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The phylogeny of *Gentianella* (Gentianaceae) and its colonization of the southern hemisphere as revealed by nuclear and chloroplast DNA sequence variation

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Abstract

The generic circumscription and infrageneric phylogeny of *Gentianella* was analysed based on *mat*K and ITS sequence variation. Our results suggested that *Gentianella* is polyphyletic and should be limited to species with only one nectary per petal lobe. *Gentianella* in such a circumscription is most closely related to one part of a highly polyphyletic *Swertia*. Within uninectariate *Gentianella* two major groups could be recognized: 1) northern hemispheric species with vascularized fimbriae at the base of the corolla lobes, and 2) northern hemispheric, South American, and Australia/New Zealand species without vascularized fimbriae. When fimbriae are present in this latter group, they are non-vascularized. Whereas ITS data suggested a sister group relationship between the fimbriate and efimbriate group, the *mat*K data suggested paraphyly of the efimbriate group with Eurasian efimbriate species as sister to the remainder of the clade. Based on the phylogeny and using geological and fossil evidence and a molecular clock approach, it is postulated that the efimbriate lineage originated in East Asia near the end of the Tertiary. From East Asia it spread via North America to South America, and from there it reached Australia/New Zealand only once by a single long-distance dispersal event. The place of origin of the fimbriate lineage remained doubtful. The high specific diversity of *Gentianella* in South America probably resulted mainly from the availability of a very large alpine area open to colonization rather than from particularly high speciation rates in comparison to other taxa.

Keywords: *Gentianella*, flower morphology, biogeography, long-distance dispersal, molecular clock, speciation rate

Introduction

Gentianella Moench (Gentianaceae-Gentianeae-Swertiinae) in its present circumscription comprises ca. 256 species (Tab. 1). The genus mostly occurs in alpine or arctic habitats in Eurasia, Northwest Africa, North, Central and South America, Australia, and New Zealand. All species are annual to perennial herbs and range from 3–100 cm in height. The flowers are 0.5–5 cm long, have entire petal margins, and one or two naked nectaries per petal lobe on the upper petal surface. A ring of vascularized or non-vascularized fimbriae in the corolla throat is sometimes present. The length of the corolla tube differs considerably. The corolla is variously coloured and the plants are usually pollinated by insects, but hummingbird pollination occurs in South America (Pringle, 1995). Most species are bisexual but some South American species are dioecious.

The circumscription of *Gentianella* has been subject to major change ever since its establishment. The last complete overview of *Gentianella* was provided by Kusnezow (1895, 1896). He divided his *Gentiana* L. subg. *Gentianella* Kusn. into eight sections. These have since been realigned into four genera (Tab. 2). Although morphological (Smith, 1936; Ma, 1951; Toyokuni, 1961), anatomical (Lindsey, 1940), palynological (Nilsson, 1967), karyological (e.g., Löve, 1953), phytochemical (Massias et al., 1982; Meszaros, 1994), and molecular (Yuan & Küpfer, 1995) evidence for the segregation of *Comastoma* (Wettst.) Toyok., *Gentianopsis* Ma, and *Megacodon* (Hemsl.) Harry Sm. has been presented, the differences between these genera and *Gentianella* were

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Table 1. Approximate no. of species of *Gentianella* s.l. in different parts of its geographical range and no. of species sampled.

	efimbriate	included in this study	fimbriate	included in this study	binectariate	included in this study
Australia/ New Zealand	32	7	–	–	–	–
South America	ca. 170	16	3 (non-vascularized)	1	–	–
North America	1 excl. *G. microcalyx* (see text)	1	ca. 8 incl. *G. microcalyx* (see text)	4	–	–
North America and Eurasia	1	1	2	2	–	–
Europe/Flora of northern GUS	2	1	ca. 9 + many microspecies	5	–	–
East/Central/West Asia	11	4	4	3	11	4
Northwest Africa	1	phylogenetic relationships unknown				

Table 2. Sectional subdivision of Kusnezow´s *Gentiana* subg. *Gentianella*, sectional heterogeneity, current names or groups as revealed by the molecular data

sectional subdivision of Kusnezow´s *Gentiana* subg. *Gentianella*	sectional heterogeneity	current name or clade in ITS tree
sect. *Megacodon*		*Megacodon*
sect. *Stylophora*		*Megacodon*
sect. *Crossopetalum*		*Gentianopsis*
sect. *Imaicola*		*Gentianopsis*
sect. *Amarella*	non-vascularized fimbriae arising from two scales	*Comastoma*
	G. thomsoni (syn. *G. pygmaea*)	binectariate (?) *Gentianella*
	G. moorcroftiana, *G. azurea*	binectariate *Gentianella*
	fimbriae separate, vascularized	*Gentianella* s.str.
	fimbriae separate, non-vascularized	*Gentianella* s.str.
sect. *Arctophila*	*G. arenaria*	binectariate *Gentianella*
	G. pygmaea	binectariate (?) *Gentianella*
	G. wislizenii	*Gentianella* s.str.
	efimbriate and uninectariate	*Gentianella* s.str.
sect. *Andicola*		*Gentianella* s.str.
sect. *Antarctophila*		*Gentianella* s.str.

sometimes regarded as not sufficient to justify generic rank (e.g., Aitken, 1999).

The remaining elements in *Gentianella* are sects. *Andicola* Griseb., *Antarctophila* Griseb., and *Arctophila* Griseb., and part of sect. *Amarella* Griseb. (= sect. *Endotrichae* Froel. p. p.) and are called *Gentianella* s.l. in the following. Of these four groups sect. *Amarella*, including the type species *G. campestris* (L.) Börner, is distinct based on vascularized fimbriae at the base of the corolla lobes (absent in *G. moorcroftiana* [Wall. ex G. Don] Airy Shaw, *G. azurea* [Bunge] Harry Sm. and *G. thomsoni* [C. B. Clarke] U. C. Bhattach.). The other three sections are only weakly characterized by morphological characters such as habit and absence/presence of hairs at the base of the filaments as well as geographical distribution. Little is known about *Gentianella* sect. *Pseudendotricha* Litard. & Maire from Northwest Africa which consists of *Gentianella tornezyana* Litard. & Maire only (Litardiére & Maire, 1924). This species was not considered in the present analysis.

Segregates of *Gentianella* s.l. include *Aliopsis* Omer & Qaiser, *Aloitis* Raf., *Arctogentia* A. Löve & D. Löve, *Chionogentias* L. G. Adams, *Eudoxia* D. Don ex G. Don, *Glyphospermum* D. Don ex G. Don, *Kurramiana*

Omer & Qaiser, *Pitygentias* Gilg, *Selatium* D. Don ex G. Don, and *Ulostoma* D. Don ex G. Don. Most of these genera were distinguished from *Gentianella* s.l. because they lack the fimbriae present in the type species *G. campestris*. Although *Gentianella* s.l. in its present circumscription is a clearly heterogeneous group, serious doubts have been expressed on whether the segregation of smaller genera is justified (e.g., Pringle, 1995) because these segregates were based on regional material only and did not consider intermediate species from other regions.

In the first important overview of the Gentianaceae (Grisebach, 1838, 1845), *Gentianella* (as part of *Gentiana* L.) was classified in tribe Swertieae together with 13 other genera based on calyx and ovary characters. Swertieae was enlarged by Bentham (1876) and further modified by Gilg (1895) who treated this taxon as subtribe Gentianinae. Using molecular characters, Struwe et al. (1998) and Thiv et al. (1999a, b) recently showed that the original concept of Swertieae by Grisebach/Bentham rather than that of Gilg was correct. The group was renamed as tribe Gentianeae and is one of six tribes of the Gentianaceae (Struwe et al., in prep.).

In molecular phylogenetic studies by Yuan & Küpfer (1995) and Struwe et al. (1998), Gentianeae were subdivided into two lineages, subtribe Gentianinae containing *Crawfurdia* Wall., *Gentiana*, and *Tripterospermum* Blume, and subtribe Swertiinae with *Gentianella* s.l. and all other genera sampled in these analyses (*Bartonia* Willd., *Comastoma*, *Frasera* Walter, *Gentianopsis*, *Halenia* Borkh., *Jaeschkea* Kurz., *Lomatogonium* A. Braun, *Megacodon*, *Obolaria* L., *Pterygocalyx* Maxim., *Swertia* L.). Earlier studies, including additional genera (*Latouchea* Franch., *Lomatogoniopsis* T. N. Ho & S. W. Liu *Veratrilla* Baill. ex Franch.) and using mainly flower morphological characters (Huxley, 1888; Gillett, 1957; Toyokuni, 1965; Ho & Liu, 1990) arrived at similar results.

In Yuan & Küpfer (1995), *Gentianella* formed a clade together with *Lomatogonium*, *Comastoma* and one species of the apparently polyphyletic *Swertia*. In Struwe et al. (1998), *Gentianella* formed a clade with *Lomatogonium*, *Comastoma* and *Jaeschkea*. In both these molecular studies few species of *Gentianella* s.l. were sampled and several taxa potentially closely related to *Gentianella* s.l. were not included (e.g., *Jaeschkea* in Yuan & Küpfer, 1995), and support of clades was weak to intermediate.

The phylogeny of *Gentianella* is of considerable interest from a biogeographical point of view. The genus is a prominent representative (170 spp. in South America [Gilg, 1916; Pringle, 1995]) of those plant taxa which have been postulated to have colonized the high mountain ranges of South America from North temperate regions. This geographical element is believed to constitute at least 30.6% (11% holarctic, 19.6% widely temperate) of the South American high mountain flora (Cleef, 1979). Other genera with northern hemispherical origin in South America are, e.g., *Berberis* L., *Ribes* L., *Potentilla* L., *Lupinus* L., *Salvia* L. and *Draba* L. Until now mainly palynological studies have been used to estimate the time of colonization and migration routes of plant taxa arriving in South America from the north (e.g., van der Hammen, 1979; Hooghiemstra, 1994). An important result of these studies was that different taxa arrived at different times from the late Pliocene onwards well into the Quaternary. However, many taxa including *Gentianella* are not well-suited for palynological investigations because their pollen is only rarely found. *Gentianella* to our knowledge has been found only in lower Pleistocene (van der Hammen, 1979) and Holocene sediments (Hansen & Rodbell, 1995) of northern South America. Detailed molecular studies investigating the mode and time of arrival of plant taxa in South America do not exist.

