

## Conflicting patterns of genetic and morphological variation in European *Gentianella* section *Gentianella*

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Genetics in European *Gentianella* Moench sect. *Gentianella* were investigated using internal transcribed spacer (ITS) nrDNA, *trnL-F* cpDNA and Amplified Fragment Length Polymorphisms (AFLP) fingerprint data to analyse relationships within the section. Both a cladistic and a phenetic approach, as well as nonhierarchical analysis (PCA) and partitioning of molecular variance (AMOVA), were used. Sequence data did not resolve groups within the section. They reflected, however, geographical isolation between central European and eastern Carpathian (*G. lutescens* and *G. bulgarica*) as well as Caucasian samples (*G. caucasea*). AFLP data revealed a strong differentiation within *G. germanica* between populations of the Alps and north-western middle Europe, although there is hardly any morphological differentiation of taxonomic significance. Morphological similarities between *G. aspera* and *G. pilosa* were not reflected in genetics, and no support was found for *G. aspera*. Genetic similarities were not in line with morphology in *G. lutescens* and *G. bulgarica*. AFLP data only partly resolve relationships among taxa. High genetic similarities, as well as partitioning of molecular variance among taxa/populations of eastern middle Europe (*G. austriaca*, *G. bohémica*, *G. germanica*, *G. stiriaca*) and intermediate morphological characters in *G. bohémica* and *G. stiriaca*, might indicate reticulate evolution. The overall low sequence divergence within the section points to a very young origin of all taxa. © 2005 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2005, 148, 175–187.

ADDITIONAL KEYWORDS: AFLP – ITS – morphological homoplasy – reticulate evolution – *trnL-F*.

### INTRODUCTION

European *Gentianella* Moench sect. *Gentianella* (= sect. *Amarella* Griseb.) comprises about 20 species that, apart from two taxa [*G. columnae* (Ten.) J. Holub and *G. crispata* (Vis.) J. Holub], have been classified into three groups representing morphologically isolated lineages of southern Europe (Wettstein, 1896; Pritchard & Tutin, 1972). The group of (i) *G. germanica*, comprising the majority of taxa (Pritchard & Tutin, 1972: nine species plus four taxa in the rank of species, which are not generally accepted), is widely distributed in western and middle Europe. In the groups of (ii) *G. campestris* (two species) and (iii)

*G. amarella* (three), mainly distributed in western, northern middle and northern Europe, species diversity and morphological differentiation between taxa is much lower. Taxa are either not generally accepted [*G. hypericifolia* (Murb.) Pritchard, included in *G. campestris* (L.) Börner by Morales *et al.*, 1996; Renobales, Lluca & Lopez, 2002] or often cannot be distinguished reliably [*G. amarella* (L.) Börner and *G. uliginosa* (Willd.) Börner; Petanidou *et al.*, 1998].

Morphological differentiation within the *G. germanica* group is higher. Because of extensive variation in vegetative and floral characters, however, systematics of the *G. germanica* group (Wettstein, 1896; Pritchard & Tutin, 1972) are based mainly on a few calyx characters: (1) shape of sinuses between the lobes; (2) degree of division (ratio lobe/tube); (3) size relations among lobes; (4) presence/absence and (5) type of papillae on margin and midrib of lobes. Delimitation

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of taxa and relationships among them are far from being resolved due, in detail, to: (i) high seasonal and ecotypic variation in all vegetative and metric floral characters (Zopfi, 1991; Wagner & Mitterhofer, 1998; Greimler & Dobeš, 2000); (ii) only modest differentiation in diagnostic calyx characters in many narrow endemics [*G. engadinensis* (Wettst.) J. Holub, *G. insubrica* (H. Kunz) J. Holub, *G. liburnica* E. Mayer & H. Kunz, *G. fatrae* (Borb) J. Holub, *G. hispanica* López Udias, Fabregat & Renob.] of the European mountain systems (Samuelsson, 1922; Kunz, 1940; Mayer, 1969; Holub, 1983; Renobales *et al.*, 2002) when compared with their widespread relatives; (iii) the presence of taxa [*G. bohémica* Skalicky, *G. stiriaca* auct. (= *Gentiana stiriaca* Wettst.)] showing intermediate characters between two or more geographically neighbouring species (Wettstein, 1892; Skalicky, 1969; Maurer, 1998); and (iv) unreliable diagnostic characters (Greimler, Hermanowski & Jang, 2004) used to distinguish between two taxa within *G. germanica* (Willd.) E.F. Warburg (Kerner & Kerner, 1882; Lauber & Wagner, 2001).

Using molecular markers, von Hagen & Kadereit (2001) estimated the splitting of *Gentianella* section *Gentianella* (= 'northern hemispheric fimbriate lineage of *Gentianella*') from other lineages to have been a Pliocene event. Molecular phylogenies either indicated monophyly (ITS) or did not resolve (*matK*), and were also contradictory in indicating Asian (ITS) or North American (*matK*) ancestry (von Hagen & Kadereit, 2001). Relationships within the section have not previously been investigated using molecular markers. Temporal estimates based on 'traditional morphological-ecological-geographic' evidence (term coined by Yurtsev, 1997) suggested a late Quaternary divergence (Braun-Blanquet, 1923; Meusel *et al.*, 1978).

DNA sequence markers proved to be powerful tools to resolve relationships among and within *Gentianella* and related genera (Yuan & Küpfer, 1995; Chassot *et al.*, 2001; von Hagen & Kadereit, 2001). However, when dealing with taxa below the generic level, nuclear and chloroplast sequence markers often fail to resolve relationships. Amplified Fragment Length Polymorphisms (AFLP, Vos *et al.*, 1995) are therefore now frequently used additionally to infer phylogenetic relationships (e.g. Hodkinson *et al.*, 2000; Koopman, Zevenbergen & van den Berg, 2001; Zhang, Comes & Kadereit, 2001; Després *et al.*, 2003).

In this study we use sequence data of the internal transcribed spacer (ITS) of nuclear ribosomal DNA and the *trnL* intron, and the intergenic spacer between the *trnL*-intron and the *trnF*-gene of the chloroplast DNA, as well as AFLP in a cladistic and phenetic approach focusing on (1) phylogenetic relationships within section *Gentianella*, (2) relationships within the *G. germanica* group and (3) congruence/

incongruence between genetic data and morphological traits in the *G. germanica* group.

