Molecular Aspects in Systematics of *Gentiana* Sect. *Calathianae* Froel.

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Par

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Gentiana, Spring Gentians, Systematics, Revision, Sequencing, maturase-K, ncpGS, 5S-NTS, genetic fingerprinting, PCR-RFLP, AFLP

Abstract

Based on results from six different molecular studies and cytology, a taxonomic revision of *Gentiana* Sect. *Calathianae* Froel. is proposed. The revised section comprises two subsections: Subsect. *Calathianae* with three taxa having relatively low chromosome numbers, *G. nivalis* L. and *G. utriculosa* L. (annuals), and *G. pumila* Jacq. (perennial); and Subsect. *Vernae* (Tzevelev) Haemmerli stat. nov. with nine species and seven subspecies including *G. verna* L. (with two subspecies, *G. verna* subsp. verna, and *G. verna* subsp. *delphinensis* Kunz), *G. tergestina* Beck (with three subspecies, *G. tergestina* subsp. tergestina, *G. tergestina* subsp. *balcanica* Haemmerli, and *G. tergestina* subsp. *pontica* Haemmerli), *G. sierrae* Briquet, *G. schleicheri* Kunz, *G. rostanii* Reuter, *G. bavarica* L. (with two subspecies, *G. bavarica* subsp. *bavarica* , and *G. bavarica* L. subsp. *subacaulis* Müller*), G. terglouensis* Hacq., *G. brachyphylla* Vill., and *G. orbicularis* Schur . These results provide indications into the biogeography of the section. While several taxa such as *G. pumila* Hacq., *G. bavarica* L. subsp. *subacaulis* Müller, *G. rostanii* Reuter or *G. verna* L. subsp. *delphinensis* Kunz, *G. terglouensis* Hacq. or *G. schleicheri* Kunz are restricted to the Alpine chain, others occur on different European Mountain ranges (*G. utriculosa* L., *G. sierrae* Briq. and *G. brachyphylla* Vill.), but only few taxa also grow outside Europe (*G. nivalis* L., *G. verna* L, *G. tergestina* Beck and *G. penetii* Romo). Two species are not represented at all within the Alps: *G. penetii* Romo from Atlas Mts. and *G. tergestina* Beck including its subspecies from Dinaric Alps eastward, throughout minor Asia to the Altai Mts. Most taxa are limited to only a limited distribution area or to only one or few mountain ranges. Widespread taxa are the annual *G. nivalis* L. and the perennials *G. verna* L. s.str. and *G. tergestina* Beck s.l.

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IMPRIMATUR POUR LA THESE

Molecular aspects in systematics of *Gentiana* Sect. *Calathianae* Froel.

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Introduction

Overview on taxonomic history of *G.* Sect. *Calathianae* Froel.

Gentiana Sect. *Calathianae* Froel. has been the battlefield for a large number of botanists who have applyed their own personal ideas to the group. History goes back in pre-Linnaean time when Ecluse (1601) identified *G. verna* L. as "*Gentianella minor verna*". Bauhin (1623) later used both the genus names *Gentiana* and *Calathiana*, and mentioned both in adaptation to Ecluse: "*Gentiana minor vern*a" and "*Calathiana verna*". Tournefort (1700) described 19 species, amongst them *G. verna*, and *G. utriculosa*. can be identified. Linnaeus (1753) then re-used these names and introduces the binominals *G. verna* L., *G. nivalis* L., *G. utriculosa L.*, and *G. bavarica* L.

Later, different strategies became evident: Froelich (1796) and Bunge (1824) conserved the Genus *Gentiana* as defined by Linnaeus. Bunge (1824) basically referred to Froelich. Meanwhile the sectional definition of Froelich (1796) comprises very diverse taxa. As such, the four first species described are *G. acaulis* L., *G. grandiflora* Laxm., *G. pyrenaica* L. and *G. verna* in that precise order. The first species is today considered as belonging to *G.* Sect. *Ciminalis* Dumort., the second and third of *G.* Sect. *Chondrophyllae* Bunge Ser. *Grandiflorae* Grossh. (Ho and Liu 2001) while not until the fourth place a taxon of actual *G.* Sect. *Calathianae* Froel can be found. De Candolle used the name *Calathia* to describe a section including *G. nivalis*, *G. utriculosa*, *G. verna*, *G. bavarica* as much as *G. pyrenaica* L. (Lamarck and Candolle 1805; Lamarck and Candolle 1806). Other authors divided the genus into smaller parts as specially Schmidt (1796) transferring taxa out of actual *G.* Sect. *Calathianae* together with others into Genus *Hippion* or Gaudin (1828) by dividing them into *Hippion* (*G. verna and G. bavarica*) and *Gentianella* (*G. nivalis* and *G. utriculosa*). The third strategy was followed by Necker (1790) or Borckhausen (1796), defining an increasing number of new genera. Necker defined *Thyrophora* covering *G.* Sect. *Calathianae*, Borckhausen named it *Ericoila*, changed by Gray (1821) into *Ericala*. Back to *G.* Sect *Calathianae* as given in 1796 by Froelich (1796) : that section got raised into the rank as genus *Calathiana* by Delarbre (1800). Grisebach (1839) overcame the problem of heterogeneity within Froelich's section in proposing a new *G.* Sect. *Cyclostigma*. Included within Grisebach's section were all taxa of the actual section known at that date, resulting into a much more homogeneous unity. Kusnezow (1894) accepted the definition of Grisebach and didn't even mention the definition of Froelich, same done by Soltokovic (1901).

The Genus *Hippion* introduced by Schmidt (1796) got redefined by Löve and Löve (1961) and applied only to Grisebach's *Cyclostigma*, Holub (1973) later restored Delarbre's genus *Calathiana* by proposing Calathiana nivalis (L.) Delarbre as type species and referred to Löve and Löve (1961) as well as to Soltokovic (1901). The earlier names *Hippion* F.W. Schmidt and *Ericoila* Ren. Ex Gray. were considered illegitimate.

The revival of G. Sect. *Calathianae* was done by Tutin (1964) and Pritchard (1977a, 1977b) by applying that section name on same taxa as Grisebach (1839) did with Sect *Cyclostigma*. Later works as Ho and Liu (2001) and Halda (1995, 1996) use the section name as proposed by Froelich. Halda (1995) in special divides the section within an annual *G.* Sect. *Calathianae* and a perennial *G.* Sect. *Vernae* embedded within *G.* Subgen. *Calathianae* . Same did few years before Tzevelev (1987) by introducing *G.* Sect. *Calathia.* As only monographer in recent time Müller (1974, 1982) referred back to *G.* Sect. *Cyclostigma* Grisebach.

Species distribution

Gentiana Sect. *Calathianae* Froel. mainly occurs in European mountain ranges, but also in fewer taxa in the surrounding flatland, preferably along streams. One taxon is reported from northern Africa. Another distinct taxon can be found in northeastern Canada, Greenland and Iceland as well as throughout Europe. While precisely defined in western Europe, the eastern parts of Europe have a series of related taxa with rather vague circumscriptions. Also within Europe, indications on species distribution are vary from author to author. The existence of different taxonomies makes the situation even more difficult, as demonstrated in Tab. 1, where a summary upon distribution of that section as reported by five different authors since 1894 is given. A precise overview on distribution based on a revised taxonomy resulting from that study is found in the last chapter.

Morphological characters in *G.* Sect. *Calathianae* Freol.

G. Sect. *Calathianae* Froel. has annual as also perennial taxa. Morphological parallels can be found amongst the entire section. Other morphological features vary between these closer groups formed by different life cycles. In general flowering stems arise singly from stolons are often creeping and rooting at lower nodes. Flowers are solitary and terminal, the calyx tube angled or winged, the corolla salverform, with very slender cylindrical tube and spreading lobes, with one major exception of dark blue color. Plicae are short and 2-cleft. Anthers are free, the stigmata lobes expanded and connate or contiguous, forming a discoid or funnelform structure. Annual taxa posses a basal leaf rosette and an often strongly branched stem. Petal lobes are in general smaller than in perennial taxa, resulting in a corolla with less in diameter. The two annual taxa differ mostly in overall plant size and size of calyx wings. Perennial taxa have an unbranched stem and a large variety of different leaf types; lanceolate with an acute apex up to broadly elliptic or rhomboid with an obtuse or sounded apex. Characters for differentiation are existence of a basal rosette, the shape of basal and stem leafs as well as the type of leaf apex and finally the size of calyx wings. An overview on morphological characters as proposed by Ho and Liu (2001) is given in Tab. 2. See also photographs in Fig. 2 and differences in calyx and leaf shape in Fig. 1.

Ecological characters in *G.* Sect. *Calathianae* Froel.

Taxa of *G.* Sect. *Calathianae* Froel. colonize mountain ranges between the subalpine and the nival altitudinal level (Aeschimann 2003) but exceptions are also known. Fewer taxa occur on the lower mountain level such as in the Maritime Alps in southern France, but also along rivers in low altitude as in case of *G. utriculosa* or along coastlines in the arctic in case of *G. nivalis* L. Occasionally alpine taxa also can be found in lower altitudes in case of special ecological conditions (see distribution of *G. tergestina* Beck. s.str., last chapter).

G. Sect. *Calathianae* has a general preference for chalky and fresh to humid soil with rather elevated pH. Exceptions are *G. brachyphylla* Growing along the Alps in decalcified underground, same as *G. sierrae* Briq. from Sierra Nevada (Spain) and *G. penetii* Romo from Atlas Mts (Morocco) (Halda and Haldovâa 1996, Ho and Liu 2001).

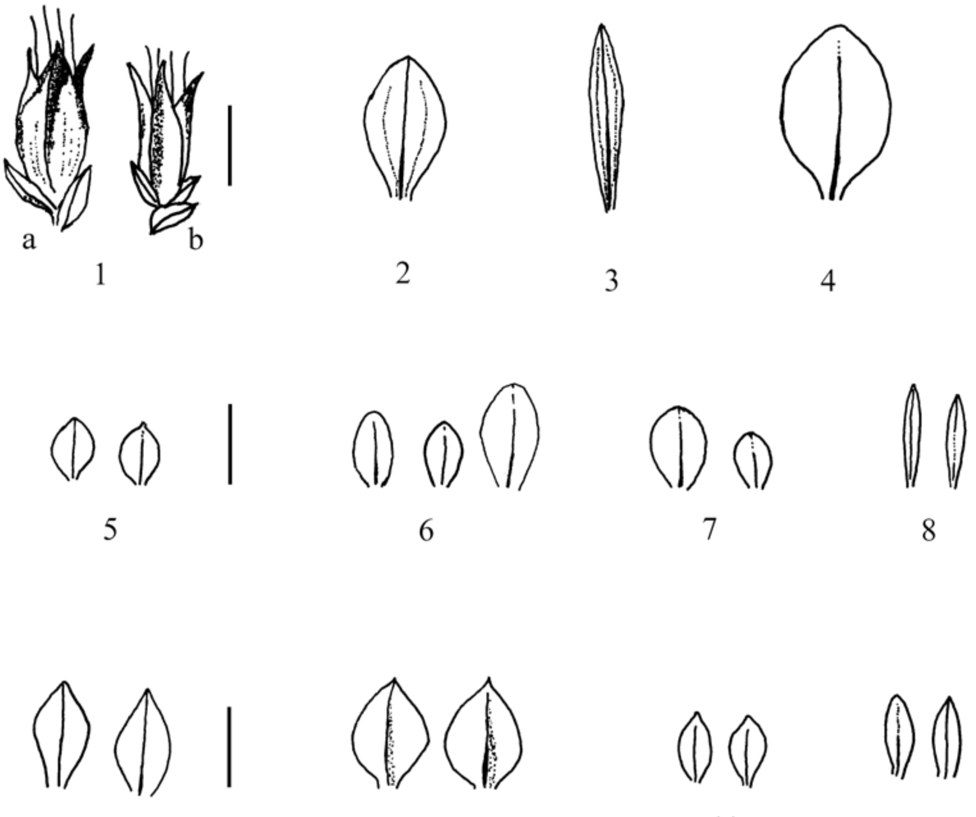


Figure 1: vertical bar 1 cm, 1: calyx shape: 1a: *G. tergestina* s.str.; 1b: G. *verna*; 2-13: leaf shapes; 2: *G. verna* s.str.; 3: *G. tergestina* s.str; 4: *G. tergestina* subsp. *pontica*; 5: *G. brachyphylla*¸6: *G. orbicularis*; 7: *G. sierrae*; 8: *G. pumila*; 9: *G. terglouensis*; 10: *G. schleicheri*; 11: *G. bavarica*; 12: *G. rostanii*; 1-3, 5, 8-12 according Pignatti (1997); 6 according Rittener (1887) , 7 according Romo (1988).