Also interesting from a biogeographical point of view is the disjunct distribution of *Gentianella* in Australia/New Zealand and South America. There exists a long list of taxa with such a distribution pattern (Moore, 1972; Thorne, 1972). For some taxa this pattern has been attributed to continental drift. For others, however, long-distance dispersal between continents in either direction has been postulated (Smith, 1986).

In summary, the traditional morphological and recent molecular studies have not succeeded in clarifying the exact phylogenetic position of *Gentianella* s.l. in Swertiinae. Also, the generic circumscription of *Gentianella* and its evolution remain unclear. In this study we provide both a new circumscription of the genus, called *Gentianella* s.str. in the following, and a well-supported hypothesis of its phylogenetic position in the Swertiinae. The intrageneric phylogeny is analysed and used to interpret the variation of flower morphological characters. Based on this phylogeny, we provide a hypothesis on the biogeography of this widely distributed genus and compare the extensive radiation of *Gentianella* s.str. in South America to that of other plant taxa.

Material and Methods

Taxon sampling

We divided our phylogenetic analysis into two parts and sampled accordingly (Tab. 3). Analysis 1 was designed to study the phylogenetic placement of *Gentianella* s.l. in the Swertiinae and to give insights into the intrageneric position of the taxonomic groups described in *Gentianella* s.l. We sampled taxa representing much of the morphological and geographical variation of Swertiinae with particular attention to those taxa identified as close relatives of *Gentianella* by Yuan

Table 3. Plant material and genebank accession numbers

taxon	collector and number	date	herbarium + Nr.	*mat*K1	*mat*K2	ITS 1	ITS 2
Comastoma cyananthiflorum (Franch.) Holub	CEE-88	8.9.1991	E 00025334	AJ406324	AJ406353	AJ294585	AJ294645
Crawfurdia speciosa Wall. ***	KEKE 1244	5.10.1989	K	AJ010512	AJ011441	AJ294586	AJ294646
Frasera albicaulis Douglas ex Griseb.	K. Gutsche 20	25.6.1995	MJG	AJ406325	AJ406354	AJ294587	AJ294647
Gentiana frigida Haenke ***	cult. in Schachen Bot. Garden, Germany	1995		AJ388166	AJ388236	AJ294588	AJ294648
Gentianella amarella EUR (L.) Börner	W. J. Schrenk	22.8.1967	FR			AJ294589	AJ294649
Gentianella amarella NA1 (L.) Börner	W. J. Corby, W. J. Wojtas	21.8.1979	E 00025297			AJ294590	AJ294650
Gentianella amarella NA2 (L.) Börner	E. W. Sullivan	22.8.1974	FR	AJ406326	AJ406355	AJ294591	AJ294651
Gentianella angustiflora Harry Sm.	Edinburgh Makalu Expedition 430	30.9.1991	E 00025322	AJ406327	AJ406356	AJ294592	AJ294652
Gentianella arenaria (Maxim.) T. N. Ho	T. N. Ho, B. Bartholomew, M. Gilbert 435	28.7.1993	E 00025340	AJ406328		AJ294593	AJ294653
Gentianella aspera (Hegetschw.) Dostál ***	K. Gutsche 45	8.9.1995	MJG	AJ010517	AJ011446	AJ294594	AJ294654
Gentianella astonii (Petrie) T. N. Ho & S. W. Liu	provided by D. Glenny/Lincoln/NZ						
Gentianella aurea (L.) Harry Sm.	H. Smith 4131	31.7.1963	E 00025348	AJ406329	AJ406357	AJ294595	AJ294655
Gentianella auriculata (Pall.) J. M. Gillett	C. Tpyrgeba	15.8.1974	K	AJ406330	AJ406358	AJ294596	AJ294656
Gentianella austriaca (A. Kern.) Holub	cult. in Mainz Bot. Garden, Germany	1995	MJG			AJ294597	AJ294657
Gentianella azurea (Bunge) Harry Sm.	T. N. Ho, B. Bartholomew, M. Gilbert 1312	17.8.1993	E 00025339	AJ406331	AJ406359	AJ294598	AJ294658
Gentianella bellidifolia (Hook.f.) Holub ***	cult. in Edinburgh Bot. Garden, Scotland	1995	19932974	AJ388162	AJ388232	AJ294599	AJ294659
Gentianella biebersteinii (Bunge) Holub **						Z48147	Z48126
Gentianella campestris (L.) Börner **						Z48104	Z48128
Gentianella canosoi G. L. Nesom & B. L. Turner	S. Gonzales, S. Acevedo 2033	4.10.1981	TEX	AJ406332	AJ406360	AJ294600	AJ294660
Gentianella caucasea (Lodd. ex Sims) Holub	J. C. Archibald 8208	29.8.1986	E 00025347			AJ294601	AJ294661
Gentianella cerastioides (Kunth) Fabris ***	R. Greissl	1995	MJG	AJ010518	AJ011447	AJ294602	AJ294662
Gentianella cerina (Hook.f.) T. N. Ho & S. W. Liu	provided by D. Glenny/Lincoln/NZ						
Gentianella cernua (Kunth) Fabris	C. Viteri 4410	1988	MO			AJ294603	AJ294663
Gentianella cosmantha (Griseb.) J. S. Pringle	J. G. Haukes, J. P.Hjirting, K. Rahn 3569	24.2.1966	L 424359	AJ406333	AJ406361	AJ294604	AJ294664
Gentianella diemensis (Griseb.) J. H. Willis	H. Hurka	2.3.1996	MJG	AJ295332	AJ295333	AJ294605	AJ294665
Gentianella fastigiata (Benth.) Fabris	K. Gutsche		MJG			AJ294606	AJ294666
Gentianella florida (Griseb.) Holub	R. Ehrich 444	5.3.1988	MJG	AJ406334	AJ406362	AJ294607	AJ294667
Gentianella foliosa (Kunth) Fabris	cult. in Kew Bot. Garden, England	1995	1994-508			AJ294608	AJ294668
Gentianella germanica (Willd.) Warb.	J. W. Kadereit	7.1995	MJG	AJ406335	AJ406363	AJ294609	AJ294669
Gentianella hirculus (Griseb.) Fabris	J. L. Clarke 1787	21.5.1996	QCNE			AJ294610	AJ294670
Gentianella holosteoides N. M. Pritchard	Southhampton University 179		K			AJ294611	AJ294671
Gentiana (*Gentianella*) *longicarpa* Gilli *	D. Podlech 12436	17.8.1965	M			AJ294612	AJ294672
Gentianella magellanica (Gaudich.) Fabris	K. Kubitzki, T. Feuerer 99-10	6.2.1999	MJG	AJ406336	AJ406364	AJ294613	AJ294673
Gentianella microcalyx (Lemmon) J. M. Gillett	E. Joyal, J. Enrique 1853	20.10.1991	TEX	AJ406337	AJ406365	AJ294614	AJ294674
Gentianella moorcroftiana (Wall. ex G. Don) Airy Shaw	R. McBeath 2093	11.8.1988	E 00025318	AJ406338	AJ406366	AJ294615	AJ294675
Chionogentias (*Gentianella*) *muelleriana* L. G. Adams *	provided by D. Glenny/Lincoln/NZ						
Gentianella narcissoides (Gilg) T. N. Ho & S. W. Liu	L. Naessany 14	7.4.1989	MJG			AJ294616	AJ294676
Gentianella patula (Kirk) Holub	cult. in Edinburgh Bot. Garden, Scotland	1996	19932978	AJ406339	AJ406367	AJ294617	AJ294677
Gentianella peruviana (Griseb.) Fabris ***	cult. in Edinburgh Bot. Garden, Scotland	1996	19950534	AJ388163	AJ388233	AJ294618	AJ294678
Gentianella propinqua (Richardson) J. M. Gillett	G. Halliday A 333/75	15.7.1975	E 00025300	AJ406340	AJ406368	AJ294619	AJ294679
Gentianella quinquefolia (L.) Small	Bozeman, Ramseur, Radford 45200	10.9.1966	E 00025241	AJ406341	AJ406369	AJ294620	AJ294680