## MATERIAL AND METHODS

### PLANT MATERIAL

Leaf samples of European *Gentianella* were collected in the field with a focus on the Alps as the centre of diversity (Table 1). Outgroup samples include *G. umbellata* (*Gentiana umbellata* M.B. in Grossgeim, 1967) of section *Arctophila* and species of the related genera *Gentiana*, *Swertia*, *Gentianopsis* and *Comastoma*. Vouchers of the sampled populations are on deposit in the Herbarium of the Institute of Botany, University of Vienna (WU). Taxonomic assignment within section *Gentianella* follows Pritchard & Tutin (1972) with two exceptions: we follow (1) Hayek (1911–1914) and Maurer (1998), assigning *G. germanica* populations of the north-eastern Alps to a separate species, *G. stiriaca*, and (2) Holub (1983) assigning western Slovak populations of *G. lutescens* (Velen.) Holub to *G. fatrae*. *Gentianella germanica* samples from the Alps and north-western middle Europe are referred to below as 'Alps' and 'Outside', respectively. For AFLP, five individuals per population (only four available in Norwegian *G. campestris* and Slovak *G. fatrae*) were analysed.

### SEQUENCE ANALYSIS

Total genomic DNA was extracted from silica gel-dried leaf material in 700 µL CTAB buffer (2% CTAB, 100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 0.2% mercaptoethanol, pH 8.0) for 30 min at 60 °C. Then, 500 µL chloroform/isoamylalcohol (24/1) were added and the extraction mix was incubated for 15 min at 4 °C. After centrifugation, the DNA was precipitated with 500 µL isopropanol. The pellet was washed with 70% ethanol and dissolved in 200 µL TE buffer.

The *trnL*(UAA)-intron and the intergenic spacer (IGS) between the *trnL*-(UAA)-3'-intron and the *trnF*-(GAA) gene of the chloroplast DNA were amplified using the primers of Fangan *et al.* (1994). The fragment comprising part of 18S rDNA, the entire ITS region and part of 26S rDNA was amplified using the primers of Sun *et al.* (1994). Amplified double-stranded DNA fragments were purified using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences). The purified fragments were sequenced directly on a PRISM 377 DNA automated sequencer (Perkin Elmer) following the DYEnamicET cycle sequencing protocol (Amersham Pharmacia). The *trnL-F* strands were sequenced using nested sequencing primers (Pfosser & Speta, 1999). The sequencing primers of ITS were the same as those used in the PCR reaction.

**Table 1.** The investigated taxa with their population sample identities (ID), locations, country (A, Austria; CH, Switzerland; CZ, Czech Republic; D, Germany; GE, Georgia; HR, Croatia; I, Italy; N, Norway; NL, the Netherlands; PL, Poland; RO, Romania; SK, Slovak Republic), coordinates and EMBL gene bank accession numbers for sequences

Taxon	ID	Location	Country	°East	°North	Seq.	AFLP	Accession no. ITS	Accession no. <i>trnL-F</i>
<i>Gentiana asclepiadea</i>	Ge09	Prein-Rax	A	15,72	47,67	+		AJ580549	AJ580515
<i>Swertia perennis</i>	Ge13	Hochschwung	A	14,35	47,40	+		AJ580550	AJ580516
<i>Gentianopsis ciliata</i>	Ge10	Prein-Rax	A	15,72	47,67	+		AJ580551	AJ580517
<i>Comastoma tenellum</i>	66	Hierkinn	N	9,50	62,52	+	+	AJ580552	AJ580518
<i>Gentianella umbellata</i>	65	Kasbegi	GE	44,48	42,53	+	+	AJ580553	AJ580519
<i>G. amarella</i>	Ge12	Chata Vratna	SK	19,03	49,20	+		AJ580573	AJ580516
	86	Sudslavice	CZ	13,78	49,08		+		
<i>G. amarella agg.</i>	94	Haarlem	NL	4,57	52,45		+		
<i>G. anisodonta</i>	Ge01	Pizzo Redorta	I	9,90	46,05	+		AJ580558	AJ580524
	84	Heukareck	A	13,17	47,30		+		
	59	Snjeznik	HR	14,58	45,43		+		
<i>G. aspera</i>	14	Hinterstoder	A	14,12	47,70	+	+	AJ580560	AJ580527
	68	Loser	A	13,78	47,67		+		
	17	Kocelovice	CZ	13,83	49,47		+		
<i>G. austriaca</i>	3	Rax	A	15,75	47,73		+		
	4	Schneeberg	A	15,83	47,73		+ <sup>3</sup>		
	5	Pernitz	A	15,93	47,88		+		
	7	Gaaden	A	16,22	48,05	+	+ <sup>3</sup>	AJ580549	AJ580540
<i>G. bohemica</i>	15	Onsovice	CZ	13,77	49,12	+	+	AJ580570	AJ580537
	16	Javornik	CZ	13,65	49,13		+		
<i>G. bulgarica</i>	76	Sinaia	RO	25,47	45,40	+	+	AJ580569	AJ580536
<i>G. campestris</i>	Ge03	Fisser Joch	A	10,58	47,07	+		AJ580557	AJ580523
	25	Maloja	CH	9,67	46,42		+		
	30	Ofenpaß	CH	10,30	46,65		+		
	89	Pilatus	CH	8,27	46,95		+		
	36	Slettvollen	N	9,33	62,50		+		
<i>G. caucasea</i>	60	Kasbegi	GE	44,77	42,57	+	+	AJ580549	AJ580520
<i>G. columnae</i>	Ge16	Monte Argentella	I	13,25	42,85	+		AJ580555	AJ580521
<i>G. engadinensis</i>	Ge02	Ofenpaß	CH	10,30	46,65	+		AJ580559	AJ580526
	29	Sulden	I	10,58	46,52		+		
<i>G. fatrae</i>	38	Tlsta	SK	18,97	48,93	+	+	AJ580575	AJ580542
<i>G. germanica</i> Alps	21	Nassfeld	A	13,05	47,05		+		
	24	Fuldera	CH	10,37	46,62	+	+ <sup>2</sup>	AJ580566	AJ580533
	50	Bösenstein	A	14,42	47,43	+ <sup>1</sup>	+	AJ580567	AJ580534
	71	Vent	A	10,90	46,85		+		
	23	Plawenn	I	10,58	46,77		+		
<i>G. germanica</i> Out	87	Dittingen	CH	7,52	47,43	+ <sup>1</sup>	+	AJ580563	AJ580530
	88	Diegten	CH	7,82	47,42	+ <sup>1</sup>	+	AJ580565	AJ580532
	20	Lengerich	D	7,88	52,19	+ <sup>1</sup>	+	AJ580562	AJ580529
	93	Kunderberg	NL	6,00	50,88	+	+	AJ580564	AJ580531
<i>G. insubrica</i>	27	Monte Generoso	I	9,05	45,93	+	+	AJ580561	AJ580528
<i>G. lutescens</i>	32	Kopa Magury	PL	19,98	49,25		+		
	73	Zarnesti 1	RO	25,28	45,57	+	+ <sup>2</sup>	AJ580568	AJ580535
	74	Zarnesti 2	RO	25,28	45,57		+		
<i>G. pilosa</i>	18	Cave del Predil	I	13,57	46,43	+	+	AJ580554	AJ580525
<i>G. ramosa</i>	Ge11	Campo Moro	I	9,90	46,30	+		AJ580556	AJ580522
	26	Maloja	CH	9,68	46,40		+		
	90	Oberalp	CH	8,77	46,67		+		
<i>G. stiriaca</i>	12	Hochschwab	A	15,22	47,62	+ <sup>1</sup>	+	AJ580577	AJ580544
	13	Lugauer	A	14,70	47,55	+	+	AJ580578	AJ580545