taxa according Tutin (1964)	Kusnezow (1894)	Soltokovic (1901)	Müller (1982)	Halda (1996)	Ho and Liu (2001)
G. bavarica L. s.str.	Alps, Apennine, Carpathians	Alps, Apennine	Alps except SW Alps	Alps, Apennine; alpine meadows, tundra, snowfields, moraines, 1600-3500 m	Alps; damp places, meadows, 1500-3600 m
G. bavarica L. subsp. *subacaulis* Müller	Alps, Apennine, Carpathians			Alps, Apennine; alpine meadows, tundra, snowfields, moraines, 1600-3500 m	
G. brachyphylla Vill. s.str.	E Pyrenees, Alps, Carpathians	Alps	Alps, Pyrenees	Alps, Pyrenees; calcifuge, alpine & subalpine meadows, snowfields, rocky slopes, 1800-2400 m	Throughout the range of the species except Turkey; calcifuge, 1800-4200 m
			G. sierrae Briq.: Sierra Nevada, Atlas	*G. penetii* Romo: Atlas; alpine pastures, porphyric grassy slopes, 3000-3800 m	
		G. nevadensis Solt.: Sierra Nevada		*G. sierrae* Briq.: Sierra Nevada; gravelly pastures, rocky slopes, grassy moraines on silicate or decalcified soil, 2100-3500 m	
G. brachyphylla Vill. subsp. *favratii* Tutin	Sierra Nevada, Alps, Taurus, W Caucasus, Elbours	Alps, Carpathians, Pyrenees, Apennine	Alps, Apennine, Carpathians	Alps, Spain, Balkan, Turkey, Caucasus, Iran; snowfields, alpine moraines, 2000-3800 m	Alps, Carpathians, Turkey; wet places, meadows, calcicole, 2000-2800 m
				G. orbicularis Schur: S Carpathians; grassy moraines, tundra, 1800-2400 m	
G. nivalis L.	Labrador, Greenland, Iceland, arctic Europe, C & S European Mts., Pontic Alps, Caucasus		Labrador, Greenland, Iceland, Scandinavia Scotland, Pyrenees, Alps, Apennine, Dinaric Alps, Balkan, Carpathians, Caucasus	Eurasia, N. America; wet meadows, stream banks, subalpine & alpine tundra	Scandinavia, Scotland, C European Mountains, Pyrenees, Apennine, Dinaric Alps, Balkan, Caucasus, W Asia, Carpathians, Britain, Iceland, Greenland, Arctic European Russia, Canada
G. pumila Jacq. s.str.	Pyrenees, Alps, Apennine	E & SE Alps	E Alps	Alps, Pyrenees, Apennine; moist meadows, grassy slopes, snowfields; 1800-2450 m	E Alps, France; grassland, alpine pastures, 1250-2500 m
G. pumila Jacq. subsp. *delphinensis* Fourn.			SW Alps, S Pyrenees	SW Alps, Pyrenees	E Pyrenees, SW Alps
G. rostanii Reuter	SW Alps	SW Alps, Pyrenees	SW Alps	SW & SC Alps; alpine pastures, gravelly slopes, 1600-2300 m	SW & SC Alps, ca. 1700 m
G. terglouensis Hacq. s.str.	E Alps, Abruzzi	SC & SE Alps	SE Alps	SE Alps; calcareous rocks & slopes, 1900-2700 m	S&E Alps; stony slopes, calcicole, 1900-2800 m
G. terglouensis Hacq. subsp. *schleicheri* Tutin			Pyrenees, SW & W Alps	Maritime Alps to W Switzerland	Maritime Alps to W Switzerland; calcicole 1200-2800 m

taxa according Tutin (1964)	Kusnezow (1894)	Soltokovic (1901)	Müller (1982)	Halda (1996)	Ho and Liu (2001)
G. utriculosa L.	Subalpine to alpine altitudes of C & S European Mts. , C European lowland marshes		C & E Alps, Apennine, Dinaric Alps, Balkan, Carpathians, along Rhine & Danube, Ukraine	C. Europe, Balkan; grassy slopes, moist meadows, open woods	Alps, Dinaric Alps, Balkan, Carpathians, NW Ukraine; manly Mts., rocky or gravel places, bogs, meadows, pine muggy woodland, 400-2500 m
G. verna L. s.str.	N European flatland, Pyrenees, Alps, Dinaric Alps, Balkan, Tatra, Caucasus, arctic European Russia	Alps, Carpathians	Pyrenees, Cantabric Mts, Alps & & northern neighboring ranges, Carpathians, British Islands, Ireland *G. arctica* Grossh.: Arctic European Russia	Europe, W Asia, NW Siberia, C Asia (Tienschan) ; dry rocky slopes, calcareous rocks, 200-3000 m	European Mts.; bogs, grassland, calcicole, alpine pastures, rocky limestone turf, headland, pine woodland, 320-3500 m
G. verna L. subsp. *oschtenica* Halda	W Caucasus		Pontic Alps, Taurus, Caucasus	W Caucasus	W Caucasus, SW Russia; scree, subalpine meadows, 2400 m
G. verna L. subsp. *balcanica* Pritch.	*G. verna* L. var. *angulosa* Wahlbg.: Altai, Tarbagatai, Tienschan, Caucasus, Taurus, Balkan, Dinaric Alps, Carpathians, Alps, C European flatland, Auvergne, Abruzzi, Pyrenees, arctic European Russia	Apennine, Dinaric Alps, Balkan	Dinaric Alps, Balkan, Pontic Alps	Balkan, S Europe	Balkan peninsula, Turkey; alpine pastures, limestone slopes & scree, 1800 – 2250 m
G. verna L. subsp. *tergestina* Hayek			Dinaric Alps	Dinaric Alps, SW Bulgaria, France, Italy	C & W Balkan Peninsula, Italy; Hillsides, grassland, limestone pastures, 400-2600 m
G. verna L. subsp. *pontica* Hayek		Turkey, C & S Caucasus	S. Caucasus, Elbours	Caucasus, Iran, Iraq, Turkey	C & E Balkan Peninsula, Caucasus, W & C Asia; grasslands, limestone slopes, subalpine meadows, alpine turf, 1830-3660 m
	G. verna L. var *tschichatschevi* Kusn.: Pontic Alps, Taurus, Caucasus	*G. angulosa* Bieb.: N Caucasus, C Asia	*G. angulosa* Bieb.: N Caucasus		
			G. uniflora Georgi: C Asia	*G. uniflora* Georgi: Altai, Mongolia; subalpine to alpine meadows, tundra, grassy moraines, 800-2700 m	

Table 1 (2 pages): Species distribution according to different authors

	life cycle	plant	stem	basal leaf	stem leaf	calyx	corolla
G. bavarica L.	perennial	4-20 cm	ascending to erect, simple	3-10 x 1.5-5 mm, ovate to spatulate, apex obtuse to rounded, no rosette	2-5 pairs, similar to basal leafs	15-18 mm, angled or winged with very narrowly wings on angles	dark blue, 20-33 mm, lobes spreading, 8-15 mm, oblong, ovate-elliptic to obovate, apex acute to rounded
G. brachyphylla Vill.	perennial	3-6(-15) mm	erect, slender, simple	5-11 x 4-6 mm, rhomboid, ovate to suborbicular, apex rounded, obtuse to subacute	1(-2) pairs, 5-6 x 2-3 mm, ovate, apex obtuse	10-12 mm, angled -winged with very narrowly wings on angles	dark blue, 15-30(-35) mm, lobes spreading, 8-12 x 3-5 mm, oblong, apex obtuse, base narrowed
subsp. *brachyphylla*				rhomboid, apex obtuse to subacute		not winged on angles	lobes narrow, about as long as wide
subsp. *favratii* Tutin				obovate to suborbicular, apex rounded		winged on angles	lobes as wide or wider as long
G. nivalis L.	annual	(2-) 6-15(-25) cm	ascending to erect, branched	9-12 x 6-8 mm, ovate to elliptic, apex obtuse to acute, crowded into rosette	6-11 x 3-6 mm, ovate to elliptic, apex obtuse to acute,	12-16 mm, angled-winged, very narrow wings on angles	dark blue, 15-20(-25) mm, lobes spreading, 4-6 mm, ovate, apex acute
G. pumila Jacq.	perennial	3-7(-15) cm	erect, slender, angled, simple	8-12 x 1.5-2.5 mm, linear to linear lanceolate, apex acute, rosette	1-3 pairs, similar to basal leafs, but smaller	11-20 mm angled or narrowly winged on angles	15-25 mm, lobes spreading, 5-8 x 2.5-4 mm, ovate to oblong
subsp. *pumila*						11-14 mm	lobe apex acute
subsp. *delphinensis* Fourn.						16-20 mm	lobe apex obtuse to apiculate
G. rostanii Reuter	perennial	10-16 cm	erect, slender, simple	8-20 x 2.5-5 mm, linear, no rosette	2-4 pairs, similar to basal leafs	15-18 mm, winged with very narrowly wings on angles	dark blue, lobes spreading, 6-10 mm, oblong to ovate, apex obtuse to rounded
G. terglouensis Hacq.	perennial	3-8 cm	erect, slender, simple	3-6 x 1.5-3.5 mm, ovate to ovate-lanceolate, apex acute, with incurved cartilaginous Tip; densely crowded, overlapping	none	10-14 mm, winged with very narrowly wings on angles	dark blue, 20-35 mm, lobes spreading, 8-10 x 3-5 mm, ovate to oblong, apex obtuse
subsp. *terglouensis*	perennial			more or less erect, not forming an apparent rosette			
subsp. *schleicheri* Tutin				spreading or erect spreading, forming an apparent rosette			
G. utriculosa L.	annual	6-25 (-35) cm	erect, slender, branched	7-12 x 5-10 mm, broadly ovate to obovate, apex obtuse, base narrowed	5-12 x 2-4 mm, lanceolate to elliptic, apex acute	13-22 mm, strongly winged, wings 2-4 mm wide	dark blue, 15-20 mm, lobes spreading, 5-7 mm, ovate-ovate-lanceolate, apex acute
G. verna L.	perennial	2-20 cm	erect, simple	8-20 x 3-8 mm, elliptic or lanceolate, apex acute, rosette	1-3 pairs, similar to basal leafs but 2-3 times shorter	12-22 mm, angled or more or less winged	dark blue, 15-25 mm, lobes spreading, dark blue, 8-10 mm, ovate-orbicular to ovate-elliptic, apex rounded to acute and mucronate
subsp. *verna*				lanceolate to elliptic, apex acute		wings 1-2 mm wide	15-25 mm, lobe apex obtuse
subsp. *oschtenica* Halda				about twice as long as wide, broadly ovate, apex obtuse		wings 2-3 mm wide	yellow
subsp. *balcanica* Pritch.				ca. 4 times longer as wide, narrowly elliptic to lanceolate, apex acuminate		wings c a 4 mm wide	(20-) 25-28 mm, lobe apex acute
subsp. *tergestina* Hayek				ca. 4 times longer as wide, narrowly elliptic to lanceolate, apex acuminate		wings c a 4 mm wide	38-40(-46) mm, lobe apex acute
subsp. *pontica* Hayek				about twice as long as wide, broadly ovate, apex obtuse		wings 2-3 mm wide	30-35 mm, lobe apex obtuse

perennial	> 10 cm	simple	lanceolate-elliptic	rosette	apex obtuse	different from basal leafs	wings > 2 mm	color dark blue
annual	- 10 cm	branched	Ellip.-ov./rhomb.	no ros.	apex acute	similar bo basal leafs	wings up to 2 mm	color yellow

Table 2: Morphological characters (including life cycle) according Ho and Liu (2001).