Gentianella rapunculoides (Willd. ex Schult.) J. S. Pringle	R. Greissl 616	2.95	MJG			AJ294621	AJ294681
Gentianella ruizii (Griseb.) Holub	Weigend & Weigend 2000/386	19.2.2000	NY	AJ406342	AJ406370	AJ294622	AJ294682
Gentianella rupicola (Kunth) Holub	cult. in Edinburgh Bot. Garden, Scotland	1995	199930516			AJ294623	AJ294683
Gentianella saxosa (G. Forst.) Holub	Gutsche		MJG	AJ406343	AJ406371		
Gentianella saxosa (G. Forst.) Holub	provided by D. Glenny/Lincoln/NZ						
Gentianella splendens (Gilg) Fabris	J. L. Clarke 1855	12.1995	QCNE	AJ295336	AJ295337	AJ294624	AJ294684
Gentianella stoliczkai (C. B. Clarke) Holub	O. Anders 8178	8.8.1971	M 50043	AJ406344	AJ406372	AJ294625	AJ294685
Gentianella sulphurea (Gilg) Fabris	J. L. Clarke 1833	12.1995	QCNE			AJ294626	AJ294686
Gentianella thyrsoidea (Hook. f.) Fabris	D. N. Smith, F. Escalona 10134	18.3.1986	MO			AJ294627	AJ294687
Gentianella tristicha (Gilg) Fabris ex T. N. Ho & S. W. Liu	D. N. Smith, F. Escalona 10125	18.3.1986	MO			AJ294628	AJ294688
Gentianella umbellata (M. Bieb.) Holub **						Z48102	Z48132
Gentianella wislizenii (Engelm.) J. M. Gillett	M. Lavin 4947	22.9.1984	TEX			AJ294630	AJ294690
Gentianopsis crinita (Froel.) Ma	cult. in Mainz Bot. Garden, Germany	1995	MJG	AJ406345	AJ406373	AJ294631	AJ294691
Halenia palmeri A. Gray ***				AJ388169	AJ388239		
Halenia palmeri A. Gray	K. B. v. Hagen 98/41	11.9.1998	MJG			AJ294632	AJ294692
Jaeschkea oligosperma (Griseb.) Knobl. ***	R. McBeath 2300	3.8.1990	E 00025275	AJ388171	AJ388241	AJ294633	AJ294693
Lomatogonium carinthiacum (Wulfen) Rchb.	V. Zuev 6649	17.8.1985	BR	AJ406346	AJ406374	AJ294634	AJ294694
Lomatogonium oreocharis (Diels) C. Marquand ***	CLD-90 1106	10.10.1990	K	AJ388174	AJ388244	AJ294635	AJ294695
Megacodon stylophorus (C. B. Clarke) Harry Sm. ***	Kuming, Edinburgh, Gothenburgh Exp. 1378	15.6.1993	E 00025279	AJ388177	AJ388247	AJ294636	AJ294696
Swertia crassiuscula Gilg	U. Hecker 1094	13.2.1973	MJG	AJ406347	AJ406375	AJ294637	AJ294697
Swertia franchetiana Harry Sm. **						Z48112	Z48138
Swertia japonica (Schult.) Makino	cult. in Kyoto Bot. Garden, Japan	1996	KYO	AJ406348	AJ406376	AJ294638	AJ294698
Swertia macrosperma (C. B. Clarke) C. B. Clarke	J. H. de Haas 2765	16.9.1974	U 500099	AJ406349	AJ406377	AJ294639	AJ294699
Swertia perennis L. ***	K. Gutsche 56	25.8.1995	MJG	AJ010528	AJ011457		
Swertia perennis L.	K. B. Hungerer	18.8.1995	MJG			AJ294640	AJ294700
Swertia punicea Hemsl.	cult in Edinburgh Bot. Garden, Scotland	1995	19943574	AJ406350	AJ406378	AJ294641	AJ294701
Swertia racemosa (Wall. ex Griseb.) C. B. Clarke	J. H. de Haas 2725	9.9.1974	U 500131	AJ406351	AJ406379	AJ294642	AJ294702
Swertia volkensii Gilg	U. Hecker 1093	13.2.1973	MJG	AJ406352	AJ406380	AJ294643	AJ294703
Veratrilla baillonii Franch. ***	Kuming, Edinburgh, Gothenburgh Exp. 1326	16.6.1993	E 00025273	AJ388196	AJ388266	AJ294644	AJ294704

* These species have not yet been formally transferred to *Gentianella*.
** ITS sequences published in Yuan & Küpfer (1995).
*** *mat*K sequences published in Thiv et al. (1999a) and Struwe et al. (in press).

& Küpfer (1995), Struwe et al. (1998), and Thiv et al. (1999b). We included species of *Gentianella* s.l. from all taxonomic groups described (except sect. *Pseudendotricha*) and from all continents where these groups occur. *Gentiana frigida* Haenke and *Crawfurdia speciosa* Wall. of Gentianinae were used as outgroups (Struwe et al., 1998). Unfortunately, DNA of *Bartonia*, *Latouchea* and *Obolaria* (all three part of Swertiinae) was not available. For analysis 1 the nuclear ITS regions of the rDNA and two adjacent regions of the chloroplast *mat*K gene were sequenced and 43 species (incl. 25 members of *Gentianella* s.l.) were sampled.

For analysis 2 as many species as possible of a redefined (uninectariate) *Gentianella* s.str. representing all parts of its geographical range and most morphological groups recognized were sampled and their nuclear ITS regions were analyzed. Taxa closely related to *Gentianella* s.str. in analysis 1 were used as outgroup in analysis 2. For this analysis we chose nuclear ITS because variation in *Gentianella* s.str. was low in *mat*K. Our sample in analysis 2 comprised 44 accessions of *Gentianella* s.str. plus ten species in the outgroup.

DNA extraction, amplification, sequencing and sequence alignment

Plant material was taken from herbarium specimens or fresh and dried with silica gel. Total genomic DNA was extracted from leaves using the Nucleo Spin Plant Kit (Macherey-Nagel GmbH, Düren, Germany). Amplification reactions were performed with standard concentrations (Palumbi, 1996) and various thermostable polymerases in a water thermocycler (autogrant II). The temperature profile for all *mat*K and ITS amplifications was: 55 °C, 15 s; 72 °C, 1 min; 94 °C 6 s; 32 cycles. Primers (5′-3′) used for the nuclear ITS region were ITS A (ggaaggagaagtcgtaacaagg), ITS B (cttttcctccgcttattgatatg), ITS 2 (gctacgttcttcatcgatgc) and ITS 3 (gcatcgatgaagaacgcagc) following White et al. (1990) and Blattner (1999). Primers used for the amplification of two neighbouring fast-evolving regions of *mat*K were *mat*K 1198F (ctgtgttagatatacgaatacc), *mat*K 1581R (cttgatacctaacataatgcat), *mat*K 1729F (aagggtctatataaagcaatt) and *mat*K 2053R (ttagcrcaagayagtcgaagta) following Thiv et al. (1999a). When possible, all genes were amplified using the external primers. In old plant material, however, internal primers had to be used regularly. The region between *mat*K 1581R and *mat*K 1729F was not available for the majority of species and was excluded from further analysis.

Each PCR product was electrophoresed in agarose and excised and eluted using the Nucleo Spin Extract Kit (Macherey-Nagel GmbH, Düren, Germany). Cycle sequencing reactions were performed with the PCR products, the same primers as used for the PCR reactions and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Norwalk, Connecticut, USA) under conditions as specified by the manufacturer. Products were resolved on ABI PRISM 310 and 377 automated sequencers (Perkin-Elmer, Norwalk, Connecticut). All sequences were edited and a consensus was made of forward and reverse reactions using the computer program Sequencher 3.0 (Gene Codes Corp., Ann Arbor, Michigan, USA). 80% or more positions were determined on both strands. Manual alignment was easy except for small regions of ITS in analysis 1, where multiple insertion/deletion events obscured assessment of homology. Inclusion or exclusion of these resulted in identical topologies. Therefore, these regions were included in the phylogenetic analysis. The limits of both ITS regions were determined following Yuan & Küpfer (1995). The 5.8S rDNA region between ITS1 and ITS2 was removed because it was not available for the majority of taxa (non-overlapping internal primers, not available for EMBL accessions). Where available, it contained only three potentially informative sites supporting clades clearly present in the ITS data alone. Both spacer regions were combined and are called ITS in the following. For *mat*K we started the alignment at that point where all sequences were readable. All sequences are deposited at EMBL (Tab. 3) and all alignments and trees are deposited in TreeBase (submitted).

Maximum parsimony analysis

All sequence data were analyzed using PAUP 4.0 b4a (Swofford, 2000) and the maximum parsimony criterion on a Power Macintosh computer. Computing options unless otherwise stated were: Random taxon addition (100 reps), TBR, steepest = off, mulpars = on, collapse = on, keep = all, indels treated as missing data. For analysis 1, the *mat*K- and ITS-sequences were analyzed separately at first. We conducted bootstrap analyses of the separate datasets using 100 resampled data sets with 10 full heuristic searches using random taxon addition on each. Decay values (Bremer, 1988) were estimated using a converse constraint method. A PAUP command file and the interpretation of the result was computed by AutoDecay 4.0.2 (Eriksson, 1998). We used 10 replicates and turned off mulpars in the PAUP analysis of the command file.

We conducted the partition homogeneity test (Farris et al., 1995) provided by PAUP 4 for a combined data set using 499 random partitions (level of significance $P \leq 0.05$). Calculation options used were similar to the main analysis except that closest addition sequence of taxa was used and the number of cladograms retained was limited to 2000. This partition homogeneity test was also performed omitting most species of *Gentianella* s.str.

Maximum parsimony cladograms and bootstrap and decay support were calculated in a combined analysis as described for the separate analyses. We tested several alternative topologies using Templeton´s significantly less parsimonious test (SLP test) as implemented in PAUP 4 (Templeton, 1983; Johnson & Soltis, 1998). For this purpose we constructed constraint trees in MacClade 3.07 (Maddison & Maddison, 1997), where only the branch of interest was resolved. We then applied the constraints in PAUP 4 and compared the constrained and unconstrained trees using the non-parametric tests in PAUP ($P \leq 0.05$ as the level of significance).

In analysis 2 we saved 20000 shortest cladograms (PAUP options as above) and stopped the search because of computer limitations. The strict consensus tree of these cladograms served as a topological constraint in a further heuristic search using the inverse constraint approach of Catalán et al. (1997). 5000 searches were initiated saving no more than two cladograms per replicate. Only those cladograms that did not fit the constraint tree were saved. No additional cladograms shorter

or equal to those previously obtained were found. This suggests that the strict consensus tree is a good summary of tree space although not all shortest cladograms are known. For bootstrap analysis of the second data matrix, 100 resampled data sets were generated. 500 random addition sequence heuristic searches were initiated for each, saving no more than two cladograms per search, and no more than 100 cladograms per bootstrap replicate. The frequencies of groups were then calculated over all cladograms saved. Decay values were calculated as in analysis 1. We also performed Templeton´s SLP test on alternative topologies as described above.