<sup>1,2</sup>Not shown in trees. <sup>3</sup>Used for AMOVA, not shown in trees and PCA.

The programmes Sequence Navigator and Autoassembler (Perkin Elmer, Applied Biosystems) were used to edit and to assemble the complementary strands. Alignment of sequences was carried out by eye following the recommendations of Kelchner (2000). Totals of 628 and 913 characters were included in the matrices for phylogenetic analyses of the ITS (including ITS1, 5.8S rDNA and ITS2) and of the *trnL-F* region, respectively. Gaps were coded as missing data, except for three informatively coded gaps at sites 295, 341 and 520 in the ITS dataset and site 928 in the *trnL-F* dataset. All sequences have been deposited in EMBL (Table 1).

Individual and combined maximum parsimony (MP) analyses of the ITS and *trnL-F* datasets were performed using PAUP\* v.4.0b10 (Swofford, 2002) on a Power Macintosh G4. Substitutions at each nucleotide position were treated as independent, unordered, multistate characters of equal weight (Fitch parsimony; Fitch, 1971). Heuristic searches were performed using addition sequence set at 1000 random additions of taxa, tree bisection-reconnection (TBR) branch swapping and MulTrees on. Robustness of clades was estimated using the bootstrap method (Felsenstein, 1985) with 1000 replicates with simple sequence addition, TBR branch swapping and MulTrees on.

#### AFLP ANALYSIS

AFLP procedures followed the protocol provided with a commercial AFLP analysis kit (Invitrogen Life Technologies) with minor modifications. Genomic DNA (200 ng) was digested with two restriction enzymes, *EcoRI* and *MseI*, for 3 h in a total volume of 5 µL, then ligated to added double-stranded *EcoRI* and *MseI* adapters (5 µL) for 5 h at 37 °C and overnight at 17 °C. A 0.5 µL aliquot of the ligate was preamplified in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) with preselective primers *MseI-C* and *EcoRI-A* using the following programme: 2 min at 72 °C, followed by 20 cycles of 1 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C, and extension of 30 min at 60 °C. The PCR products were diluted 1 : 10 and selective amplification was carried out using three different selective primer combinations: *Eco-ATC(FAM)/Mse-CTC*, *Eco-AGG(HEX)/Mse-CAC*, and *Eco-AGC(NED)/Mse-CAC*. Selective amplification was performed using a touchdown cycling profile starting with 20 s at 94 °C, 20 s at 65 °C, 50 s at 72 °C, followed by 12 cycles with a decreasing annealing temperature of 0.7 °C per cycle. During the remaining 23 cycles, the annealing temperature was kept at 56 °C. The fluorescence-labelled selective amplification products were resolved on a 5% denaturing polyacrylamide gel together with an internal size standard (GeneScan-500 ROX, PE Applied Biosystems) on a PRISM 377 DNA automated

sequencer (Perkin Elmer). Raw data were collected and aligned with the internal size standard using the ABI Prism GeneScan Analysis Software (PE Applied Biosystems). GeneScan files were imported into Genographer (version 1.1.0, J. Benham, Montana State University, 1998) for scoring the fragments. Presence or absence of peaks (i.e. fragments) was scored for each accession within a readable range of 50–500 bp and assembled as a binary (1/0) matrix.

Pairwise genetic distances for all accessions were calculated from the presence/absence matrix according to the complementary value of Nei & Li's (1979) similarity coefficient implemented in PAUP\* v.4.0b10 (Swofford, 2002). A neighbour-joining (NJ) phenogram (Saitou & Nei, 1987) was calculated based on the distance matrix. Robustness of clades was estimated using the bootstrap method (Felsenstein, 1985) with 1000 replicates. In addition, MP analyses were performed (PAUP\* v.4.0b10; Swofford, 2002). Heuristic searches were carried out using addition sequence set at 1000 random additions of taxa, tree bisection-reconnection (TBR) branch swapping, MulTrees option not in effect. Robustness of clades was estimated using the bootstrap method (Felsenstein, 1985) with 1000 replicates, fast stepwise addition option in effect.

A principal components analysis (PCA) was performed using the program SPSS for Windows 10 (Option factor analysis: extraction method principal components). Outgroups and *G. caucasea* (Loddiges ex Sims) J. Holub were removed in the final analysis to increase resolution within section *Gentianella*. The AMOVA (analysis of molecular variance) procedure of the programme Arlequin ver. 2.000 (Schneider, Roessli & Excoffier, 2000) was used to examine the partitioning of molecular variation into variance components due to differences among individuals, among populations and among taxa or groups of populations using taxa samples consisting of at least two populations (*G. germanica* was separated into two regional groups: Alps and Outside).