A


B


C


D


E


F


G


H


I


J


K


L

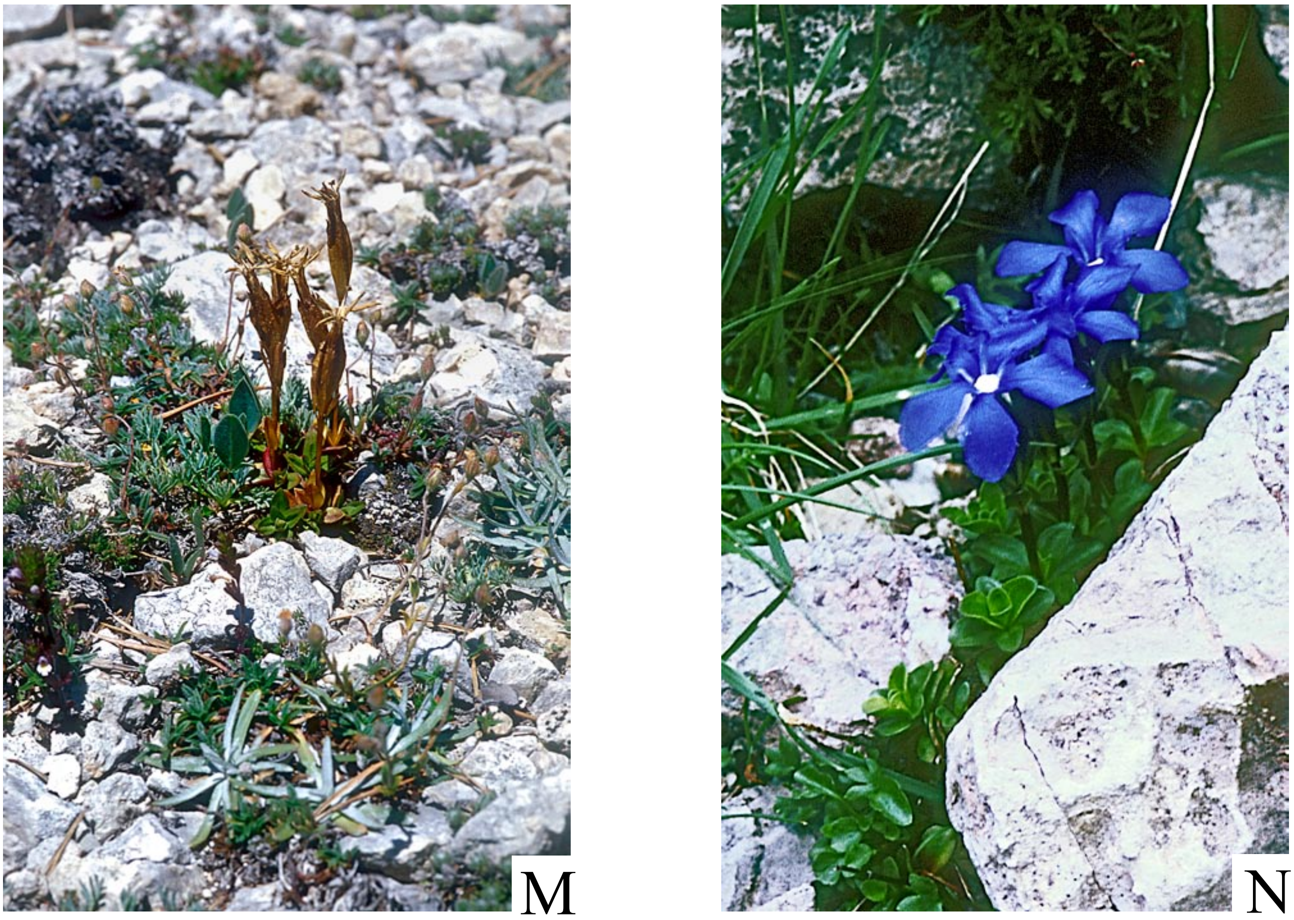


Figure 2 (4 pages): Different taxa of G. Sect. Calathianae Froel.

A: *G. nivalis* L., Hochbrett, Berchtesgaden, Germany
B: *G. utriculosa* L., Risnjak, Croatia
C: *G. pumila* Jacq. s.str., Wiener Schneeberg, Austria
D: *G. terglouensis* Hacq. s.str., Triglav, Slovenia
E: *G. bavarica* L. s.str., Hochbrett, Berchtesgaden, Germany
F: *G. bavarica* L. subsp. *subacaulis* Müller, Flims, Switzerland
G: *G. rostanii* Reuter, Col de Vars, France
H: *G. brachyphylla* Vill. s.str. Flims, Switzerland
I: *G. verna* L. s.str., Mangart, Slovenia
J: *G. pumila* Jacq. subsp. *delphinensis* Fourn., Mtgne de Lure, France
K: *G. verna* L. subsp. *tergestina* Hayek, Velika Alan, Croatia
L: *G. verna* L. subsp. *balcanica* Pritch., Olympos, Greece
M: *G. verna* L. subsp. *pontica* Hayek, Ilgaz Dağ, Turkey
N: *G. brachyphylla* Vill. subsp. *favratii* Tutin, Mongioie, Italy

Problems within *G.* Sect. *Calathianae* Froel. and aim of present project

G. Sect. *Calathianae* has a confusing distribution pattern by comprising a number of largely scattered taxa. Also morphological criteria seem to be not enough sufficient to deduce the taxonomy as reflected by a large number of different sometimes contradicting classifications. A need for new characters to enlarge the knowledge about the section and to enlighten the taxonomy of that group became evident. The present project was initialized to revise that taxonomy on so far unused molecular markers. Three principal goals were designated:

1) The large scale study of taxonomic structures within G. Sect. *Calathianae*
Under the assumption of *G.* Sect. *Calathianae* being a monophyletic unit: can closer related groups of taxa be identified?

2) A revision of the taxonomy within *G.* Sect. *Calathianae*
As different classifications are in use today, there is a need for new insights. Taxa of special interest are:
    a) *G. rostanii* Reuter ≡ *G. bavarica* L. subsp. *rostanii* Coste
    b) *G. terglouensis* Hacq. subsp. *schleicheri* Tutin ≡ *G. schleicheri* Kunz
    ≡ *G. verna* L. subsp. *schleicheri* Bolos et Vigo
    c) *G. brachyphylla* Vill. subsp. *favratii* Tutin = *G. orbicularis* Schur = *G. magellensis* Tammaro
    d) *G. brachyphylla* Vill. from Sierra Nevada (Spain) and Atlas (Morocco) = *G. sierrae* Briq.
    = *G. verna* L. subsp. *penetii* Lit. et Maire = *G. penetii* Romo
    e) *G. pumila* Jacq. subsp. *delphinensis* Fourn. ≡ *G. delphinensis* Beauv. ≡ *G. verna* L. subsp. *delphinensis* Kunz

3) Development of an evolutionary model to explain the distribution of one of the widespread taxa. Basically two taxa are showing a widespread distribution: *G. nivalis* and *G. verna* s.l. While the first one, distributed from Europe westward over Iceland and Greenland toward the Canadian coast of Labrador, shows a low amount of morphological variation, the second one, occurring from Europe eastward till Northern Central Asia is characterized by a number of distinct morphological types, varying in plant size, leaf shape, flower size and size of calyx wings.

In a preliminary assumption it was decided to base the present study on the taxonomy proposed by Tutin (1964) and Ho and Liu (2001) with exceptions: as such *G. bavarica* subsp. *subacaulis* Müller was collected as independent taxon as also the Sierra Nevada population of *G. brachyphylla* s.str. (also recognizes as *G. sierrae* Briquet).

Selection of methods and parameters

After preliminary studies covering pollen and seed morphology as well as cytology to evaluate the potential of these criteria, it was decided to focus fully on molecular biological markers to achieve the goals as above.
Choices were:

1) Chloroplast PCR-RFLP

Fingerprinting methods with different selectivity upon genotype variation were done. To possess a rough overview on variation within the chloroplast genome PCR-RFLP was chosen, enlightening variability between distant taxa and geographic pattern (Comes and Kadereit 1998). Such a uniparental marker is estimated to reflect different processes as isolation and range fragmentation in an efficient way (Halliburton 2004).
Chloroplast PCR-RFLP was selected as initial marker fast providing uniparental inherited information over a potentially large part of chloroplast genome. The usefulness of that method for developing a phylogeny between close related identities was been proofed in studies upon *Saxifraga oppositifolia* L. (Gabrielsen, Bachmann et al. 1997) , but also in *Zea mays* L. (Gauthier, Gouesnard et al. 2002) and *Brassica oleracea* L. (Panda, Martin et al. 2003) , but also by more distant taxa in different sections of *Musa sp.* L.(Nwakanma, Pillay et al. 2003) or between different conifers (Tsumura, Yoshimura et al. 1995). The experimental setting used in that study was designed to detect as much polymorphic RFLP sites in a reproducible way as possible. Amplified chloroplast sites were selected in regard of their length to provide enough digestion sites afterwards, but also in regards or reproducibility over the entire range of taxa. Parameters represented therefore a compromise between selectivity of amplification to prevent from undesired amplification products and quantity of amplified DNA to provide as much material as possible for afterward digestion. Enzymes used for digestion were selected in regard of a high number of polymorphic sides between the examined taxa and the uniformity of reaction conditions. Gel electrophoresis and staining conditions in the final step were designed to provide an as high degree of separation as possible over a long range of different digestion fragments and at same time guarantee the detection even of difficult (such as very short) fragments in a way as efficient as possible.

2) Chloroplast matK sequence

As all other sequences included, sequencing of matK provided a potentially co dominant marker represented by a limited number of loci (in case of matK a single one) and a known function and precise localization on the chloroplast genome in between conserved regions. As chloroplast sequence present marker is estimated to provide only one allele and therefore direct sequencing without preliminary cloning was done.

Usefulness of matK by Bremer, Bremer et al. (2002) to enlighten relationships between Asterids and related groups, or between distant plant families (Kusumi and Tachida 2000) respectively to establish a taxonomy within a family (Samuel, Kathriarachchi et al. 2005) , but was also part of studies concentrating on close taxonomic relationship within Japanese *Fagus* L. (Fujii, Tomaru et al. 2002) or studying the relationships around Canarian *Ixanthus sp.* Griseb. within the *Gentianaceae* (Thiv, Struwe et al. 1999, Struwe, Kadereit et al. 2002) or in *Myosotis sp.* L.(Winkworth, Grau et al. 2002).

In a preliminary study going out from a primer system developed by (Endress, Sennblad et al. 1996) leaded into a new and highly specific primer set for *G.* Sect. *Calathianae* matK. Main aim was the reproducibility within different taxa under selective conditions.

Sequencing of the complete sequence was rejected after about ¾ were known. Reasons therefore were the relatively high cost to get the remaining 400 to 600 base pairs and the low amount of expected additional information.

3) Nuclear encoded chloroplast expressed Glutamine Synthetase sequence (ncpGS)

NcpGS is reported as reported to be a single copy gene (Emshwiller and Doyle 1999, 2002). That nuclear and therefore biparental inherited single gene marker used also by Emshwiller and Doyle (1999, 2002) for *Oxalis sp.* L. and Perret, Chautems et al. (2003) in *Gesneriaceae* respectively alfalfa by Tischler, Dassarma et al. (1986) and different distant taxa by Pérez Vicente, Dorado et al. (1996). Perret, Chautems et al. (2003) showed the elevated level of resolution a phylogenetic analysis based on ncpGS compared to plastid markers. Emshwiller and Doyle (1999, 2002) compare ncpGS markers to ITS markers by providing an elevated resolution for the first marker. With the use of an autosomal marker it was estimated to obtain closer insights into eventual hybridization and exchange processes by be loss of resolution efficiency (see above).

NcpGS promised to provide a higher amount of variation than matK by covering also non coding parts and finally to access information inherited different than chloroplast markers. Also in discussion were sequences for nuclear Adh1 and Adh2 (Cummings and Clegg 1998; Ge, Sang et al. 1999) as much as Chalcon Synthetase (ChS) (Farzad, Soria-Hernanz et al. 2005). Parallel approaches for all 3 sequences (ncpGS, Adh and ChS) indicated a better efficiency for the first sequence.

Based on primers introduced by Emshwiller and Doyle (1999, 2002) an own primer set was designed leading into a sequence significantly longer than in Oxalis and equipped both coding as also noncoding sections with polymorphic intron.