Maximum likelihood, genetic distances, molecular clock

We analysed the large ITS data matrix of analysis 2 with a maximum likelihood approach using a HKY85 model with gamma distributed among site variation. We first estimated all model parameters in PAUP 4 using one randomly chosen cladogram of the pool obtained in the parsimony analysis. Missing and ambiguous characters were excluded for the evaluation of the parameters. Model parameters estimated were: ti/tv ratio = 1.5276; estimated base frequencies: A = 0.261, C = 0.258, G = 0.267, T = 0.214; proportion of invariable sites = 0; gamma shape parameter = 0.9960 (molecular clock not enforced). The likelihood of the randomly chosen cladogram was evaluated with these parameters under either a molecular clock constraint or no constraint in PAUP 4. These were compared using a Chi-square test (52 degrees of freedom, $P \leq 0.01$ as the level of significance) with model-test 3.0 (Posada & Crandall, 1998). After pruning all outgroups except *Swertia punicea* Hemsl. and keeping all members of *Gentianella* s.str. we conducted a second test for clock-like behaviour (43 degrees of freedom) with the same model parameters.

Maximum likelihood pairwise distances (model parameters as described, molecular clock not enforced, all characters included) were computed in PAUP 4. Mean distances of selected groups and standard deviations were computed with Excel 97 (Microsoft, Seattle, Washington, USA). Mean mutation rates were calibrated with geological data (high alpine areas in South America appearing ca. 3 mya [Simpson 1975, van der Hammen 1979]) or fossil data (first pollen of *Gentianella* s.str. found in South America ca. 1.6 mya [van der Hammen 1979]). The calibrated node of the phylogeny was the split between North American and South American efimbriate species. Divergence time of other groups were then calculated with T = D/2r (T = time, D = Maximum Likelihood distance, r = mutation rate). In order to compare our mutation rates with other published rates we additionally computed and calibrated Kimura pairwise-distances using Paup 4.

Speciation rates

Speciation rates for South American and Australia/New Zealand species of *Gentianella* s.str. were estimated using the following formulas provided by McCune (1997): $SR_{lin} = n / t$, $SR_{ln} = (ln\ n) / t$, $TFS_{lin} = t / (n - 1)$, $TFS_{ln} = (t \times ln\ 2) / ln\ n$ (n = number of species, t = time since last common ancestor, SR = speciation rate, TFS = time for speciation). For t of *Gentianella* we used the diversification time as derived from geological or fossil data. It is not resolved in our phylogeny how many times *Gentianella* s.str. may have reached South America. For this reason we calculated speciation rates for one or two arrivals.

The linear speciation rate (SR_{lin}) is strongly dependent on the absolute number of species in a group and therefore difficult to compare between taxa. It provides an easily understandable estimate of how many species evolved on average per unit time in a group. The linear time for speciation parameter (TFS_{lin}) is roughly the inverse of SR_{lin}. The logarithmic parameter SR_{ln} and its roughly inverse TFS_{ln} assume a balanced phylogenetic tree and use an exponential model of speciation. The absolute number of species is less important. Although our unresolved phylogeny does not allow to determine whether *Gentianella* s.str. followed an exponential model of speciation, the logarithmic parameters are biologically more meaningful than the linear parameters and are here used to compare speciation rates between taxa.

Biogeographic methods

For identifying the area of origin of *Gentianella* s.str., we initially tried the Ancestral Area Analysis (Bremer, 1992; Ronquist, 1994). This method, however, works best with fully bifurcating trees which are not available for *Gentianella* s.str. Instead of making several arbitrary assumptions to resolve the polytomies, only standard Fitch parsimony considerations were used to suggest an area of origin and likely migration routes.

Results

Analysis 1

31 sequences of *mat*K are new and 12 sequences were obtained from EMBL. We were not able to amplify the second region (1729F/2053R) of *mat*K of *Gentianella arenaria* (Maxim.) T. N. Ho for unknown reasons. This region was treated as missing data for this species. The alignment of the two *mat*K regions is 676 bp long. It contains 80 potentially informative, 120 variable but uninformative, and 476 invariant sites. All positions were easy to align. The one potentially informative indel found in *mat*K was from a repeat with apparently multiple insertion/deletion events and was not included in the calculations.

42 sequences of ITS are new and 1 sequence was obtained from EMBL for analysis 1. The alignment of the ITS region in analysis 1 is 495 bp long. The physical length of ITS1 and ITS 2 is between 219 and 234 bp long (mostly 230 bp each). The ITS data contain 162 potentially informative, 132 variable but uninformative, and 201 invariant sites. The five potentially informative indels found decreased resolution and support of branches when coded because they were apparently from positions with multiple insertion/deletion events

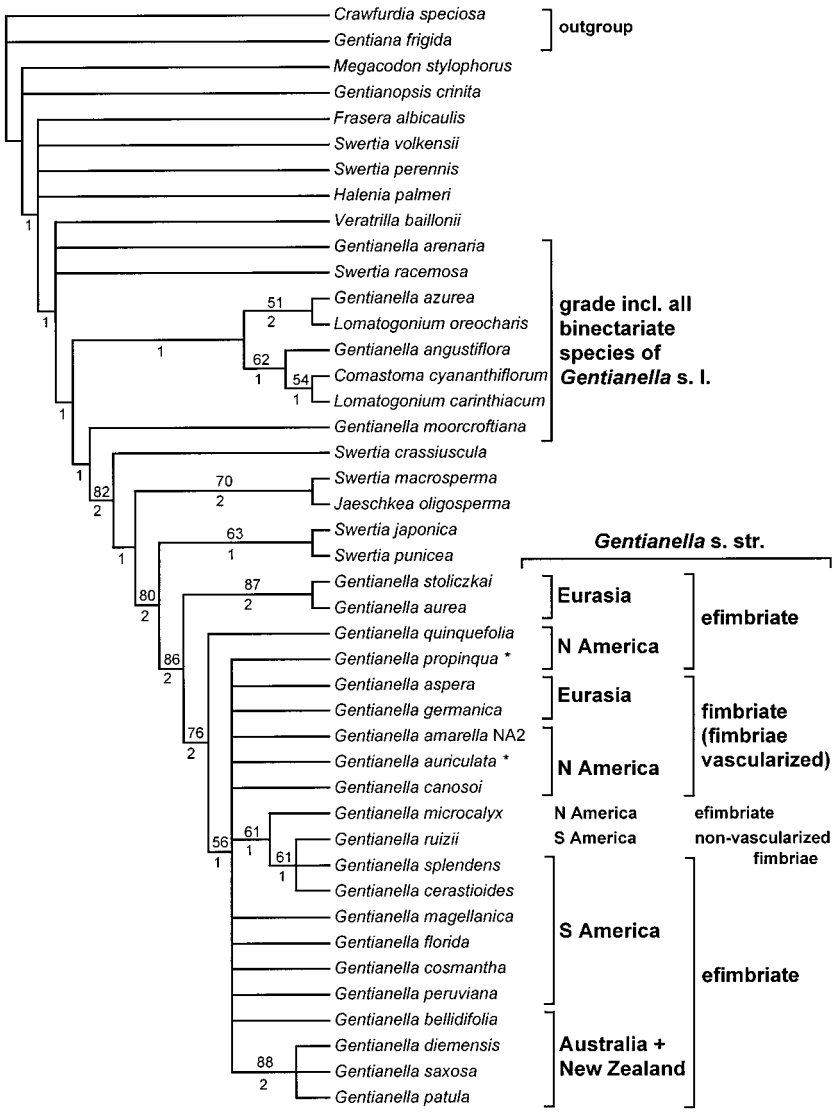

Fig. 1. Strict consensus tree of 48 shortest cladograms (178 steps) of the *mat*K data of analysis 1. Decay values are indicated below branches, bootstrap support (>50%) above branches. * *Gentianella auriculata* occurs in northwestern North America and Northeast Asia, and the mainly North American *G. propinqua* has one known locality in Northeast Asia.

and they were not included in the calculations. The combined data matrix contained 242 potentially informative characters for 43 taxa.

The parsimony analysis of the *mat*K data resulted in 48 cladograms of length 178 (uninformative characters excluded, CI = 0.573, RI = 0.759, RC = 0.435). Their strict consensus tree is shown in Fig. 1. The parsimony analysis of the ITS data resulted in 1127 shortest cladograms of length 550 (uninformative characters excluded, CI = 0.476, RI = 0.541, RC = 0.258; not shown). The consensus trees of the individual analyses differed from each other in a few places but the overall arrangement of the different lineages of *Gentianella* s.l. was similar. The combined analysis resulted in 1814 most parsimonious cladograms 744 steps long (uninformative characters excluded, CI = 0.489, RI = 0.597, RC = 0.292). The bootstrap support values were generally higher in the combined analysis than in the separate analyses.

The members of *Gentianella* s.l. fell into two clades in the combined analysis (Fig. 2). One clade consisted of all *Gentianella* species with two nectaries per petal lobe intermingled with *Lomatogonium*, *Swertia racemosa* and *Comastoma*. All other species of *Gentianella* s.l. have one nectary per petal lobe and were in a clade containing only species of this genus (incl. relatives of *G. campestris* as its type, see analysis 2). The latter clade is referred to as *Gentianella* s.str. In *Gentianella* s.str. two morphological groups can be defined. These are a fimbriate group with mostly vascularized fimbriae in the corolla throat and an efimbriate group lacking such fimbriae. It is important to note that in the *mat*K data the Eurasian efimbriate species of *Gentianella* s.str. were sister to the remainder of *Gentianella* s.str. (Fig. 1). In contrast, the fimbriate lineage was sister to the efimbriate lineage with ITS and in the combined data set (Fig. 2).