## RESULTS

#### ANALYSIS OF ITS

Alignment of all ITS region sequence positions resulted in a matrix of 628 characters. Length of the entire ITS region, including ITS1, 5.8S rDNA and ITS2, varied among the *Gentianella* DNAs from 624 to 625 bp. ITS1 and 5.8S rDNA had a uniform length of 233 bp and 164 bp, respectively; ITS2 ranged from 227 to 228 bp. Forty-four characters, including three coded gaps at positions 295, 341 and 520 in the alignment, were parsimony-informative. MP analysis yielded 18 trees of 167 steps [consistency index (CI) = 0.64, retention index (RI) = 0.61]. In the bootstrap consensus tree

(not shown), the only strongly supported clade within genus *Gentianella* besides the outgroup relationships was the one uniting *G. lutescens* and *G. bulgarica* (Velen.) J. Holub (BP 98). Another weakly supported (BP 53) group was the clade comprising all species of *Gentianella* except *G. umbellata* and *G. caucasea*.

#### ANALYSIS OF CPDNA

Alignment of all sequence positions resulted in a matrix of 997 characters (*trnL* intron: 449 characters; *trnL-trnF* IGS: 548 characters), 913 of which were included in the analysis. Thirty characters, including one coded indel at site 928 in the alignment, were parsimony-informative. MP analysis produced three shortest trees of 116 steps with a CI of 0.84 and RI of 0.90. As for the analysis of ITS, in the bootstrap consensus tree of the *trnL-F* region (not shown) the only strongly supported clade within genus *Gentianella* was the one uniting *G. lutescens* and *G. bulgarica* (BP 87). Weak support (BP 61) was found for a clade uniting the two *G. germanica* accessions with the *G. lutescens* and *G. bulgarica* subclade.

#### COMBINED ANALYSIS OF ITS AND CPDNA

As we did not observe any strongly supported (>BP 85) incongruence in either of the two separate MP analyses of ITS and *trnL-F*, we proceeded with direct combination of the ITS and *trnL-F* data sets. MP analysis of the combined matrix produced 84 shortest trees of 284 steps, with a CI of 0.73 and RI of 0.78. Figure 1 shows one of the most parsimonious trees with branch lengths (ACCTRAN) and bootstrap percentages (BP). Monophyly of genus *Gentianella* was supported by BP 100. *Gentianella umbellata* (section *Arctophila*) was sister to a clade (BP 96) comprising all accessions of section *Gentianella*, followed by *G. caucasea* which was again sister to the rest (BP 67). *Gentianella lutescens* and *G. bulgarica* formed the only strongly supported (BP 100) clade within genus *Gentianella*.

#### AFLP

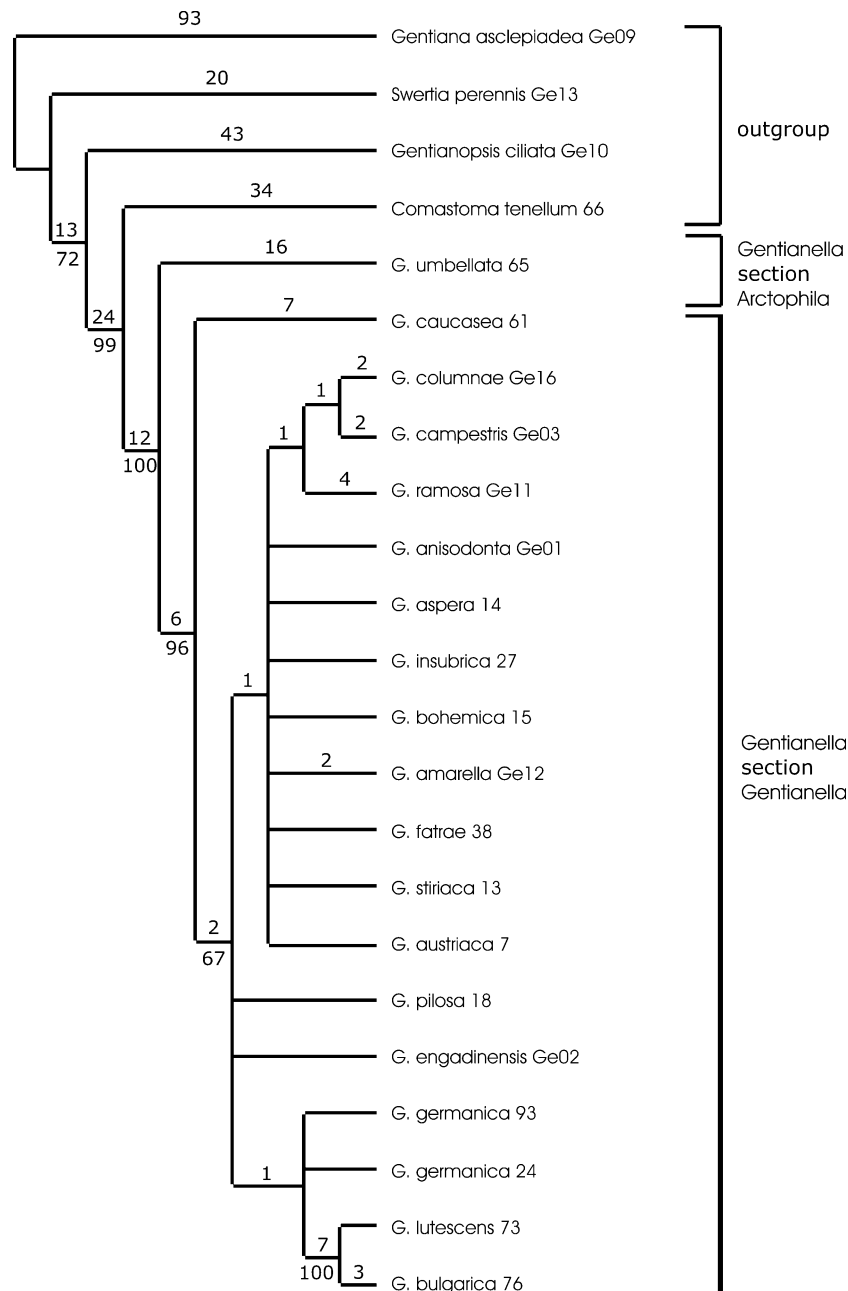
In total, 797 markers ranging between 50 and 500 bp were found in 183 individuals of *Gentianella* and *Comastoma*. For the outgroup taxa, 32 markers were unique. After removing the outgroups, 765 markers, of which 17 (2.22%) were monomorphic, remained for a sample of 173 individuals of 16 taxa of *Gentianella* section *Gentianella*. The highest number of 21 private markers was found in *G. caucasea*, followed by *G. campestris* (12), accessions of *G. germanica* from the Alps (ten), and *G. ramosa* (Hegetschw.) J. Holub and *G. anisodonta* (Borbás) Á. & D. Löve (nine each).