4) Nuclear 5S-NTS sequences

5S-NTS represents a multi locus short sequence with a high amount of variation compared to ITS for some taxa. Cloning genetic accessions do discover potential different alleles were rejected in regard of the potential low amount of gained supplementary information in opposite to high investments necessary. Elaborated sequences represent therefore the most represented sequence respectively the one most preferred by the sequencing process. Since in between the different alleles of 5S-NTS homogenization events take rarely place (Kellogg et al. 1996) , the presence of a certain number of alternative alleles has to be expected.

The usefulness in phylogeny was shown by Playford, Appels et al. (1992) for *Acacia* L., by Becerra (2003) for *Bursera* Jacq. or for some *Gentianaceae* by Gould and Struwe (2004). A main advantage 5S-NTS is its shortness, facilitating a fast sequencing by doing few effort, but since no cloning technique was applied to discover a wider range of possibly different alleles, the gathered sequences represent the most represented respectively the best amplified one, but not the totality of existing different 5S-NTS sequences within each accession.

Since flanked by conserve 5S coding regions, the NTS was gathered by using the primers as proposed by Cox, Bennett et al. (1992) with an afterward removal of undesired side products. Sequences were obtained from direct sequencing using the backward directed primer as proposed by Cox, Bennett et al. (1992).

5) Amplified fragment length polymorphism study (AFLP)

As second fingerprinting accession AFLP (Vos et al 1995) was chosen since being a highly resolving universal method (Stehlik et al. 2001). It is estimated to deliver a fine scale analysis of phylogeographical relationships respectively of genetic diversity in between close related accessions.

AFLP suffers the deficit of being a dominant marker. As such polymorphisms are represented either by band presence or absence but not by a co dominant coexistence of both states (Robinson and Harris 1999) combined with a loss of efficiency (Lynch and Milligan 1994). With its manner of digesting DNA and selectively reproducing fragments provides a large number of selection criteria by consuming only a small amount of genomic DNA of high quality. The multiplex ratio of AFLP (number of genetic different loci) is the highest of all methods included in present work.

Previous sequences showed a polymorphism within *G. verna* representing one of the two largely distributed taxa, while the second, *G. nivalis* has few variable sites in the examined markers so far. Also *G. nivalis* was subject to an AFLP study done by the "Intrabiodiv" project (Commission of the European Union, Sustainable Development, Global Change and Ecosystems Program - GOCE-CT-2003-505376). Due to these two circumstances and also the higher availability of sampled populations *G. verna* was preferred over *G. nivalis*

AFLP was chosen to visualize the genetic diversity of *G. verna* within the western European Mountains. Also included as out group taxa were several populations of eastern European as much as Turkish *G. verna* and subspecies based on knowledge gained from studies above. Primer combinations chosen were selected for an enough high number of amplifications sites dispersed as regular as possible along the entire scanning range to facilitate the identification of loci and to obtain enough variable sites. Applying that setting the initially chosen number of four different primers resulted into a satisfying amount of variation.

Pollen and seed morphology

Nilsson (1967) did a very exhaustive study on pollen morphology within *Gentiana* L. describing for *G.* Sect. *Calathianae* subprolate to prolate, tricolporate pollen of 30-45 x 24-36 µm with a striate to reticulate sexine (often transitional in between the two states). Same conclusions can be found in Endress (1999) and Nilsson, Hellbom et al. (2002). Ho and Liu (2001) even proposes for *G. nivalis* from Greenland a rugulate-reticulate sexine. Vinckier and Smets (2003) described the presence of orbicules on the surface of *Gentiana* L. pollen grains.

An own study on pollen morphology was carried out on the scanning electronic microscope facilities of SMN, Université de Neuchâtel. A selection of dried pollen from herbarium sheets was examined to get information about shape, size and surface structure (see Fig. 4 and 5).

Pollen grains were generally of same shape as proposed by Nilsson (1967) , but in some cases larger up to 58 µm in case of one grain from *G. terglouensis* Hacq. subsp. *schleicheri* Tutin respectively pollen grains that were rather spheroid than prolate. Sexine structure varied between striate and reticulate. No clear type for a particular taxon was discovered.

Taxa as *G. verna* and *G. terglouensis* subsp. *schleicheri* tend to provide a rather prolate pollen while taxa as *G. bavarica*, *G. rostanii* and *G. terglouensis* s.str. provide rather spheroid pollen while *G. nivalis*. had no preference in particular (Fig. 3). But we have to face the fact that pollen of these taxa show a certain tendency of shrinking in an irregular way while dried (compare Fig. 4). Also are these pollen sticky and application to supporters for microscopy is only possible by touching these supporters with anthers, a artificial deformation of the examined grains has to be taken in account. Due to these two facts pollen morphology seems not to be a reliable criterion for species differentiation within *G.* Sect. *Calathianae*, in contrast to other authors as Moser (1996) who showed in other taxa even the possibility to deliver pollen type differences between subspecies.

Seeds of *G.* Sect. *Calathianae* visualized the same way as pollen above are reticulate and elliptic, sometimes shrunken and therefore elongated. Length is between 0.5 to 1.1 mm (Miège and Wüst 1984). The seeds are slightly winged at one end. Seeds of *G. nivalis* are comparably small . Miège and Wüst (1984) , same as *G. verna* and *G. pumila* Jacq. s.str.(Fig. 6) , while *G. terglouensis* Hacq. subsp. *schleicheri* Tutin, *G. brachyphylla* subsp. *favratii* Tutin and *G. utriculosa* provide rather large seeds (Fig. 6). Studies covering that subject were also done by Halda 1(995) and Boumann, Cobb et al. (2002).

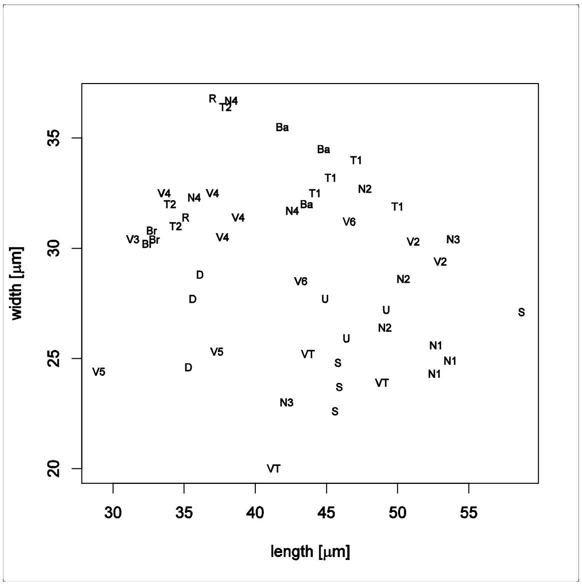


Figure 3: pollen measurements of different species:

Ba: *G. bavarica*
D: *G. pumila* subsp. *delphinensis*
N: *G. nivalis*
S: *G. terglouensis* subsp. *schleicheri*
T: *G. terglouensis* s.str.
V: *G. verna* s.str.
VT: *G. verna* subsp. *tergestina* Hayek.

Each letter marks one pollen grain, letters with additional numbers mark pollen grains from different herbarium sheets.

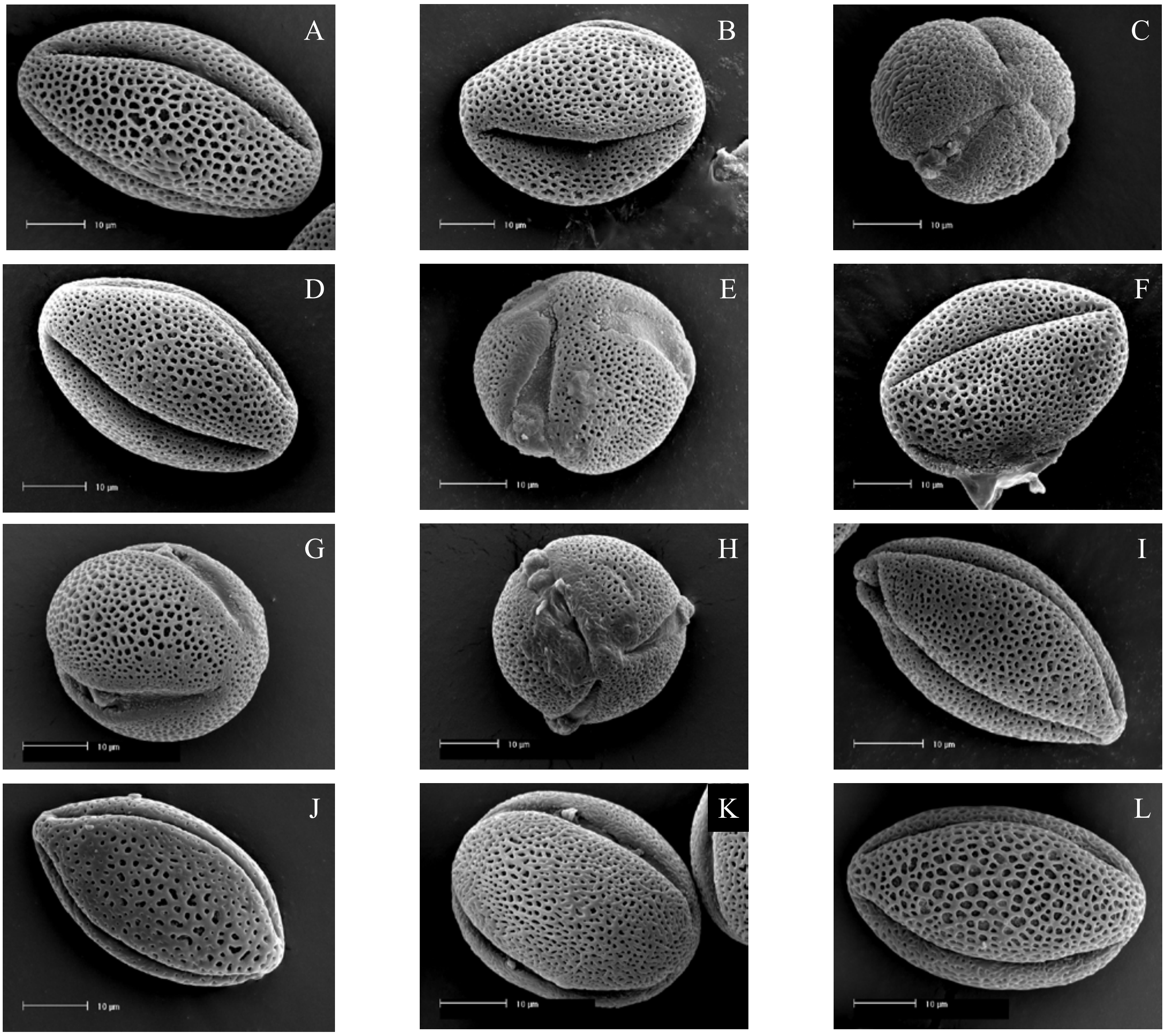
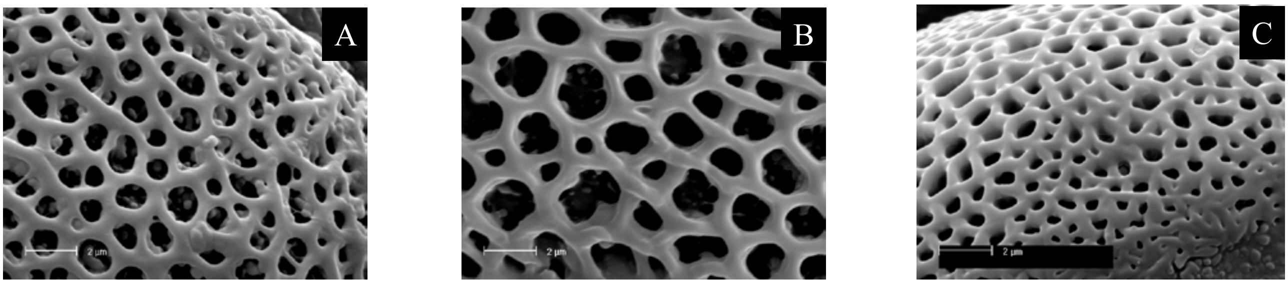