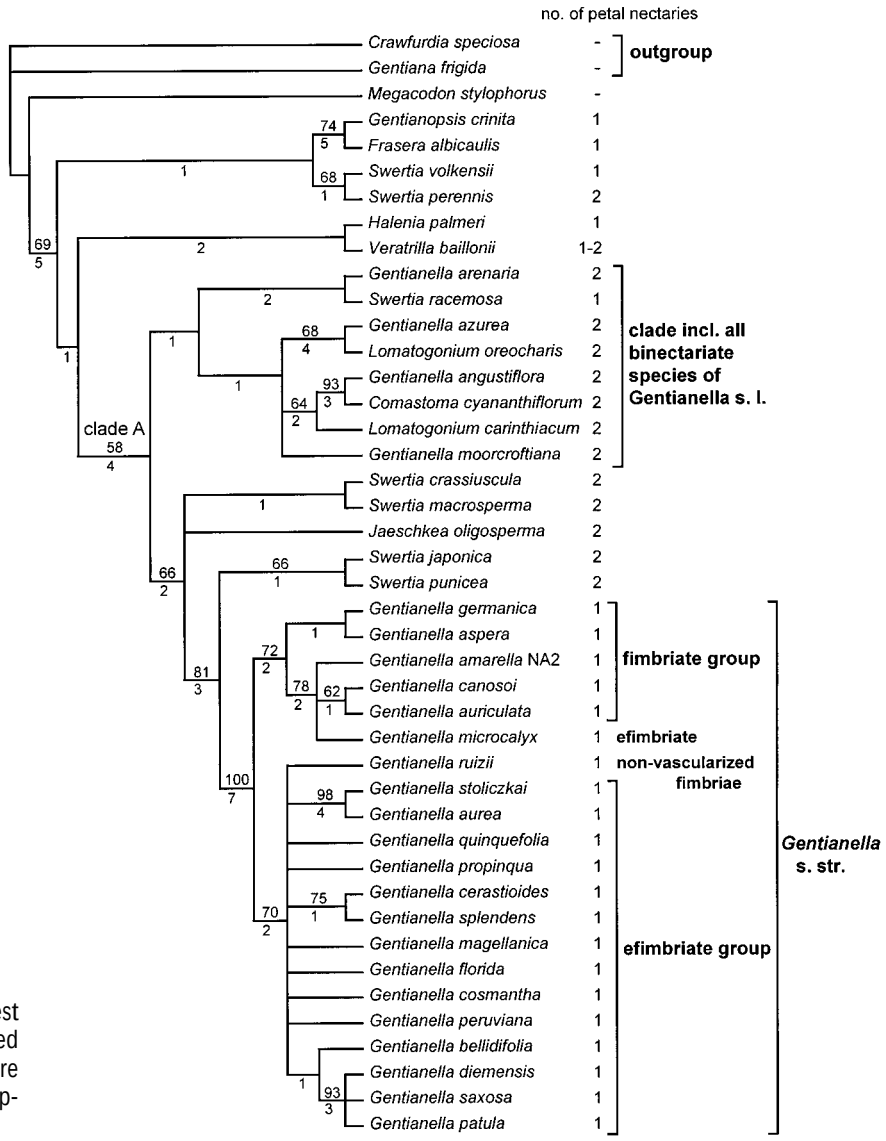

Fig. 2. Strict consensus tree of 1814 shortest cladograms (744 steps) of the combined *mat*K/ITS data of analysis 1. Decay values are indicated below branches and bootstrap support (>50%) above branches.

The partition homogeneity test revealed significant heterogeneity between the nuclear and chloroplast parts of the complete combined data set ($P < 0.01$). When we excluded most members of *Gentianella* s.str. but retained all other members of Swertiinae, the test was not significant ($P = 0.29$).

The results of the SLP test with different constraints are given in Tab. 4. Forced monophyly of *Gentianella* s.l., all binectariate species of *Gentianella* s.l., all binectariate species of *Gentianella* s.l. excluding *G. arenaria*, or of all species of *Swertia* or of all *Swertia* in clade A (see Fig. 2) resulted in significant differences from our most parsimonious trees. Only potential monophyly of *Lomatogonium* could not be rejected. We also explored the major topological difference within *Gentianella* s.str. between the two data sets. When the topology of the lower branches of *Gentianella* s.str. as found with ITS (fimbriate and efimbriate clades as sister) was used as a constraint in a separate *mat*K analysis, the SLP-test revealed non-significant differences in 80% of the trees. The highest value was $P = 0.24$. When the *mat*K topology (Eurasian efimbriate species as sister to the remainder of *Gentianella* s.str.) was used as a constraint in an ITS analysis, 68% of the cladograms were not significantly different. The highest value was $P = 0.13$. In both tests the values for most cladograms were marginally above or below the level of significance ($P \leq 0.05$).

Analysis 2

In addition to the 21 ITS sequences of *Gentianella* s.str. used in analysis 1, 20 more sequences were generated and three more were taken from EMBL for analysis 2. The alignment of this data set was 503 bp long. It contained 99 potentially informative, 130 variable but uninformative, and 274 invariant sites. Seven informative

Table 4. Templeton´s SLP test for constrained alternative topologies. P ≤ 0.05 indicates a significant difference from the most parsimonious cladograms.

constraint	data set	P values
Swertia monophyletic	analysis 1: combined	< 0.01
Swertia of clade A monophyletic	analysis 1: combined	0.01–0.04
Lomatogonium monophyletic	analysis 1: combined	0.11–0.13
Gentianella s.l. monophyletic	analysis 1: combined	< 0.01
binectariate *Gentianella* monophyletic	analysis 1: combined	0.01–0.02
binectariate *Gentianella* excl. *G. arenaria* monophyletic	analysis 1: combined	0.01–0.03
fimbriate group sister to efimbriate group	analysis 1: *mat*K	0.04–0.24
Eurasian fimbriate species sister to remainder of *Gentianella* s.str.	analysis 1: ITS	0.02–0.13
	analysis 2: ITS	0.49–0.54
Gentianella amarella monophyletic	analysis 2: ITS	0.05–0.11

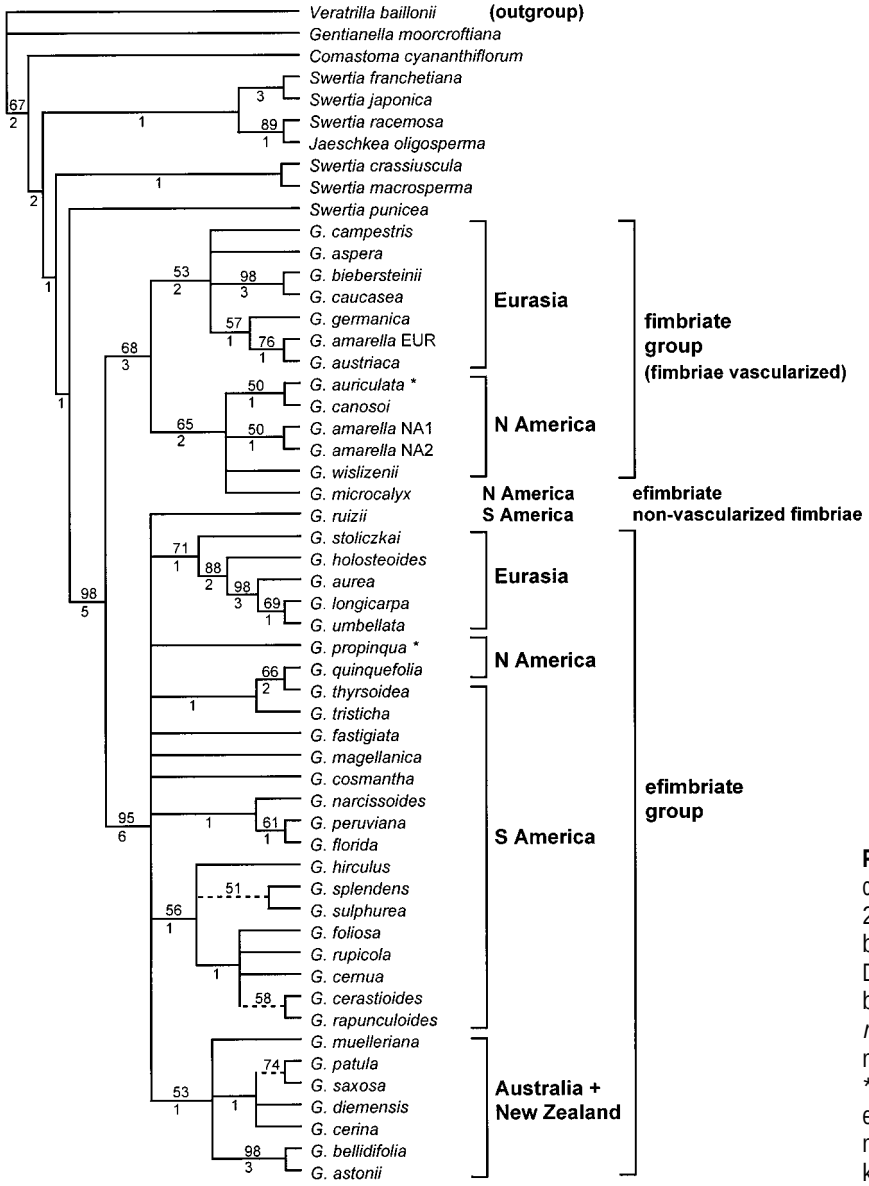

Fig. 3. Strict consensus tree of 20000 shortest cladograms (268 steps) of ITS data of analysis 2. Decay values are indicated below branches, bootstrap support (>50%) above branches. Dashed branches additionally appear in the bootstrap tree. Note the position of *Gentianella ruizii* and *G. microcalyx* which have a flower morphology differing from their close relatives. * *Gentianella auriculata* occurs in northwestern North America and Northeast Asia, and the mainly North American *G. propinqua* has one known locality in Northeast Asia.

indels were detected, coded as binary characters and were used additionally in the phylogenetic analysis. Within *Gentianella* s.str., 57 potentially informative sites were found.

The parsimony analysis produced an unknown number (20000 saved) of most parsimonious cladograms 268 steps long, CI = 0.541, RI = 0.726 RC = 0.393. The large number of cladograms is reflected in the several large polytomies of the strict consensus tree (Fig. 3). The lower branches of *Gentianella* s.str. in analysis 2 were identical with the branches resolved with ITS or the combined data in analysis 1. When we used the topology as revealed by *mat*K in analysis 1 as a constraint for the ITS data in analysis 2, the trees obtained were not significantly different from the unconstrained analysis (Tab. 4). Forced monophyly of the potentially polyphyletic *G. amarella* (L.) Börner did also not result in significantly longer cladograms (Tab. 4).

Molecular clock and genetic distances of ITS in *Gentianella* s.str.