In *G. amarella*, we found only four private fragments. No private fragment was found in *G. bulgarica*.

Figure 2 shows the AFLP NJ tree based on Nei & Li (1979) genetic distances. Numbers indicate bootstrap support values down to interpopulational level. All conspecific accessions, except *G. germanica*, *G. aspera* (Hegetschw. & Heer) Dostal ex Skalicky, Chrtek & Gill, *G. bulgarica*, *G. lutescens* and *G. stiriaca*, clustered together with BP 66–100. All single populations were found in separate clusters except for one population of *G. bulgarica*. In contrast to the combined ITS/*trnL-F* tree, *G. amarella* (BP 97) was sister to all the remaining accessions, albeit with no bootstrap support. Most terminal groupings in the NJ tree were well resolved and supported, but no support was found for most of the very basal nodes.

Figure 3 illustrates one of the three AFLP MP phylogenetic trees, with bootstrap support values plotted on the tree down to interpopulational level. As for the NJ tree, conspecific accessions, except *G. germanica*, *G. aspera*, *G. bulgarica*, *G. lutescens* and *G. stiriaca*, grouped together (BP < 50–100). All single populations were found in separate clades except one population each of *G. bulgarica* and *G. austriaca*. In contrast to the NJ tree, *G. caucasea* (BP 88) was sister to all the remaining accessions (not supported). As in the NJ tree, most terminal groupings in the MP tree were well resolved and supported but again, most of the very basal nodes received no bootstrap support. Removing *G. stiriaca* and *G. bohémica* did not change relationships among remaining taxa in any of the trees. Various character states (intermediate used for intermediate state and/or occurrence of both other states in a taxon) of five characters were mapped according to the MP tree (Fig. 3). Variation among taxa in these morphological characters was found to be highly incongruent with the genetic pattern.

The first three axes of the PCA explained nearly 56% (46.9%, 4.8% and 3.9%) of the total variation in the data. In the diagram (Fig. 4), clusters of individuals belonging to one taxon or to closely related taxa [*G. anisodonta*, *G. engadinensis*, *G. pilosa* (Wettst.) J. Holub] could be identified. High intraspecific variation (as in *G. aspera*) was indicated in the PCA by high variation on both axes. High PCA loadings on the first axis were found in *G. germanica* (Outside), *G. aspera* and *G. insubrica*, in contrast to *G. ramosa* and *G. amarella*. On the second axis, highest differentiation was found between *G. amarella* and *G. germanica* (Outside) plus *G. campestris*. *Gentianella germanica* (Outside) was well separated from the other taxa, whereas the *G. germanica* populations of the Alps occurred in a diffuse group containing other taxa of the eastern Alps (*G. austriaca*, *G. stiriaca*) and Bohemia (*G. bohémica*) together with Carpathian taxa (*G. lutescens*, *G. bulgarica*). The Carpathian taxa were



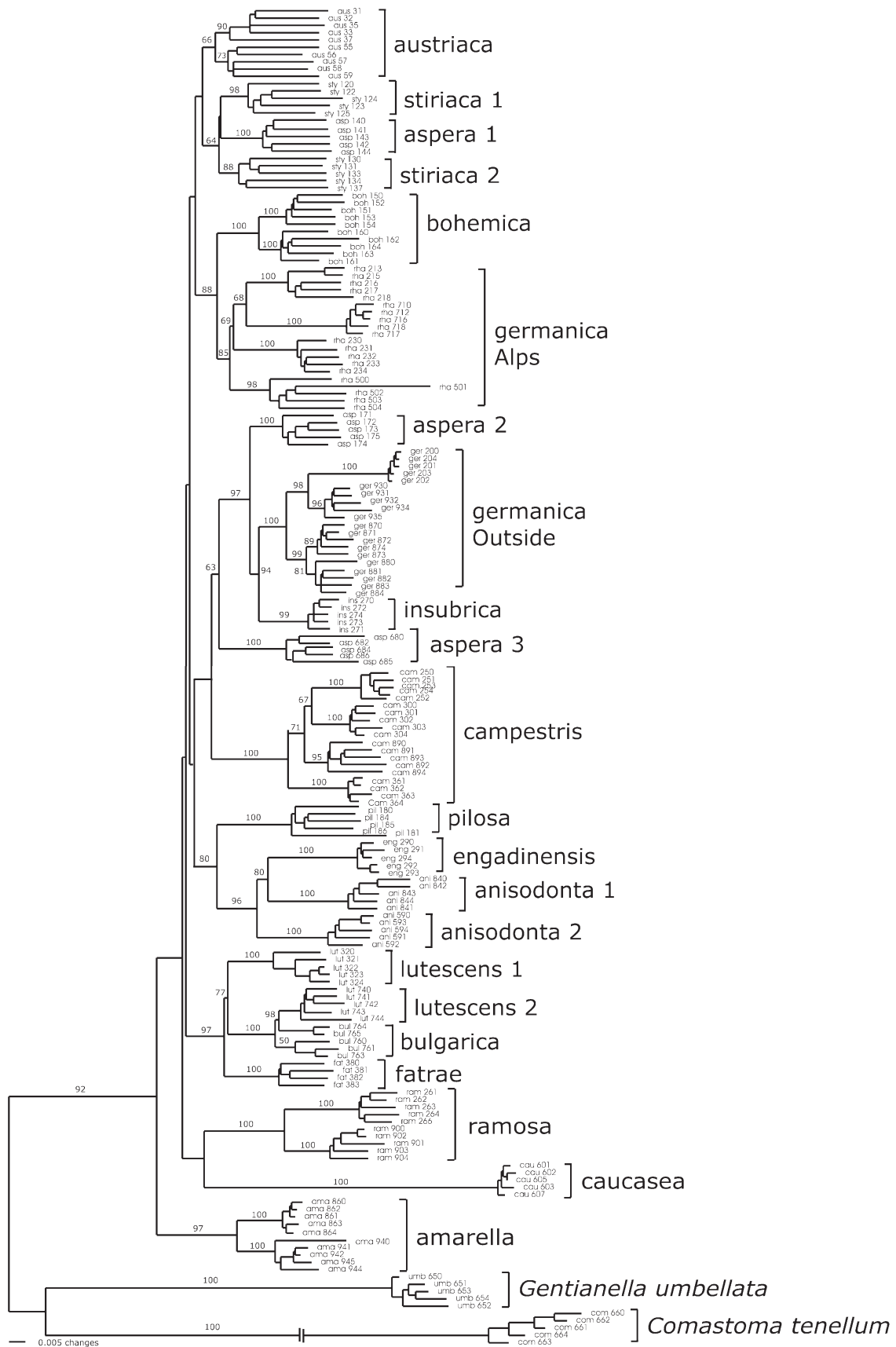
**Figure 1.** One of the 84 most parsimonious trees obtained from MP analysis of the combined data set (ITS nrDNA, *trnL-F* cpDNA). Numbers above branches are estimated branch lengths, numbers below branches are bootstrap percentages.