Figure 4: scanning electron microscope images of G. Sect. Calathianae pollen grains.
A: G. nivalis L. Piz Umbrail, Switzerland (NEU 079785)
B: G. bavarica L. Binntal, Switzerland (NEU 079441)
C: G. rostanii Reuter, Mt. Gondran, France (NEU 399320)
D: G. utriculosa L. Binntal, Switzerland (NEU 079435)
E: G. brachyphylla Vill. s.str., Zermatt, Switzerland (NEU 079438)
F: G. terglouensis Hacq. s.str., Passo Sella, Itlay (NEU 399297)
G: G. terglouensis Hacq. s.str., Tre Cime, Itlay (NEU 399298)
I: G. terglouensis Hacq. subsp. schleicheri Tutin, Binntal, Switzerland (NEU 079442)
J: G. verna L. subsp. tergestina Hayek, Caven, Slovenia (NEU 398271)
K: G. verna L. s.str., Passo Sella, Italy (NEU 398406)
L: G. verna L. s.str., Gasterntal, Switzerland (NEU 079800)



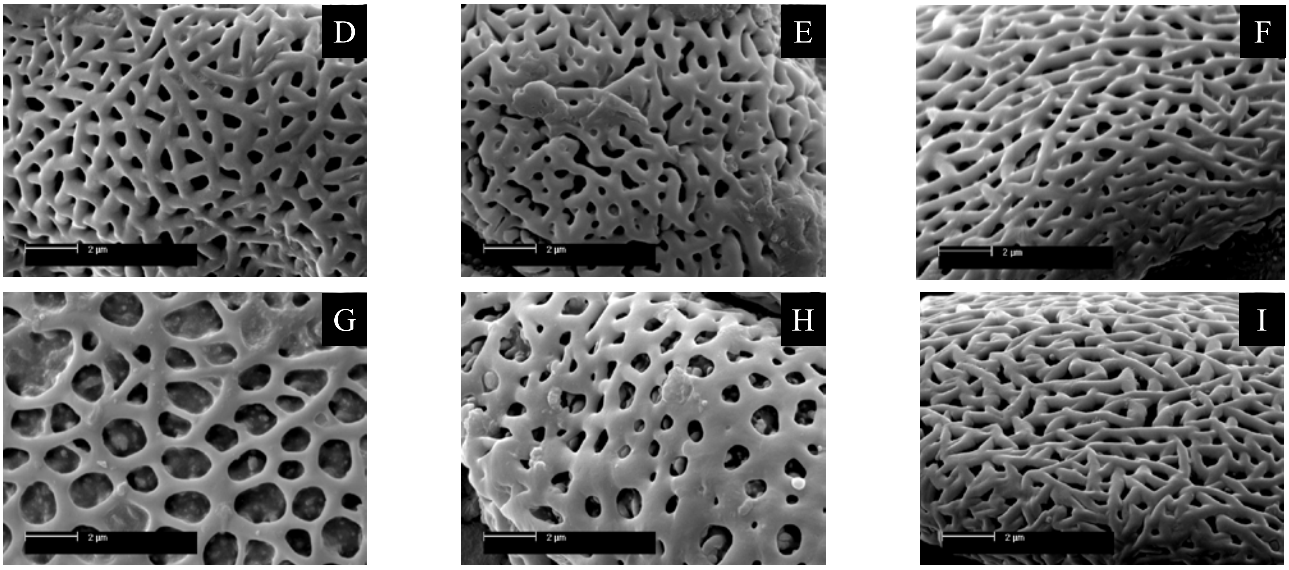


Figure 5: scanning electron microscope images of G. Sect. Calathianae pollen grain sexine structure.
A (prev. page): G. nivalis L., Cormet de Roseland, France (NEU 399329)
B (prev. page): G. nivalis L., Piz Umbrail, Switzerland (NEU 079785)
C (prev. page): G. terglouensis Hacq. s.str., Tre Cime, Itlay (NEU 399298)
D: G. brachyphylla Vill. subsp. favratii Tutin, Lötschenpass, Switzerland (NEU 079792)
E G. terglouensis Hacq. subsp. schleicheri Tutin, Binntal, Switzerland (NEU 079442)
F: G. verna L. s.str., Passo Sella, Italy (NEU 398406)
G:G. verna L. s.str., Gasterntal, Switzerland (NEU 079800)
H: L: G. verna L. s.str., Hourquette d'Ossue, France (NEU 398293)
I: G. verna L. s.str., Mt. Mongioie, Italy (NEU 398295)

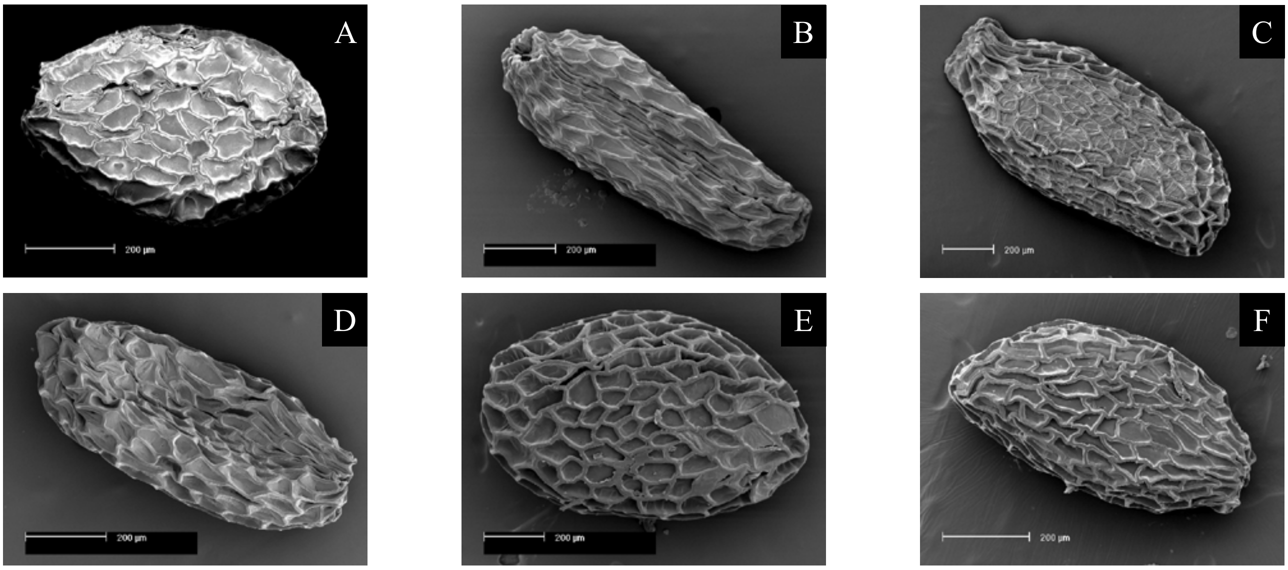


Figure 6: Surface electron microscope pictures of seeds
A: G. pumila Jacq. s.str., Wiener Schneeberg, Austria (NEU 398289)
B: G. utriculosa L. Binntal, Switzerland (NEU 079435)
C: G. brachyphylla Vill subsp. Favratii Tutin, Col de Restefonds, France (NEU 398303)
D: G. verna L. s.str., Ofenpass, Switzerland (NEU 079799)
E: G. terglouensis Hacq. subsp. schleicheri Tutin, Binntal, Switzerland (NEU 079442)
F: G. verna L. s.str., Pt. St. Bernard, France (NEU 398300)

Chemotaxonomy

A detailed report facing the phytochemical compounds of Gentiana L. was done by Jensen and Schripsema (2002) mentioning a series of iridoids in G. verna as much as some xanthone in G. nivalis and G. verna L. The presence of magniferin was proved by Hostettmann and Jacot-Guillarmod (1974, 1977) for G. nivalis, G. utriculosa and G. verna as much as for G. brachyphylla subsp. favratii Tutin by Massias, Charbonnier et al. (1982) , same as C-glucoflavones in G. bavarica, G. brachyphylla s.str. and subsp. favratii Tutin, G. rostanii Reuter, G. utriculosa (Hostettmann and Jacot-Guillarmod 1977; Massias, Charbonnier et al. 1982) , G. nivalis (Hostettmann, Hostettmann-Kaldas et al. 1981) and G. verna (Hostettmann and Jacot-Guillarmod 1974). Further work were done by Rivaille and Raulais (1969), Mpondo and Garcia (1989) , Mpondo, Garcia et al. (1989), Mpondo and Garcia (1990) and Mpondo and Garcia (1990).

Cytology

G. Sect. Calathianae has been subject to cytological studies for a long time. A series of studies were done, comprising in general only few taxa of that section. The work of Müller (1974, 1982) contains all taxa also included in the present work. An overview is given in Tab. 4

New counts were done as following: plants were grown in culture in a chalky substrate. Root tips of 2-3 mm length were collected and treated for 1h in a saturated aquatic solution of α-bromonaphthalene and immediately fixed afterward in Carnoy fluid (ethanol-acetic acid 3:1, v/v) with few acetic carmine and iron acetate added. Fixations were stored for at least one month prior to examination. Therefore the root tips were transferred to carmine acetate solution and stained during two hours, afterwards hydrolyzed by heating within the coloring liquid for two minutes to the boiling point and immediate transfer to cold 45% acetic acid. Squashes were made and observed under a Leitz Dialux 20 EB microscope (Leica Microsystems, Wetzlar, Germany). Drawings were done using a drawing apparatus. Results are given in Tab. 3. No further counts were made since not of all populations of living specimens were obtained and the results above conformed basically to what was given in Müller (1974, 1982).

G. verna L. s.str.	Alps, Switzerland, Jaunpass	2n=28
	Alps, Switzerland, Ofenpass	2n=28
	Jura, Switzerland, Mt. Tendre	2n=28
	Alps, Italy, Passo Sella	2n=28
	Apennine, Italy, Majella	2n=28
	Apennine, Italy, Gran Sasso	2n=28
G, brachyphylla Vill. subsp. *favratii* Tutin	Alps, Italy, Mt. Mongioie	2n=32
G. rostanii Reuter	Alps, France, Col de Vars	2n=30
	Alps, France, Mt. Gondran	2n=30
	Alps, France, Col de la Mautière	2n=30-31
G. terglouensis Hacq. subsp. *schleicheri* Tutin	Italy, Alps, Mt. Mongioie	2n=30
G. verna L. subsp. *tergestina* Hayek	Croatia, Velebit Mts.	2n=(28) -30