A summary of the likelihood pairwise distances of the ITS data of analysis 2 is given in Tab. 5. Although only ITS data were used, mean distances are given for groups as found in the ITS or in the *mat*K topologies. The split of the southern hemispheric species from the North American species was used to calibrate mutation rates. Using geological evidence (slow rate), this resulted in r = $(4.48 \pm 2.25) \times 10^{-9}$ per site per year; using fossil evidence (fast rate) this resulted in r = $(8.41 \pm 4.22) \times 10^{-9}$ per site per year. With Kimura 2-parameter distances (2.71% ± 1.27 between North and South American efimbriate species) the mutation rates were r = $(4.52 \pm 2.12) \times 10^{-9}$ and $(8.47 \pm 3.97) \times 10^{-9}$ per site per year, respectively.

Based on this calibration, the split of *Gentianella* s.str. and *Swertia punicea* as a member of its sistergroup dates back to between 4.4 ± 0.6 mya to 8.2 ± 1.2 mya. The last common ancestor of *Gentianella* s.str. can be dated to between 2.6 ± 0.8 mya to 4.9 ± 1.4 mya. The incongruent topologies of ITS or *mat*K give almost identical results for the age of the last common ancestor of *Gentianella* s.str.

Speciation rates

The different speciation rates calculated for the radiations of *Gentianella* s.str. in South America and Australia/New Zealand are shown in Tab. 6. For comparison, we also have shown speciation rates for various other genera for which speciation times have been postulated. *Gentianella* s.str. in South America has by far the highest estimated number of species appearing per unit time (SR_{lin} = 58.6 species / my, TSF_{lin} = 17.000 y / new species assuming an invasion of South America 3 mya or SR_{lin} = 106.3 / my and TSF_{lin} = 9.000 y assuming an invasion of South America 1.6 mya). Even when assuming that *Gentianella* s.str. entered South America twice and that both lineages diversified equally, each lineage was faster for SR_{lin} than the other genera of Tab. 6. The parameters of an exponential speciation model (SR_{ln} and TSF_{ln}) for *Gentianella* s.str. from South America are comparably high but lower or similar to *Argyranthemum* Webb from the Canary Islands (Francisco-Ortega et al., 1997) and *Dendrosenecio* (Haumann ex Humbert) B. Nord. from tropical African mountains (Knox & Palmer, 1995). Assuming repeated migration

Table 5. Mean genetic distances of the ITS region calculated with a Maximum Likelihood model (see Material and Methods for parameters) between and within species groups. The time of divergence between groups was calculated with r = (4.48 (2.25) x 10^{-9} per site per year (slow rate based on geological evidence) or r = (8.41 (4.22) x 10^{-9} per site per year (fast rate based on fossil evidence).

lineage comparison	topology	mean distances ± standard error	mimimum age of lineagesplit (fast rate)	maximum age of lineage split (slow rate)
Swertia punicea vs. all *Gentianella* s.str.	both	7.38% ± 1.05%	4.4 my ± 0.6 my	8.2 my ± 1.2 my
Eurasian efimbriate vs. all other	*mat*K	4.30% ± 1.50%	2.6 my ± 0.9 my	4.8 my ± 1.7 my
fimbriate vs. efimbriate	ITS	4.38% ± 1.29%	2.6 my ± 0.8 my	4.9 my ± 1.4 my
fimbriate vs. non-Eurasian efimbriate	*mat*K	4.18% ± 1.23%	2.5 my ± 0.7 my	4.7 my ± 1.3 my
among all fimbriate species	both	2.44% ± 1.19%		
among North American fimbriate species	both	2.64% ± 1.33%		
among Eurasian fimbriate species	both	1.90% ± 0.96%		
among all efimbriate species	ITS	2.73% ± 1.31%		
among efimbriate species from the northern hemisphere	ITS	3.25% ± 1.42%		
southern hemisphere vs. North American efimbriate species	ITS	2.69% ± 1.35%	1.6 my (fossils)	3.0 my (geology)
among South American efimbriate species	both	2.38% ± 1.43%		
South American vs. Australia/New Zealand species	both	2.43% ± 0.98%	1.4 my ± 0.6 my	2.7 my ± 1.1 my
among Australia/New Zealand species	both	1.19% ± 0.65%		

Table 6. Speciation rates (SR) and time for speciation (TFS) following McCune (1997) of *Gentianella* in the southern hemisphere and comparison with other taxa (ordered by SR_{lin})

taxon	number of species	estimated time since divergence (my)	SR_{lin} (sp/my)	SR_{ln} (sp/my)	TFS_{lin} (my/sp)	TFS_{ln} (my/sp)
average speciation time in plant fossils (Niklas 1997)					0.38	
average speciation time in Hawaiian plants (Niklas 1997)					0.42	
Robinsonia DC./ Juan-Fernandez Islands (Sang et al. 1995)	7	max. 4	1.8	0.49	0.67	1.43
Dendroseris D. Don / Juan-Fernandez Islands (Sang et al. 1994)	11	2.6	4.2	0.92	0.26	0.75
Silversword alliance Hawaii (Baldwin 1997)	28	6	4.7	0.56	0.22	1.25
Dendrosenecio (Hauman ex Humbert) B. Nord /	11.0	1	11.0	2.40	0.10	0.29
tropical African mountains (Knox & Palmer 1995)	17 (incl. subsp.)	1	17.0	2.83	0.06	0.24
Eucalyptus L´Hér. (Rickleffs 1989)	600	40	15.0	0.16	0.07	4.33
Gentianella in Australia/New Zealand	32	max. 2	16.0	1.73	0.07	0.40
Argyranthemum Webb / Canary Islands	24	1.2	20.0	2.65	0.05	0.26
(Francisco-Ortega et al. 1997)	39 (incl. subsp.)	1.2	32.5	3.05	0.03	0.22
Gentianella in South America (two dispersal events)	2 x 85	max. 3.0	28.3	1.48	0.036	0.47
Gentianella in South America (one dispersal event)	170	max. 3.0	56.7	1.71	0.018	0.40
		min. 1.6	106.3	3.21	0.009	0.22
Gentianella incl. South America and Australia/ New Zealand	202	max. 3.0	67.3	1.77	0.015	0.39

of *Gentianella* s.str. into South America and equal diversification of both lineages resulted in still comparatively high SR_{lin} and TSF_{lin} values. The SR_{ln} and TSF_{ln} values were then average among the other taxa.

Discussion

Phylogenetic relationships of Gentianella s.l.

Gentianella in its current circumscription is a polyphyletic genus. This is clearly shown by all separate and combined phylogenetic analyses of the molecular data (Fig. 1, Fig. 2), and constrained monophyly of *Gentianella* s.l. resulted in significantly longer trees. Following these results, *Gentianella* s.l. can be divided into two different groups. *Gentianella* s.str. is monophyletic and contains all species with one nectary per petal lobe (Figs 4c–4f). *Gentianella* s.str., containing the type species *G. campestris*, is unambiguously defined by this flower morphological character. The remaining four species of *Gentianella* s.l. included in our sample all have two nectaries per petal lobe (Fig. 4a). These four species group intermingled with species of *Swertia*, *Lomatogonium* and *Comastoma* in all analyses.

Based on the combined analysis (Fig. 2) all genera of Swertiinae not part of clade A are not likely to be close relatives of *Gentianella* s.l. These are *Megacodon*, *Swertia* p. p., *Frasera*, *Halenia*, *Veratrilla* and *Gentianopsis* (including *Pterygocalyx* [Smith in Nilsson, 1967; Yuan & Küpfer, 1995]). This verifies the exclusion of *Megacodon* and *Gentianopsis* from *Gentianella* s.l. as discussed in the introduction. Although *Bartonia*, *Latouchea* and *Obolaria* could not be included in our analysis, the presence of nectaries at the base of the ovary of the two latter genera, as also found in *Megacodon*, make it likely that they are not close relatives of *Gentianella* s.l. Nectaries are not known from *Bartonia*. From the position of this genus in the analysis of Struwe et al. (1998), however, it can be concluded that *Bartonia* also is not closely related to *Gentianella* s.l.

In addition to all species of *Gentianella* s.l., *Swertia* p. p., *Lomatogonium*, *Jaeschkea*, and *Comastoma* are part of clade A. We can not name a single morphological character supporting this clade. Also, the partly well-supported position of the binectariate species of *Gentianella* s.l. among *Lomatogonium* and *Comastoma* or as sister to *Swertia racemosa* (Wall. ex Griseb.) C. B. Clarke, and the negative result obtained when enforcing a group of monophyletic binectariate *Gentianella* are difficult to explain from a morphological point of view. A thorough discussion of these relationships and the morphological evolution in this part of the phylogenetic tree requires better sampling of the relevant groups.