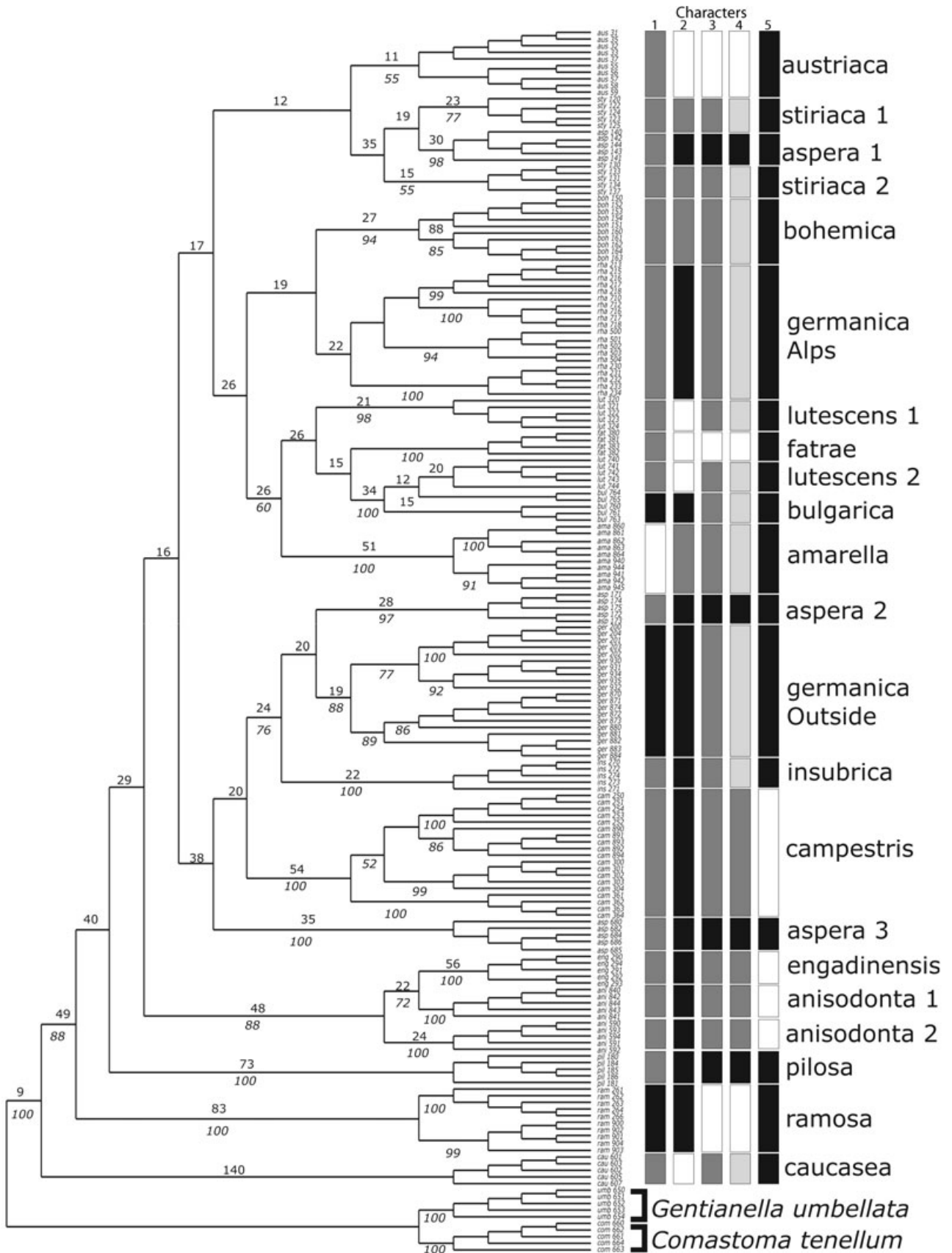
separated from this group along the fourth axis (not shown).

The partitioning of molecular variance (AMOVA) was calculated for the largest taxa samples (Alps and

Outside separately in *G. germanica*) (Table 2). Differentiation among the sample groups of *G. germanica* (37.36%) was on the level of differences between other species. Differentiation among *G. germanica* (Alps)

**Figure 2.** Neighbour-joining tree of 173 *Gentianella* accessions from 16 species of section *Gentianella* and two outgroup taxa (*Gentianella umbellata*, *Comastoma tenellum*) based on AFLP data. Numbers above branches are bootstrap percentages.





**Figure 3.** One of three most parsimonious trees obtained from the MP analysis (sample as in Fig. 2) based on AFLP data. Numbers above branches are branch lengths (given down to the basal node of a taxon, down to populations for ‘polyphyletic’ taxa), below branches bootstrap percentages. Character states plotted in black/dark grey white/ indicate: 1. gynophore (as long as calyx tube/half as long/sessile or nearly so); 2. calyx sinus (acute/intermediate/obtuse); 3. papillae on sepal (margin and midrib/only margin/missing); 4. type of papilla (black: long cylindrical, dark grey: long conical, light grey: short conical, white: not applicable); 5. white/black: two lobes much wider/all ± equal.