Table 3: Chromosome numbers counted within present study

G. bavarica L. s.str.	-2n=28 Mattik in (Tischler 1950) -Slovenia, Karawanken: 2n=28+0-3B (Love 1986) -Alps, different proveniences: 2n=30 (Müller 1974; Müller 1982)
G. bavarica L. subsp. *subacaulis* Müller	-Alps, different proveniences: 2n=30 (Müller 1974; Müller 1982)
G. brachyphylla Vill. s.str.	-Switzerland, Simplon: 2n=28 (Favarger 1965) -Switzerland, Graubünden: 2n=28 Scholte G. in (Love 1977) -Alps, different proveniences: 2n=28 (Müller 1974; Müller 1982) -Spain, Pyrenees: 2n=28+0-2B (Love 1986) -Marokko, Haut Atlas: 2n=30 (Müller 1982) -Spain, Sierra Nevada: 2n=30 (Müller 1982) -2n=28 Mattik in (Löve 1953)
G. brachyphylla Vill. subsp. *favratii* Tutin	-Switzerland, Vaud: 2n=30 (Favarger 1965) -Slovenia, Julische Alpe: 2n=28+0-4B (Love 1986) -Spain, Sierra Nevada : 2n=28+3B and 2n=30+1B (Küpfer 1968) -Alps, Abruzzi, different proveniences: 2n=32 (Müller 1974; Müller 1982) -France, Col du Tronchet and Col d'Izoard: 2n=32 (Küpfer 1969) -France, Col de Traversette: 2n=28-29 (Küpfer 1969)
G. nivalis L.	-Norway, Hemsedal: 2n=14 (Knaben 1950) -Alps: 2n=14 (Favarger 1949; Favarger 1952; Müller 1974) -Iceland: 2n=14 (Love 1986) -Norway: 2n=14 (Love 1986) -Different proveniences: 2n=14 (Löve 1953; Müller 1982)
G. pumila Jacq. s.str.	-Austria, Wiener Schneeberg: 2n=20 (Favarger 1965; Müller 1982) -Slovenia, Mangart: 2n=21 (Love 1986)
G. pumila Jacq. subsp. *denphinensis* Fourn.	-France, southern Alps: 2n=28 (Guinochet 1967; Müller 1982)
G. rostanii Reuter	-France, Hautes Alpes: 2n=30 Dent, T. C. in (Love 1969) -Italy, Valle Germenasca: 2n=30 (Müller 1974) -SW Alps, different proveniences: 2n=30 (Müller 1982) -Italy, Cottian Alps: 2n=28+0-2B (Love 1986) -France, Hautes-Alpes: 2n=30 (Favarger 1969)
G. terglouensis Hacq. s.str.	-Italy, Rossalpe: 2n=40 (Favarger 1965) -Slovenia, Julische Alpe: 2n=42 (Love 1986) -SE Alps, different proveniences: 2n=38(-40) (Müller 1982)
G. terglouensis Hacq. subsp. *schleicheri* Tutin	-Switzerland, Wallis: 2n=42 (Love 1986) -Alps, different proveniences: 2n=30-31 (Müller 1982) -Pyrenees, Puigmal: 2n=30-33 (Müller 1982)
G. utriculosa L.	-Slovenia, Julische Alpe: 2n=28+0.6B (Love 1986) -Alps, different proveniences: 2n=22 (Müller 1974; Müller 1982) -Alps, Mt. Torrione: 2n=22 (Favarger 1952)
G. verna L s.str	-2n=26, Tatra Mts. (Skalinska 1950) -Spain, Pyrenees: 2n=28+0-3B (Love 1986) -Alps, Jura, Abruzzi, different proveniences: 2n=28 (Favarger 1949; Müller 1974; Müller 1982) , Quézel in (Löve and Löve 1961) , Matitick in (Löve 1953) *G. arctica* Pall. 2n=24 Krogulevich R. E. in (Gagnidze and Gviniaschvili 1984)
G. verna L subsp. *tergestina* Hayek	-Slovenia, Primorsko: 2n=28+0-2B (Love 1986) -Former Yugoslavia, different proveniences: 2n=30 (Müller 1982)
G. verna L subsp. *balcanica* Pritch.	-Bulgaria, Rhodope Mts: 2n=28+0-2B (Love 1986) -FYR Macedonia and Greece, different proveniences: 2n=30 (Müller 1982)
G. verna L subsp. *pontica* Hayek	-*G. angulosa* Bieb (Caucasus) 2n=30 (Gagnidze, Küpfer et al. 1992) -*G. uniflora* Georgi 2n=26 (Krasnobrow, Rostovtseva et al. 1980) , Krasnobrov J.M. in (Gagnidze and Gviniaschvili 1984) -Iran, Elbours: 2n=30 (Müller 1982)
G. verna L subsp. *oschtenica* Halda	-2n=26 Gagnidze, R.I. in (Takhtajan and Agapova 1990) -Caucasus: 2n=30 (Müller 1982)

Table 4: Chromosome numbers in literature with provenience, if known.

Conservation Status

Calathianae are generally known elements of a decorative alpine flora. Beside human impact and destruction of the natural environment these plants are potentially endangered by exhaustive sampling activities. As consequence a protection by law is known from regional or national level such as G. verna for Basel Land, Switzerland (SGS Basel Land, 790.11) or G. utriculosa Steiermark, Austria (NSchG Steiermark, § 13 Abs.2, 1976) , for *Gentiana* in general for Germany (Verordnung zum Schutz wild lebender Tier- und Pflanzenarten, Germany, Anlage 1). (Danton, Baffray et al. 1995) also mentions G. utriculosa as protected for France and indicates her only for the Vanoise (Savoie) , while its said to be extinct in the departments "Haute-Rhin", "Bas-Rhin" and "Haute-Savoie".

On the course of our plant collections in the alpine region we have been able to refind several stations, described from herbarium sheets with more than 150 years in age (France, Col du Galibier, "*Gentiana brachyphylla*, Galibier (Dauph.) , 22. Août 1846, Verlot" [P]; Germany, Berchtesgaden, *Gentiana brachyphylla* s.str. "Pelouses sur la terre micaschisteuse du sommet de la montagne Hochbret, à 2432 m, dans les Alpes calcaires", Einsele, 4.8.1850 [G]). On the other hand as indicated above for G. utriculosa some rather less elevated occurrences have disappeared in the last decades. As such (Meusel, Jäger et al. 1978) reports extinct populations of *G. utriculosa* from along the Rhine around Mannheim, Germany and for *G. verna* from the Harz Mountains, Germany. We made similar experiences by searching *G. verna* subsp. *tergestina* Hayek at Bassovizza (Trieste, Italy) as reported from different herbarium specimen ("Triest: Karst-Wiesen um Bassovizza, Kalkboden, ca 350m, April 1892", Steurer [P, G, BM]) which we were not able to find in nature. Only few described places from alpine elevation we were not able to re-find. Possibly a severe case we have to face for the north African type of G. brachyphylla, also described from the High Atlas as G. penetii Romo, where the its best known occurrence close to Ref. Neltner, (Djebel Toubkal) seems to be degraded by intensive touristic and agricultural use and no samples of that taxon was found.

Nevertheless several taxa are estimated to have a somehow threatened status in Europe. Red Lists covering the Alpine chain have been done for France (Olivier, Galland et al. 1995) , Switzerland (Moser, Gygax et al. 2002) , Liechtenstein (Broggi, Waldburger et al. 2006) , Italy (Conti,

taxon	Liechtenstein (Broggi, Waldburger et al. 2006)	Switzerland (Moser, Gygax et al. 2002)	Italy (Conti, Manzi et al. 1997)	France (Olivier, Galland et al. 1995)	Germany (Ludwig and Schnittler 1996)	Austria (Niklfeld and Grims 1999)
G. utriculosa	**rare**	CH: **LC** MP: **EN**; NA: **VU**; WA: **NT**; EA: **LC**; SA: **LC**	Em: **VU**; La: **VU**		D: **CR!**; RP: **EX**; BW: **CR**; BY: **EN**	**rare** V, T, S, K Rh, KB
G. brachyphylla	**rare**	CH: **LC**; NA: **LC**; WA: **LC**; EA: **LC**; SA: **LC**				
G. verna		CH: **LC**; JU: **LC**; MP: **EN**; NA: **LC**; WA: **LA**; EA: **LC**; SA: **LC**	Cal: **VU**		D: **EN**+; HE: **EX**; TH: **CR-EX**; ST: **EX**; BW: **EN**; BY: **EN**	**rare** V, T, S, K, St, O, N, B Rh, KB, n+sölVL, Pann
G. bavarica		CH: **LC**; NA: **LC**; WA: **LC**; EA: **LC**; SA: **LC**				
G. nivalis		CH: **LC**; JU: **EN**; NA: **LC**; WA: **LC**; EA: **LC**; SA: **LC**	Em: **EN**; La: **VU**			
G. brachyphylla subsp. favratii		CH: **LC**; NA: **LC**; WA: **NT**; EA: **LC**, SA: **NT**	It: **LR**; Ma: **VU**; La: **VU**; Ab: **LR**; Pi: **LR**		BY: **VU**	
G. terglouensis subsp. schleicheri		CH: **NT**; WA: **NT**	It: **LR**; Pi: **LR**	**rare**		

Table 5: Conservation cathegories affecting *G.* Sect *Calathianae* for Alpine range

Austria: V: Vorarlberg; T: Tirol; S: Salzburg; K: Carinthia; O: Oberoesterreich; N: Niederoesterreich and Vienna; St: Styria, B: Burgenland; Rh: Rheintal, KB: Kärntner Becken- und Tallandschaften, nVL: Vorland nördlich der Alpen, söVL: Vorland südöstlich der Alpen

Germany: D: overall Germany; RP: Rheinland-Pfalz; HE: Hessen; TH: Thüringen; ST: Sachsen-Anhalt; BW: Baden-Würtemberg; BY: Bayern; **+**: regionally stonger endangered; **!**: important responsability

Italy: **LR**: lower risk; It: overall Italy; Pi: Piemonte; La: Lazio; Ma: Marche; Em: Emilia Romagna; Cal: Calabria; Ab: Abruzzo

Switzerland: JU: Jura; MP: Mittelland; WA: westliche Zentralalpen; EA: Östrliche Zentralalpen; SA: Südalpenflanke; NA: Nordalpenflanke

IUCN code: **EX**: extinct; **CR**: Critical; **EN**: endangered; **VU**: vulnerable; **NT**: near threatened; **LC**: lest concern (IUCN-The World Conservation Union 1994, IUCN-The World Conservation Union 2001).

New insights into *Gentiana* Sect. *Calathianae* Froel. (*Gentianaceae*) based on chloroplast PCR-RFLP

Abstract

Gentiana Sect. *Calathianae* Froel. represents a small species group that occurs in the mountains of Europe and Asia. Based on a chloroplast PCR-RFLP we are able to establish a new detailed phylogeny of the section. *G.* Sect. *Calathianae* Froel. is composed of an annual and a perennial group of taxa, supported by results from chloroplast PCR-RFLP. As only perennial taxon placed in between the two annual species *G. pumila* Jacq. s.str. gets separated from the other perennial taxa. The group covering remaining perennial taxa becomes divided into tree parts: one containing all non-*G.-verna*-taxa, one comprising of western European *G.-verna*-taxa, and the last formed of eastern-*G.-verna*-taxa, supported by morphological and also geographical considerations. Several taxa have to be rearranged. As such *G. pumila* Jacq. subsp. *delphinensis* Fourn. gets separated from *G. pumila* Jacq. s.str.. Same is the case In *G. terglouensis* Hacq. s.str. and subsp. *schleicheri* Tutin respectively *G. brachyphylla* Vill s.str. and subsp. *favratii* Tutin. The taxon *G. brachyphylla* Vill s.str. sensu Ho and Liu also turns out to be polyphyletic by providing one type covering alpine populations and another one populations from Spanish Sierra Nevada. G. verna L. itself is also polyphyletic by being divided into an alpine/western European and an eastern part, corresponding mainly to the distribution areas of subsp. *verna* L. for the western and subspecies t*ergestina* Hayek, *balcanica* Pritch. and *pontica* Hayek for the eastern part.

Keywords

Phylogeny, Gentiana, Chloroplast PCR-RFLP, PCR, Restriction, Taxonomy

Introduction

Gentiana Sect. *Calathianae* Froel. is comprised of 15 taxa found mainly in alpine regions in Europe, less commonly in Asia, and one species each in North America and Africa. There are two annual taxa, and the remaining are perennials. Common characters include sympodial stems, the flowering stems arising singly from stolons, solitary flowers, and a salver-shaped corolla subtended by an angled or winged calyx. The stigma lobes are expanded and connate or contiguous, forming a discoid structure. Seed coats are reticulate and winged on some side.

Kusnezow (1894) did an early revision of that section by placing it as an outstanding group distant from the other sections of *Gentiana*, neighboring nowadays *G.* Sect. *Ciminalis* Dum. Halda (1995) used the presence of two different life-cycles to postulate a division this group into two sections. Müller (1982) did a recent work covering the section by counting chromosome numbers for all taxa by proposing several basal groups with common chromosome evolution. These were each one covering the annuals *G. nival*is L. (2n=14), and *G. utriculosa* L. (2n=22), a third one for *G. bavarica L.* and *G. rostanii* Reuter (both 2n=39), colonizing as vicariant taxa almost the same ecology, a fouth one for the two vicariant *taxa G. terglouensis* Hacq. (2n=38) and subsp. *schleicheri* Tutin(2n=30) as well *as G. pumila* Jacq. (2n=20). A last group hosted *G. verna* L. and its subspecies (2n=28-30), but also *G. brachyphylla* Vill. s.str. and in isolated positions *G. brachyphylla* Vill subsp. *favratii* Tutin and *G. sierrae* Briq. (all 2n=30). Later Halda (1995) and Ho and Liu (2001) focused for that section mainly on morphological criteria. Ho and Liu postulated one only section comprising of a large central species covering *G. verna* L. and its subspecies (Tutin 1964), a eastern and western alpine, disjunct species *G. pumila* Jacq. including subsp. *delphinensis* Fourn., a high alpine species covering the two vicariant taxa *G. terglouensis* Hacq. and subsp. *schleicheri* Tutin as well as a large taxon *G. brachyphylla* Vill. including the southern Spanish and northern African taxa, beside some smaller species. Halda (1995), some years before, proposed to separate the perennial taxa in an new *G.* Sect. *vernae* Halda and to arrange that one together with the remaining *G.* Sect. *Calathianae* Froel., hosting only the annual taxa, under the roof of *G.* subgen. *Calathaiane* (Froel.) Halda. Gielly and Taberlet (1996) derived a taxonomy on European Gentians from trnL (UAA) sequences, covering also some taxa of *G.* Sect*. Calathianae* Froel.