The well-supported (81% bootstrap) closest relatives of *Gentianella* s.str. in the combined *mat*K/ITS analysis are species of *Swertia* with two long-fimbriate nectaries per corolla lobe (Fig. 4b). In view of the enormous size and variation of *Swertia* (135 species, Shah, 1990, 1992) it is not certain whether those species identified as sister to *Gentianella* s.str. in our analysis indeed are its closest living relatives. The provisional inclusion of more species of *Swertia* (ITS only, personal data), however, resulted in identical relationships. We do not know any morphological character in support of the unexpectedly close relationship of these species of *Swertia* with

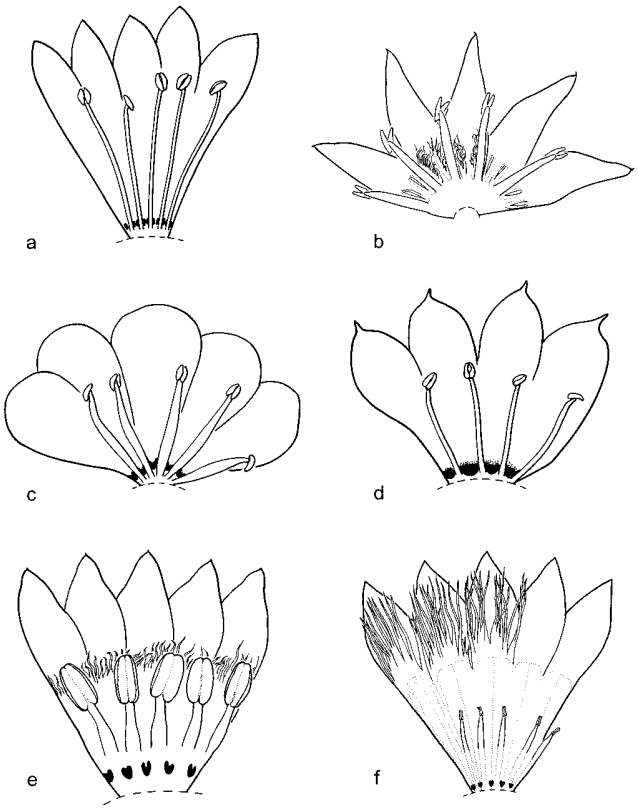

Fig. 4. Representative flower morphology of different subgroups of *Gentianella* s.l. and relatives. a) *Gentianella* s.l. with two nectaries per corolla lobe and a long corolla tube. b) *Swertia punicea,* representing the sistergroup of *Gentianella* s.str., with paired petal nectaries with fimbriate margins and a short corolla tube. c) *Gentianella* s.str. from New Zealand, uninectariate and efimbriate with obtuse corolla lobes and a short corolla tube. d) *Gentianella* s.str. from the northern hemisphere, uninectariate and efimbriate with a long corolla tube and often acute corolla lobes. e) *Gentianella* s.str. from South America, uninectariate and fimbriate, fimbriae non-vascularized. f) *Gentianella* s.str. from the northern hemisphere, uninectariate and fimbriate, fimbriae vascularized.

two fimbriate nectaries per petal lobe to *Gentianella* s.str. with its single and naked nectary per petal lobe. Also, the fimbriae in *Swertia* p. p. and *Gentianella* s.str. are not homologous because the fimbriae of all species of *Swertia* examined are non-vascularized (Fig. 4b) in contrast to the fimbriae of the corolla throat of *Gentianella* s.str. (Fig. 4f). The non-vascularized fimbriae of few South American species of *Gentianella* s.str. (Fig. 4e) probably evolved independently from an efimbriate South American progenitor.

Phylogeny of *Gentianella* s.str.

Of the ca. 244 species described in *Gentianella* s.str. (Tab. 1), we sampled 42 species (44 accessions) in our ITS analysis (Fig. 3), including deviating species such as the dioecious *G. florida* (Griseb.) Holub, the bird-pollinated *G. splendens* [Gilg] Fabris and *G. cosmantha* [Griseb.] J. S. Pringle, and *G. ruizii* (Griseb.) Holub with non-vascularized fimbriae. Because all uninectariate species that were not included are morphologically similar to those sampled (Kusnezow, 1895; Gilg, 1916; own observations), we consider it highly unlikely that the inclusion of more species would break the monophyly of this group. Also the members of the many segregated genera, as named in the introduction, are part of our *Gentianella* s.str. and therefore these names are superfluous.

The topologies within *Gentianella* s.str. revealed by ITS and *mat*K are in conflict with each other. In the ITS analysis (analysis 1 and 2), the fimbriate and efimbriate species are well-supported monophyletic groups (68% and 95% bootstrap in analysis 2) and sister to each other. In contrast to this, *mat*K suggests paraphyly of the efimbriate species in relation to the fimbriate species (72% + 56% bootstrap). In addition to the strong branch support in the separate analyses the following reasons also argue that conflict between the two datasets is concentrated in *Gentianella* s.str.: 1) The combined analysis supports the ITS topology but support of the critical branch of the efimbriate group is reduced (70% bootstrap instead of 85% in separate ITS of analysis 1 [not shown]). 2) The amount of heterogeneity between data sets in analysis 1 is significant only when all species sampled of *Gentianella* s.str. are included. Nevertheless, forcing the ITS topology onto the *mat*K data and vice versa (analysis 1) did not result in significantly longer trees. Also forcing the *mat*K topology of analysis 1 on the ITS data of analysis 2 gave non-significant results. In general, conflicts between gene trees can arise from unequal rates of molecular evolution, shifting base composition, lineage sorting or introgression (e.g., Li, 1997).

The monophyly of the fimbriate species could not be demonstrated with *mat*K but was not ruled out either. In conclusion, the monophyly of the fimbriate group as suggested by ITS is likely but its exact phylogenetic position in relation to the efimbriate group remains unclear.

The problem of monophyly or paraphyly of the two major subgroups of *Gentianella* s.str. is further complicated by two species which were not placed where expected from a morphological point of view. The efimbriate *G. microcalyx* (Lemmon) J. M. Gillett from Mexico grouped among the fimbriate species with ITS but was sister to a group of South American efimbriate species with weak support (61% bootstrap) with *mat*K. Because a very similar and probably closely related species with fimbriae was described from Mexico (*G. tarahumarae* G. L. Nesom, Nesom, 1991; not sampled), we here assume that fimbriae simply were lost in *G. mi-*

crocalyx. The weakly supported *mat*K topology is probably misleading in this case because *G. microcalyx* grows far distant from other efimbriate species and hybridisation between *G. microcalyx* and an efimbriate species as a possible explanation for the *mat*K topology seems unlikely. A similar morphological transition is visible within *G. wislizenii* (Engelm.) J. M. Gillett from Mexico which was clearly part of our fimbriate group. In this usually fimbriate species flowers with only few or even without fimbriae are regularly found (Nesom, 1991; Pringle, pers. comm.).

Also the position of the fimbriate *G. ruizii* from South America among different efimbriate (ITS) or South American efimbriate species (*mat*K) can easily be explained because the fimbriae of *G. ruizii* are not vascularized (Fig. 4e). Therefore, these structures are not homologous with the vascularized fimbriae of the large fimbriate group (Fig. 4f). This difference between vascularized fimbriae in northern hemispheric and non-vascularized fimbriae in some South American species of *Gentianella* s.str. has never been recognized before. After having observed non-vascularized fimbriae in *G. nitida* (Griseb.) Fabris (not sampled) and *G. ruizii* (sampled), we predict that *G. crossolaema* (Wedd.) T. N. Ho & S. W. Liu as the third fimbriate species from South America also has no vascular bundles in its fimbriae. In conclusion, the morphological distinction between a fimbriate and an efimbriate group in *Gentianella* s.str. is justified because the few existing exceptions can be explained convincingly.

The phylogeny within the large group of efimbriate species is not well resolved (Fig. 3) because of a lack of ITS variation. There is, however, information from morphological characters which supports further phylogenetic resolution. All northern hemispheric members of the efimbriate group have corolla tubes longer than the corolla lobes (Fig. 4d) and the corolla lobes are often acute. This is also true for the fimbriate species (Fig. 4f). A long corolla tube is only rarely found in South America in, e.g., bird-pollinated species (*G. splendens* and *G. cosmantha* in our sample). In contrast, most species in South America and all species from Australia/New Zealand have obtuse corolla lobes and a short corolla tube (Fig. 4c). A close similarity between Australia/New Zealand and some South American species was also found in pollen morphology by Nilsson (1967). A coarsely striate pollen surface pattern is unique for the Australia/New Zealand and some South American species, e.g., *G. foliosa* (Kunth) Fabris from Ecuador. The latter species and its close relatives in the ITS tree (Fig. 3), e.g., *G. rupicola* (Kunth) Holub and *G. cerastioides* (Kunth) Fabris, also have very short corolla tubes and obtuse corolla lobes. These flower and pollen morphological characters suggest that the monophyletic Australia/New Zealand species are derived from South American species rather than from species of other continents although this could not be resolved with the molecular data.

The fimbriate group is divided into a North American and a Eurasian subgroup as sister clades (Fig. 3). Only our accession of *G. auriculata* (Pall.) J. M. Gillett from Asia clearly grouped with American species. This species, however, occurs in both Northeast Asia and Northwest America and may have migrated to Asia only recently.

Gentianella amarella from the fimbriate group is non-monophyletic in the strict consensus tree (Fig. 3) and the accessions from North America and Europe group with species in their geographical neighbourhood. Although constrained monophyly of the three accessions did not result in significantly less parsimonious cladograms (Tab. 4), polyphyly of *G. amarella* was not surprising considering that several subspecies sometimes raised to specific rank have been described in this species.

Biogeography of *Gentianella* s.str.

In clade A of our combined analysis (Fig. 2) the majority of taxa including binectariate *Gentianella* are exclusively Asian in distribution. Only one of the 24 Asian species of *Lomatogonium* also grows in North America (*L. rotatum* [L.] Fr. ex Nyman), *Comastoma* is distributed more or less evenly in Eurasia and North America, and the only non-Asian *Swertia* of clade A sampled is the African *Swertia crassiuscula* Gilg which belongs to a small group of morphologically similar African species (Sileshi, 1998).

For the biogeography of *Gentianella* s.str., two hypotheses must be provided because two incongruent phylogenetic hypotheses were obtained from ITS and *mat*K (simplified area cladograms in Fig. 5). As judged from the geographical distribution of the sistergroup of *Gentianella* s.str. and the other lineages of clade A (Fig. 2), the progenitor of *Gentianella* s.str. is likely to have grown in East or Central Asia. Following the ITS phylogeny, the efimbriate and fimbriate groups would have dispersed independently from there through the Holarctic (Fig. 5a). In contrast to this, the *mat*K phylogeny suggests that only efimbriate *Gentianella* s.str. originated in Asia and dispersed from there to North America (Fig. 5b). The *mat*K phylogeny can not indicate the area of origin of fimbriate *Gentianella* s.str. because the North American and Eurasian lineages are contained in a polytomy with other regions (Fig. 5b). If, however, the fimbriate lineage should be monophyletic and consist of a Eurasian and a North American clade as shown by ITS (Fig. 3), then it is more parsimonious in the *mat*K topology to assume that the fimbriate lineage originated in North America and not in East Asia (Fig. 5b).