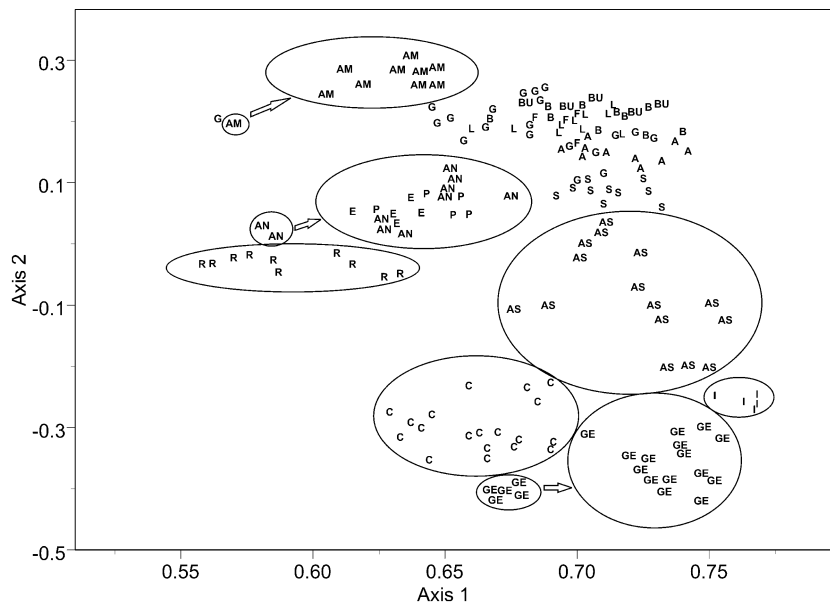
and *G. austriaca*, however, was much lower (16.73%). A similar result (16.79%) was obtained when analysing these two taxa together with *G. bohemica* and *G. stiriaca*.

DISCUSSION

The high genetic differentiation within *G. germanica* as revealed by AFLP data (Table 2, Figs 2–4) is a very surprising result as this is not in line with patterns of morphological variation. However, this result supports an older classification scheme (still within genus *Gentiana*) by Kerner & Kerner (1882), assigning the alpine populations to a separate taxon, *G. rhaetica* A. & J. Kerner. Nearly all differential characters given by the authors (relative leaf size and shape, corolla size) and ‘calyx with wings’ (Lauber & Wagner, 2001), however, are too variable to separate the populations from the Alps and the regions of northern middle Europe (Greimler *et al.*, 2004). Another differential character given by Kerner & Kerner (1882), the length ratio gynophore/calyx tube, showed a significant difference

in the means (*t*-test, *P* < 0.001) due to the more delicate calyx in *G. germanica* from outside the Alps, but combined with considerable overlapping (25%) between the two samples (Greimler *et al.*, 2004). The fact remains that there is no clear morphological differentiation between the two samples. In most floras covering the distributional area of both taxa (Hess, Landolt & Hirzel, 1972; Pritchard & Tutin, 1972; Guinochet & de Vilmorin, 1975; Jäger & Werner, 2002) they were not recognized as biogeographical entities.

The pronounced genetic split into a group of populations of the Alps and a group of Outside populations within *G. germanica* probably mirrors patterns of isolation between middle European ‘lowland’ (including lower mountain ranges) and the mountain populations of the Alps, as found in *Saxifraga paniculata* (Reisch, Poschlod & Wingender, 2003), and, enhanced by different ploidy levels, in *Biscutella laevigata* (Tremetsberger *et al.*, 2002). The striking discordance between AFLP and morphological data might be a consequence of conservative morphological evolution in both groups during this isolation. Different speed of



**Figure 4.** Principal components analysis (scale with principal component loadings) without outgroups and *Gentianella caucasea*. Axes 1 and 2 explain 46.9 and 4.8%, respectively, of the total variance (A *G. austriaca*, AM *amarella*, AN *anisodonta*, AS *aspera*, B *bohemica*, BU *bulgarica*, C *campestris*, E *engadinensis*, F *fatrae*, G *germanica* (Alps), GE *germanica* (Outside), I *insubrica*, L *lutescens*, P *pilosa*, R *ramosa*, S *stiriaca*).

**Table 2.** Results of AMOVA of AFLP data (based on squared Euclidean distances) calculated from populations of *Gentianella amarella*, *G. anisodonta*, *G. aspera*, *G. austriaca*, *G. bohemica*, *G. campestris*, *G. germanica* (Alps), *G. germanica* (Outside), *G. lutescens*, *G. ramosa* and *G. stiriaca*. N, number of groups; d.f., degrees of freedom; SSD, sum of squared deviations

Grouping	N	Source of variation	d.f.	SSD	Variance components	% variation
(a) <i>G. germanica</i> (Outside)/ <i>G. germanica</i> (Alps)	2	among regions	1	786.63	30.63	37.36*
		among populations	6	1044.35	30.68	37.42***
(b) <i>G. germanica</i> (Outside)/ <i>G. campestris</i>	2	within populations	32	661.60	20.68	25.22***
		among taxa	1	829.46	35.07	45.48*
		among populations	6	871.92	26.70	34.62***
		within populations	31	475.70	15.35	19.90***
(c) <i>G. germanica</i> (Alps)/ <i>G. campestris</i>	2	among taxa	1	802.37	31.83	36.70*
		among populations	6	1087.77	32.67	37.67***
		within populations	31	689.30	22.24	25.64***
		among taxa	1	754.73	30.87	38.77*
(d) <i>G. germanica</i> (Outside)/ <i>G. austriaca</i>	2	among populations	6	824.45	22.16	27.84***
		within populations	32	850.80	26.59	33.39***
		among taxa	1	419.70	12.32	16.73*
		among populations	6	1040.30	28.02	38.08***
(e) <i>G. germanica</i> (Alps)/ <i>G. austriaca</i>	2	within populations	32	1064.40	33.26	45.19***
		among taxa	1	769.38	32.03	38.01*
		among populations	6	867.87	23.89	28.35***
		within populations	31	878.50	28.34	33.63***
(f) <i>G. campestris</i> / <i>G. austriaca</i>	11	among taxa	10	5307.85	26.34	32.91***
		among populations	20	3308.09	28.20	35.24***
		within populations	123	3135.70	25.49	31.85***
		among taxa	3	982.97	11.88	16.79***
(g) 11 largest taxa (groups) samples	4	among populations	8	1248.30	24.29	34.32***
		within populations	48	1660.80	34.60	48.89***
		among taxa	3	982.97	11.88	16.79***
		among populations	8	1248.30	24.29	34.32***
(h) <i>G. germanica</i> (Alps)/ <i>G. austriaca</i> / <i>G. bohemica</i> / <i>G. stiriaca</i>	4	among populations	8	1248.30	24.29	34.32***
		within populations	48	1660.80	34.60	48.89***
		among taxa	3	982.97	11.88	16.79***
		among populations	8	1248.30	24.29	34.32***

\*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ .

genetic and morphological evolution has been suggested, e.g. in *Paeonia* (Sang, Crawford & Stuessy, 1997). Morphological homoplasy, however, could be another reason for the patterns observed. Hedrén, Fay & Chase (2001) assume that similar ecotypes occur in *Dactylorhiza* due to similar selection pressures at different places. In a review of macro- and microevolution, Kellogg (2002) concludes that morphological homoplasy is apparently high among closely related taxa, as developmental constraints or selection repeatedly seem to favour similar forms.