Present study serves to delifer more detailed informations on molecular evolution concerning that rather confusing section. We would specially ask the question wheather the section can be divided into smaller units as done by Halda (1985). Furthermore the identity of recently rearranged taxa such as *G. pumila* Jacq. subsp. *delphinensis* Fourn. of *G. terglouensis* Hacq. subsp. *schleicheri* Tutin is a point of question as much as the extention of G. brachyphylla Vill s.str. by the integration of *G. sierrae* Briq and *G. penetii* Romo. We also would answer the question about the relatedness of distinct Alpine and Carpathian populations of *G. brachyphylla* Vill. subsp. *favratii* Tutin.

As working basis we accept the taxonomy proposed by Tutin (1964) and Ho and Liu (2001). Our sampling comprises of individuals from the mountain ranges of

Northern Spain and the Central Spain Sierra Nevada, the Central and Southern Pyrenees, the entire Alpine range as well as the Swiss Jura, Scandinavia and Island, the Dinaric Alps and the Balkan range, the southern Carpathians, the Northern and Southern Pyrenees, the Pindus and the Pontic Mountains as well as few material from the Ural and Altai Range. Not included is material from Northern Africa, the Apennine, the British and Irish Islands, Greenland or North America. On the taxonomic level we are missing *G. verna* L. subsp. *oschtenica* Halda, a *verna*-like taxa with lemon-yellowish corolla, growing endemic in the Caucasus and southwestern Russia (Komarov 1967; Halda 1995) and *G. penetii* Romo (Romo 1988), a *brachyphylla*-like taxon from the Higher Atlas. Ho and Liu (2001) treated *G. penetii* Romo as *G. brachyphylla* Vill. s.str.. We are also missing sample material clearly identified as taxon *G. angulosa* Bieb. from Eastern Turkey and the Caucasus synonymized under *G. verna* L. subsp. *pontica* Hayek by Ho and Liu (2001).

In studies parallel to these uniparental inherited marker system, chloroplast and nuclear sequences got elaborated, such as maturase K (Haemmerli and Küpfer in publication a), nuclear encoded glutamine synthethase (Haemmerli in publication b) or 5S-NTS (Haemmerli in publication c). Chloroplast PCR-RFLP as presented provides a basic screan for cpDNA variability as used in several comparable studies (Holderegger at al. 2002, Parducci and Szmidt 1999). Since ist potential lack of recombination (Comes and Kadereit 1998) in opposite to nuclear encoded sequences, that marker is estimated to illustrate different processes as isolation and range fragmentation (Halliburton 2004).

Material and Methods

Sampling sites were selected by studies of the herbaria NEU, G, P, K, BM and ZA/ZAHO, but also the CRSZ-database for Switzerland (Baumler 1999). The taxa and populations collected for this study are listed in sample table (Annex). As outgroup *G. prostrata* Haenke (*G.* Sect. *Chondrophylla* Bunge) and *Gentianopis ciliate* (L.) Ma have been selected due to their ability to produce suitable amplification products for all desired chloroplast regions included into that study. Between 5 and 10 individuals with a distance of more than 30 cm were taken from each population by preventing from sampling twice on the same individual. Leaf material was fresh dried on silica gel. Of 2 respectively 5 samples per population chosen by chance approximately 20 mg of leaf material was grounded using Tungsten Carbide beads and Qiagen TissueLyser (Qiagen, Basel),. A DNA extraction was performed using DNEasy Plant Mini Kit (Qiagen, Basel), leading into about 50ng total DNA per µl extract. Based on approximately 50ng DNA, a PCR was performed, using 1U Taq DNA Polymerase (Qiagen, Basel), 200µM of each dNTP, 0.2µM of each primer, 1x Q-solution, 2.5mM MgCl2 and 1x PCR buffer (Qiagen, Basel) in a total volume of 25µl. The procedure was carried out on a Biometra T3000 thermocycler (Biometra, Göttingen) by performing a initial denaturation of 2 min at 95°C, 40 cycles: 10s at 94°C, 1 min at the appropriate annealing temperature and 3 to 4 min at 68°C for extention, followed by final 5 min at 68 °C. Primers used were described by Demesure (1995), Dumolin-Lapegue (1997), Morton (1993) and Petit et al. (1998). For details see Tab. 2. 5µl of amplification product were digested with 2.5U of restriction enzyme (Promega, Madison), 1.25mg BSA and 1x restriction buffer (Promega, Madison) in a total volume of 12.5µl. Digestion was performed for 4 hours at 37°C with subsequent 15 min at 70°C on a Biometra T3000 thermocycler (Biometra, Göttingen). Another 5µl of amplification product was used to run a horizontal gel electrophoresis on 1% agarose in 0.5x TBE-buffer (Maniatis, Fritsch et al. 1982) as control. 2µl of digestion product was used for vertical polyacrylamide electrophoresis on 20 x 20 x 0.4 cm gels composed by 6% polyacrylamide (acryl:bisacryl = 29.1:0.9, Merck, Darmstadt) in 1x TBE buffer (Maniatis, Fritsch et al. 1982) in Protean II cells ((Biorad, Reinach). Electrophoresis was performed for 4 hours at a voltage of 150V. The gels were colored with Ethidium Bromide (Biorad, Reinach) and photographed on a Geldoc 100 (Biorad, Reinach). Bands were scored by hand and transformed into a binary presence/absence code.

chloroplast region	primer 1 (5’->3’)	primer 2 (5’->3’)	annealing temperature	elongation time	restriction enzymes
psaA-trnS [1]	ACTTCTGGTTCCGGCGAACGAA	AACCACTCGGCCATCTCTCCTA	57.5	3’30”	a
trnF-trnV [2]	CTCGTGTCACCAGTTCAAAT	CCGAGAAGGTCTACGGTTCG	57.5	3’30”	b
trnV-rbcL [2]	CGAACCGTAGACCTTCTCGG	GCTTTAGTCTCTGTTTGTGG	57.5	3’30”	c
trnK [1]	GGGTTGCCCGGGACTCGAAC	CAACGGTAGAGTACTCGGCTTTTA	57.5	3’30”	c
trnK2-trnQ [2]	TAAAAGCCGAGTACTCTACCGTTG	CTATTCGGAGGTTCGAATCCTTCC	57.5	3’30”	a
trnT-psbC [2]	GCCCTTTTAACTCAGTGGTA	GAGCTTGAGAAGCTTCTGGT	52.5	3’	d
Z1204-psaI [3]	TTTGGTGGAACTTTAGGACACCCTTGGGG	GCAATTGCCGGAAATACTAAGC	52.5	3’	c
rpoC2-rpoC1[4]	TAGACATCGGTACTCCAGTGC	AAGCGGAATTTGTGCTTGTG	51	4’	d

Restriction enzymes: a: EcoRI, HindIII, MspI, RsaI
b: AluI, HindIII, MspI, RsaI
c: AluI, EcoRI, MspI, RsaI
d: EcoRI, HaeIII, HspI, RsaI

Tab. 2: primers used as described by Demesure (1995)[1], Dumolin-Lapegue (1997)[2], Morton (1993)[3] and Petit et al. (1998)[4], pcr conditions and restriction enzymes.

Data Analysis

The molecular data were scored as binary characters: each fragment was coded as present or absent.

Parsimony analysis (Fitch 1971) was carried out with PAUP* (Swofford 1998) using random sequence addition with 1000 sequence replicates via stepwise addition, where 10 trees were held at each step, the TBR branch-swapping-algorithm in effect. A bootstrap analysis was done with the same parameters as above for 1000 replicates.

To provide more detailed information upon the relationship between these taxa, grouping together in parsimony analysis, a reduced dataset consisting of the taxa *G. terglouensis* Hacquet s.str., *G. brachyphylla* Vill., *G. bavarica* L. and *G. rostanii* Reuter including their subspecies, the mean distances were calculated upon the binary coded restriction sites and used as base for a principal component analysis on the R package (Gower 1966; Becker, Chambers et al. 1988; Chambers and Hastie 1992).

Results

A totality of 294 samples produced 42 different restriction types with a 553 restriction fragments. 316 of these fragments represent informative sites. All samples out of the same population showed an identical restriction profile.

The region trnKs-trnQ provides no restriction sites for enzyme MspI, different from the outgroup species sshowing restriction activity also within that sequence. Netherless the length of that amplification product turned out to be highly polymorphic. 7 different length classes were defined as shown in Tab. 3 and are further called K2Q-types.

A first K2Q-type (1) is unique to the annual taxa *G. utriculosa* L., while the second annual taxon *G. nivalis* L. provides two distinct types. All populations of *G. nivalis* L. (ni01 and ni02) except those froum southern Carpathians provide type 2 sharing that one with the Sierra Nevada population of *G. brachyphylla* Vill. s.str. (br01). The second ones (ni03 and ni04) are sharing type 3 togwther with *G. pumila* Jacq. s.str. and subsp. *delphinensis* Fourn. as much as *G. rostani* Reuter, the Carpathian populations of *G. brachyphylla* Vill. s.str. (bf01), *G. terglouensi* Hacq. subsp. *schleicheri* Fourn. and *G. verna* L. including all its subspecies. Type 4 was only presented by the western European populations of *G. brachyphylla* Vill. subsp. *favratii* Tutin (bf02 and bf03), type 5 was private to *G. bavarica* L. s.str. and *G. terglouensis* Hacq. s.str., while *G. bavarica* L. subsp. *subacaulis* Müller war home to type 6. Type 7 finally was home only to alpine *G. brachyphylla* Vill. s.str.

Parsimony analysis was resulting in 4 equally parsimonious trees of 904 steps each with a CI = 0.558 and RI = 0.792. A strict consensus was calculated based on these 4 trees, resulting into 911 steps and slightly lower fit measures (see fig. 1).

The parsimony consensus tree provides a first bifurcation separating *G.* Sect. *Calathianae* Froel. From the outgroup species (out1 and out2). Within the section a next node with 100% bootstrap support separates between annual (*G. nivalis* L. and *G. utriculosa* L.) and perennial taxa. Within the perennial ones *G. pumila* Jacq. s.str. gets separated with 79% bootstrap support. The remaining taxa are divided with maximum support into a group covering the eastern European and Asian *G. verna* L. subspecies (eastern-verna-group) and another composed of the western European *G. verna* L. s.str. and some other taxa in a first (western-verna-group) and a variety of other taxa (non-verna-group) in a second group. The support for that last bifurcation is somehow lower as above with only 67%.

As derived from parsimony analysis the *G.* Sect. *Calathianae* Froehl. is divided into two subclades: the one hereafter called the *Calathianae*-subclade consists of the two annual species *G. nivalis* L. and *G. utriculosa* L., the other named as *Vernae*-subclade is composed by all other taxa which are perennial. Within the *Calathianae*-subclade the Alpine *G. nivalis*-populations and the material from Iceland show all a same restriction type (ni01), while the Norwegian population (ni02) is separated by 8 differing restriction sites. The two Carpathian populations ne03 and ne04 are separated from each other by 2 differing sites. Between the Carpathian and the Alpine ones 10 respectively 8 differing restriction sites were identified. All restriction sites differing between the Alpine and the Norwegian individuals were also differing between the Norwegian and the Carpathian ones. The Alpine and Dinaric populations of *G. utriculosa* L. separate from the Carpathian by 25 different restriction sites. We can therefore declare the existence of two clearly differnet Alpine and Carpathian haplotypes for *G. utriculosa* L. and different Alpine, Carpathian and Scandinavian haplotypes for *G. nivalis* L. The K2Q-types of G. *utriculosa* L. are distinct from those of *G. nivalis* L., which is providing us a type for the western and one for the eastern populations, the eastern one in common with a large part of the perennial taxa out of the *Vernae*-subclade.