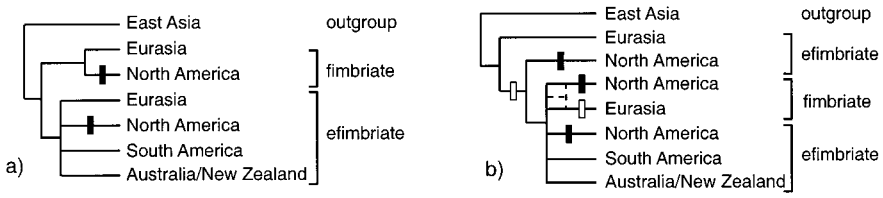

Fig. 5. Simplified area cladograms of *Gentianella* s.str. a) ITS. Following this topology, the efimbriate and fimbriate groups originated in Asia and migrated to North America independently (black bars). b) *mat*K. The efimbriate lineage clearly originated in Asia but the place of origin of the fimbriate lineage is not resolved. If the fimbriate group should be monophyletic and the North American and Eurasian clades sister to each other as revealed by ITS (dotted line), then two migration events (1. migration of efimbriate *Gentianella* s.str. from Eurasia to North America, 2. remigration of fimbriate *Gentianella* s.str. from North America to Eurasia; open bars) are required assuming a North American origin of the fimbriate lineage. If the fimbriate and efimbriate lineages would have originated in Eurasia, three independent migration events from Eurasia to North America (black bars) must be postulated.

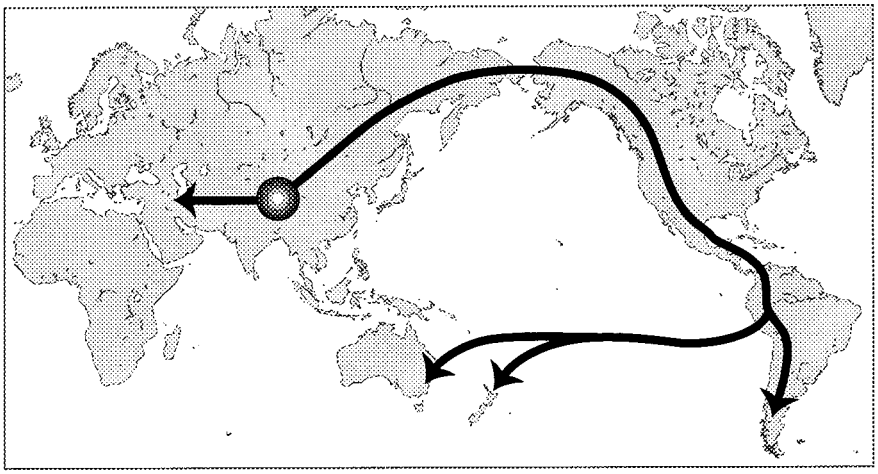

Fig. 6. Hypothetical centre of origin and main migration/dispersal route of the efimbriate lineage of *Gentianella* s.str. The centre of origin and the direction of migration of the northern hemispheric fimbriate lineage is unclear.

The biogeography of *Gentianella* s.str. is further complicated by species such as *G. auriculata* and *G. propinqua* which have a Northwest American and Northeast Asian distribution range. *Gentianella propinqua* is clearly centred in North America and only one population is known from Northeast Asia. *Gentianella auriculata* may also be of North American origin because its closest relatives all are North American in distribution.

The sistergroup relationship of the efimbriate *G. quinquefolia* from North America to the equally North American efimbriate *G. propinqua* and all southern hemispheric efimbriate (and all fimbriate) species found by *mat*K may also indicate that southern hemispheric *Gentianella* s.str. originated from North America (Fig. 5b). The ITS analysis is not informative for the origin of the southern hemispheric species (Fig. 5a). The southernmost extant occurrence of an efimbriate species in North America is Arkansas (4000 km from the Andes). Although this may suggest long distance dispersal between North and South America as demonstrated for, e.g., *Microseris* D. Don (van Heusden & Bachmann, 1992) and *Sanicula* L. (Vargas et al., 1998), migration through Central America cannot be ruled out. This possibility is supported by the gradual increase of northern temperate elements in Central and South America which has been observed in pollen profiles from the first strong temperature drop at the end of the Miocene onwards (summarized in Burnham & Graham, 1999). By whatever mechanism South America was invaded, this invasion can not have happened before the origin of alpine altitudes in the northern Andes ca. 3 mya (Simpson, 1975; van der Hammen, 1979), and may have been much easier after the closing of the Isthmus of Panama ca. 2.8 mya (Hallam, 1994).

The Australia/New Zealand group of species is monophyletic and likely to have dispersed there only once. The origin of the Australia/New Zealand species from South America (Fig. 6) was not resolved with the molecular data but is suggested by the morphological (short corolla tubes, obtuse corolla lobes) and palynological (coarsely striate pollen surface) similarities between part of the South American and all Australia/New Zealand species. Dispersal by wind is one possibility for the transport of *Gentianella* s.str. from South America to Australia/New Zealand. Prevailing winds in the rele-

vant southern latitudes, however, are from West to East and thus in opposite direction to the potential migration route, and were so throughout the climatic changes of the Quaternary (Stewart & Neill, 1984). It is well-known that large seabirds migrate between all Southern continents and potentially could carry seeds in their faeces or attached to their body (Moore, 1972). Such behaviour, however, is not known for alpine birds of South America, the potential dispersal agents of *Gentianella* s.str. In summary, we can not answer the question of how *Gentianella* s.str. dispersed from South America to Australia/New Zealand.

Timing of biogeographic events

The hypothesis of a molecular clock in ITS could not be rejected once most outgroups were deleted. We calibrated our molecular data with the average distance between the North American efimbriate and all South American species. Two dates are available for this split: 1) the oldest fossil of *Gentianella* s.str. in South America at ca. 1.6 mya (van der Hammen, 1979). This provides a minimum age for the presence of *Gentianella* s.str. in South America and its separation from the North American species and results in the fast mutation rate. 2) High alpine areas originated only ca. 3 mya (Simpson, 1975; van der Hammen, 1979). This provides a maximum age and results in a slow mutation rate. It is remarkable that the slower mutation rate obtained ($r = (4.52 \pm 2.12) \times 10^{-9}$ per site per year using Kimura distances for this comparison) is roughly average among other published rates (Sang et al., 1994, 1995; Wendel et al., 1995). A much slower mutation rate was found in Winteraceae (Suh et al., 1993) which, however, have a different life form.

Following the calibrated genetic distances (Tab. 5), the last common ancestor of *Gentianella* s.str. probably existed around 4.9 ± 1.4 mya (slow rate) or 2.6 ± 0.8 mya (fast rate) near the end of the Tertiary (late Pliocene). At this time temperatures in the northern hemisphere decreased quickly, and the temperature oscillations typical for the Quaternary had already started (Mai, 1995). These events may have allowed a Central Asian progenitor of *Gentianella* s.str. to reach new areas along newly available arctic and alpine migration routes, and to rapidly diversify.

The estimated age of the last ancestor of the Australia/New Zealand clade (Tab. 5) ranged from 2.7 ± 1.1 mya to 1.4 (0.6 mya. These values probably overestimate the age of this clade because the phylogeny did not resolve a South American sistergroup of the Australia/New Zealand clade and necessitated the inclusion of non-sistergroup species from South America in the calculation. It seems likely that the age of the Australia/New Zealand clade lies somewhere in between these dates or lower because the high alpine mountain ranges of Australia and New Zealand again are rather young, and suitable habitats for *Gentianella* s.str. originated only approximately 2 mya in this region (summarized in Smith, 1986). Such dating clearly rules out a Gondwana origin of the southern hemispheric distribution of *Gentianella* s.str. because the split of the relevant continents has been dated to > 40 mya (e.g., Linder & Crisp, 1995).

Radiation of *Gentianella* s.str. in South America

The large number of species of *Gentianella* s.str. in South America and their apparently recent origin led us to compare speciation rates in *Gentianella* s.str. with those in other genera. Ideally, the calculation of speciation rates requires that parameters such as time, competition, extinction rates, demographic structure, "speciator" properties, and structural diversity of the habitat are taken into account (McCune, 1997). These parameters are often unknown and also the application of different species concepts in different genera makes comparison among taxa difficult. We assume, however, that species concepts are comparable, and that some of the conditions of the different taxa are sufficiently similar to allow comparison (Tab. 6). In particular, we consider it reasonable to compare the colonization of the rising Andes of South America with the colonization of newly formed islands. Both habitats initially will have offered many empty niches not yet occupied by well-adapted native species, and the principally unknown extinction rate may have been rather low and should be less important for the calculation of speciation rates from the extant number of species than in occupied habitats.

The high SR_{lin} and TFS_{lin} values of *Gentianella* s.str. in South America only reflect the large absolute size of this lineage and have few implications for the tempo of diversification. When using the logarithmic parameters of an exponential model of speciation, the average values (Tab. 6) obtained for *Gentianella* s.str. indicate that the large number of species in South America is not the result of a particularly high speciation rate. Instead, it seems likely that both the greater age of *Gentianella* s.str. in comparison to *Argyranthemum* and *Dendrosenecio* and, more importantly, the vast area of Andean South America open to colonization in comparison to the Canary Islands (*Argyranthemun*), Juan-Fernandez Islands (*Robinsonia* DC, *Dendroseris* D. Don), Hawaii (silversword alliance), and African tropical mountains (*Dendrosenecio*) may be the major reason for the high specific diversity of *Gentianella* s.str. in South America. Such relationship between number of species and area has been discussed for various organisms by, e.g., MacArthur & Wilson (1967) and Rosenzweig (1995).

Our phylogeny does not allow to determine how many times *Gentianella* s.str. dispersed to South America, and the above calculations are based on a single origin. When two independent dispersal and diversification events of identical properties are assumed, the speciation rate (SR_{lin}) and time for speciation (TSF_{lin}) are still high compared to other taxa. The logarithmic parameters (SR_{ln}/TSF_{ln}) were less affected and the same general conclusions can be drawn assuming a single or double dispersal of *Gentianella* s.str. to South America.

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