Sequence divergence and genetic distance calculated from AFLP data within the section reflect geographical distance between the central European taxa and *G. caucasea* of Georgia, as well as the eastern Carpathian samples of *G. lutescens* and *G. bulgarica*. These two Carpathian taxa were not considered closely related based on morphological features (Wettstein, 1896). Instead, affinities (short calyx tube in relation to lobes) were emphasized between *G. bulgarica* and *G. ramosa* (Wettstein, 1896; Renobales *et al.*, 2002).

In both AFLP trees (NP, MP) we found strong support (BP 97–100) for taxa supposed to represent relatively older lineages (*G. amarella*, *G. ramosa*, *G. campestris*; Wettstein, 1896). Sequence data also revealed a weak divergence for *G. ramosa*, *G. campestris* and *G. columnae*. Despite conflicting basal topologies between the AFLP (NP, MP) trees (which collapse in the bootstrap consensus trees), we found strong support in both trees (BP 88–100) for two 'taxa' with samples of at least two populations, *G. bohémica* and the regional group of *G. germanica* outside the Alps. No support was found for *G. aspera*, a taxon of very high morphological polymorphism (Wettstein, 1896).

The group comprising *G. anisodonta*, *G. engadinensis* and *G. pilosa* (NJ BP 80, PCA) is consistent with the view of Kunz (1940) and Mayer (1969) on relationships among these taxa, in contrast to Wettstein (1896) who considered *G. aspera* and *G. pilosa* to be closest relatives. Again, morphology is in conflict with genetic findings as *G. aspera* and *G. pilosa* share one character, not found in other taxa (papillae occurring on midrib and margin of calyx lobes), which has been used in taxonomy since Wettstein (1896). Furthermore, we found that these papillae are of a special cylindrical type and occur only in these two taxa (Greimler *et al.*, 2004).

The nonhierarchical analysis (PCA) indicated an isolated position of *G. ramosa* (as in the AFLP trees). High differentiation was also found among the *G. germanica* populations outside the Alps plus *G. campestris* on the one hand, and *G. amarella* plus *G. germanica* of the Alps, *G. bohémica*, *G. lutescens* and *G. bulgarica* on the other. Wettstein (1896) did not consider closer relationships among *G. amarella* and the eastern European taxa.

Due to the lack of support for basal nodes in phylogenetic and phenetic trees, taxonomic conclusions on relationships among taxa within section *Gentianella* are limited. Dealing with closely related taxa, the problem has to be faced of too low variability of DNA sequences and hypervariability of fingerprint markers (such as AFLP), resulting in patterns of high variation within and among the lower hierarchical groups (e.g. populations) and only small proportions of variation to resolve basal relationships among taxa (grouping h in Table 2, see also AFLP trees in Koopman *et al.*, 2001; Kropf, Kadereit & Comes, 2003; Marhold *et al.*, 2004). Besides these technical limitations there are two possible reasons for the lack of resolution among the central European taxa of section *Gentianella*. First, gene flow between and within all three groups of the section homogenizes the gene pool. Extensive gene flow might also be responsible for some of the conflicting patterns between morphology and genetics. Second, the taxa are too young for a significant divergence in the investigated sequence markers and for establishing better resolution at basal nodes in the AFLP analysis.

Hybrid populations between and within groups have frequently been observed (Wettstein, 1896; Pritchard, 1961; Moravec & Vollrath, 1967; Winfield *et al.*, 2003). The only taxon of the middle European species pool which, to our knowledge, has never been observed in hybridization, is *G. ramosa*. The relatively high number of autapomorphies and private markers in this taxon very likely reflects a long lasting reproductive isolation. Likewise, in *G. amarella*, a taxon frequently involved in hybridization, only few autapomorphies and private markers were found.

Apart from obvious hybridization (hybrid populations with the parents nearby), reticulate evolution, i.e. large scale introgression, was suggested as a likely mode of speciation in *G. stiriaca* (Wettstein, 1892) and *G. bohémica* (Skalicky, 1969). Both taxa were considered transitional between the geographically neighbouring taxa *G. aspera*, *G. austriaca* and *G. germanica* by the respective authors. In fact, in the PCA analysis, *G. bohémica* groups with *G. germanica*, *G. austriaca* and Carpathian taxa, whereas *G. stiriaca* links *G. aspera* with this group (Fig. 4). Hybridization and repeated backcrossing during postglacial colonization and succession, as observed in oaks (Petit *et al.*, 1997) and in *Cochlearia* (Koch, Huthmann & Hurka, 1998; Koch, 2002), is a possible explanation for these genetic similarities that are in line with poor morphological characterization leading to uncertainties in taxonomic treatments of the taxa. Reticulate evolution, however, need not be a postglacial phenomenon. Kropf *et al.* (2003) in *Pritzelago alpina* point to a pattern of (i) long-lasting periods of glacial intergradation among populations in refugial areas and (ii) relatively short intervals of interglacial 'vicariance', which is a possi-

ble scenario for the establishment of intermediate *Gentianella* taxa presently covering a more or less defined distribution area.

The low sequence variation we found in section *Gentianella* might also be an indirect indicator of (rather recent) lineage splitting during the Quaternary, as suggested by Wettstein (1896) and Braun-Blanquet (1923). Based on ITS mutation rates, fossil pollen and geological evidence, von Hagen & Kadereit (2001) found the last common ancestor of *Gentianella* s.s. (before splitting into fimbriate and efimbriate lineages) to have existed some  $2.6 \pm 0.8$  to  $4.9 \pm 1.4$  million years ago. Our data do not allow temporal estimates except for the general conclusion that the taxa might be very young.

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