; Yuan and Küpfer, 1995) have demonstrated their usefulness in resolving plant phylogenies at inter- and intrageneric levels. Our present analyses support that conclusion. As can be seen from these results, both the cpDNA intron and nrDNA ITSs exhibit valuable resolution at specific levels (Figs. 2 and 3). However, the relative informativeness of cpDNA seems to be lower at this taxonomic level than ITSs: the relationships among *G. lutea*, *G. punctata*, and *G. asclepiadea* were not clearly resolved in cpDNA trees due to lower divergence of the cpDNA intron sequences (Fig. 2), but were resolved in the ITS-based trees (Fig. 3). This suggests that, at least in *Gentiana*, cpDNA *trn*L (UAA) intron sequences may be more appropriate at higher taxonomic levels, such as intergeneric, and ITSs sequences are preferable in assessing phylogeny at lower taxonomic levels, such as interspecific. The reason is that the cpDNA intron displayed less sequence divergence at intrageneric level and the level of homoplasy is low in cpDNA sequences at intra- and inter-generic levels (higher consistency index of the trees, Table 2; see also Gielly and Taberlet, submitted for publication), and that higher sequence divergence in ITSs corresponds to a higher level of homoplasy which can represent a limitation in their use at higher taxonomic levels (Kim and Jansen, 1994; Yuan and Küpfer, 1995).

Within *Gentiana*, ITS1 exhibits higher sequence divergence than ITS2 (Table 2), but gives a similar (the present results) or lower (Yuan *et al.*, in press) resolution than ITS2, since both higher sequence divergence and higher homoplasy level have been involved in this region. A similar situation has also been reported in *Calycadenia* (Asteraceae) (Baldwin, 1993) and *Arceuthobium* (Viscaceae) (Nickrent *et al.*, 1994), where the percentage of potentially informative sites is higher for ITS2. So we suppose that ITS2 alone could be sequenced for intrageneric phylogenies without losing any important phylogenetic resolution.

It is difficult to define a general rule for the choice of a particular genome (cpDNA or nrDNA) or a particular region of the given genome (e.g., ITS1 versus ITS2) to address plant phylogenies. Sequence divergences can vary greatly in chloroplast DNA, depending on genera (Gielly and Taberlet, 1994a) and even within a genus (Gielly and Taberlet, unpublished), and that could be the case for nuclear DNA also. So, due to the heterogeneity of evolutionary rate of noncoding sequences of cpDNA and also nrDNA, it is quite impossible to predict if the selected sequence(s) would evolve at a sufficient rate to provide enough resolution at the considered taxonomic level. Therefore, a preliminary study should be performed before a large-scale analysis is carried out.

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