Within the *Vernae*-subclade we can identify three different groups, a non-*verna*-group, a western-*verna*- and an eastern-*verna*-group, as well as two solitary populations (pu01 and br01) as mentioned by describing the parsimony tree (for details see fig. 1). An isolated taxon within the perennials is *G.* pumila Jacq. s.str. with a basalmost position. The two accessions of *G. pumila* Jacq. provide no possibilities for internal differentiation. Also a separate place takes the Sierra-Nevada population *of G. brachyphylla* Vill. s.str. but the low support in bootstrap analysis for the nodes involved demonstrates proximity of that taxon to the non-*verna*- and western-*verna*-group. The K2Q-type of Sierra-Nevada population of *G. brachyphylla* Vill. is similar to the one of the western *G. nivalis*-populations (n01 and n02), but different from all other populations of *G. brachyphylla* Vill

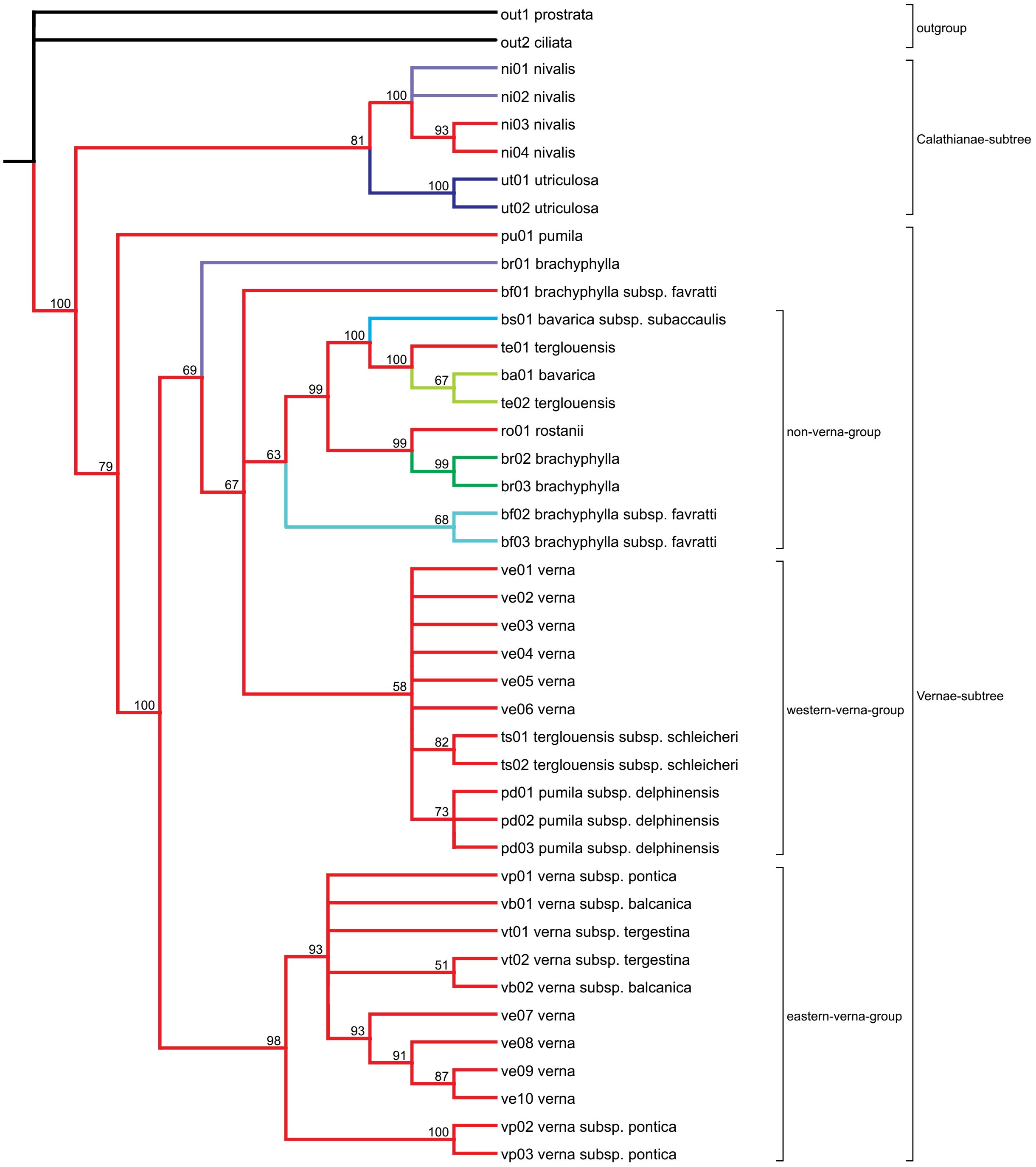


Fig. 1: Strict consensus out of 4 most parsimonious tress. Bootstrap supports above 50% are indicated.

s.str., while the other isolated taxon *G. pumila* Jacq. s.str shares his type with accessions of *G. verna* L.

Strong support is also provided by a group consisting of all south-eastern European *G. verna* L. and *verna*-subspecies including also the Turkish and the eastern Russian populations. Internally isolated are the two Turkish populations of *G. verna* L. subsp. *pontica* Hayek. (vp02 and vp03) as well as the southern Carpathian *G. verna* L. (ve07 to ve10), all other specimen, the material of *G. verna* L. subsp. *tergestina* Hayek from the Dinaric Alps up to the Istrian Peninsula, the Greek subspecies *G. verna* L. subsp. *balcanica* Pritch. from Pindus Mts. and the Russian Altai population of *G. verna* L. *subsp. pontica* Hayek (vp 01) provide no clear measures for differentiation (see fig. 1).

The Alpine, Pyrenean, Cantabric and Ural specimen of *G. verna* L. are situated within the western-*verna*-group, showing a low bootstrap support at its base, but united with *G. pumila* Jacq. subsp. *delphinensis* Fourn. (pd01 to pd03) from the south western Alps as well as *G. terglouensis* Hacq. subsp. *schleicheri* Tutin (ts01 and ts02) from the central and southwestern Alps. Both taxa are separated from *G. verna* L. by a bootstrap support of 73 respectively 82, while between the haplotypes of *G. verna* L. s.str. (ve01 to ve06) no supported structure is visible resulting into a unresolved polytomie. Within the Alps the type ve04 is most frequent, in the far eastern Alps on Raxalp it gets replaced by ve02, differing by 6 restriction sites. The Pyrenees provide type ve01, different from ve04 by 4 sites.

In the Cantabric Mountains ve04 is as well present as ve03, different by 6 sites. The Jura Mountains show both ve01 and ve04. Type ve05, different from ve04 by 2 sites, was found on the southwestern Alpine Col de Larche on the border between France and Italy. The Russian population from Ural Mountains was identified as ve06 with 20 respectively 24 different sites to the Alpine ve04 and the eastern Alpine ve02.

As last, the non-*verna*-group, is built by *G. brachyphylla* Vill., *G. terglouensis* Hacq. s.str.*,* *G. rostanii* Reuter and *G. bavarica* L. At its real base we find a separation of Carpathian populations of *G. brachyphylla* Vill. *subsp. favratii* Tutin (bf01), while the Alpine and Pyrenean populations of the same taxon (bf02 and bf03) are embeded inside the non-*verna*-group. Different to the separation between the both *verna*-groups, a bootstrap support of that non-*verna*-group at its base is weak. Highly significant support can be foud within that group for the position of taxa *G. bavarica* L. s.l. (bs01 and ba01) as well as *G. terglouensis* Hacq. s.str. (te01 and te02), *G. rostanii* Reuter (ro01) and the Alpine *G. brachyphylla* Vill. s.str. (br02 and br03). .While *G. barachyphylla* Vill. subsp. *favratii* Tutin and the Sierra Nevada population of *G. brachyphylla* Vill. s.str. (br01) are outstanding the non-*verna*-group or located at its base, the Alpine populations of *G. brachyphylla* Vill. s.str. are sister to *G. rostanii* Reuter locaed on a side branch equipped with 99% bootstrap support within the group. The other side branch with maximal support is built by the both subspecies of *G. bavarica* L. as well as *G. terglouensis* Hacq. s.str. That later *G. terglouensis* Hacq. s.str. gets therefore separated from its subspecies *schleicheri* Tutin, which is placed within the *verna*-group. Also we get a clear separation of the two vicariant taxa *G. bavarica* L. s.str. and *G. rostanii* Reuter. *G. bavarica* L. subsp. *subacaulis* Müller gets a position basal to the two taxa *G. bavarica* L. s.str. and *G. terglouensis* Hacq. s.str., separated from those two with maximal support in bootstrap analysis as well as a different K2Q-type as explained above.

Discussion

G. Sect. *Calathianae* Froel. Provides two basalmost clusters: the fist one covering the two annual species *G. nivalis* L. and *G. utriculosa* L., the second one hosting all perennial taxa of the section. The annual group represents plants without a basal leaf rosette and branched stems with usually many flowers, while the perennials provide solitary stems with few flowers and in many cases basal leaf rosettes. Cytological informations retrived by Müller (1982) also support the declaration of two different unities within the section. For most perennial taxa chromosome numbers different from the annuals got established. We support therefore the division between the two, annual and perennial groups, either in two subsections (Rouy, Foucaud et al. 1893; Müller 1982) or within two different sections (Hegi 1927; Zuev 1985; Halda 1995; Halda and Haldovâa 1996).

G. pumila Jacq. s.str.has a separated position within the perennial taxa as also indicated by chromosome counts as above. Müller (1982) provided a chromosome number of 2n=20, different from all other perennial taxa and also from its subspecies *G. pumila* Jacq. subsp. *delphinensis* Fourn. (Tutin 1964; Ho and Liu 2001). While that second grows on the western border of the alpine chain and the eastern Pyrenees, *G. pumila* Jacq. s.str is restricted to the most eastern part of the alps in Austria, Slovenia and Italy. Since only doubtfull indications for *G. pumila* Jacq. S.str. in the western Alps are existent, possibly due to confusions in morphology, *we* estimate that taxon to be strict eastern alpine and not related to *G. pumila* Jacq. subsp. *delphinensis* Fourn..

Non-*verna*-group

G. brachyphylla Vill. is a polyphyletic species, composed of the two *subsp. brachyphylla* and as subsp. *favratti* Tutin. The later of these two has a basalmost position within the non-*verna*-group providing some not highly significant differences between the Alpine and the Carpathian populations as much in parsimony analysis as in the length of the highly polymorphic K2Q-sequence. *G. brachyphylla* Vill. subsp *favratii* was several times subject of rearrangements based on morphological considerations. It has been treated as independent species (Schur 1852; Rittener 1887), but also as subspecies *to G. brachyphylla* Vill. as above (Heywood, Tutin et al. 1971), and to *G. verna* L. (Schultz and Sauter 1879; Rittener 1887; Kusnezow 1904). The Alpine and Carpahtian populations are estimated as one taxon (Aeschimann 2004, Heywood, Tutin et al. 1971, Ho and Liu 2001), but also as two independent ones (Halda 1996). Tammaro (1986) presended a number of differential criteria between the Alpine, the Carpathian and the Apennine populations of that taxon. Netherless our results propose the threatment of eastern and western populations as a single species independent from *G. brachyphylla* Vill..
G. brachyphylla Vill. s.str. itself hosts two different taxa according out data. As sister to both the western-*verna*-group and the non-*verna*-group the Spanish Sierra Nevada population of *G. brachyphylla* Vill. s.str. is significantly separated from the Alpine populations of the same subspecies. Müller (1982) supports that difference by establishing 2n=30 chromosomes for *G. brachyphylla* Vill. s.str. from Sierra Nevada, while he found 2n=28 in the Alpine populations. The Sierra Nevada populations of *G. brachyphylla* Vill. s.str. have to be excluded from *G. brachyphylla* Vill. and estimated as independent species as done by Briquet (1931) and Romo (1988) or included into *G. verna* L. as proposed by Bolos and Vigo (1984). The remaining Alpine *G. brachyphylla* Vill. s.str. becomes sister to the southern Alpine *G. rostanii* Reuter.
Another polyphyletic taxon represents *G. ter-*

glouensis Hacq. Its subspecies *schleicheri* Tutin is located within the western-*verna*-group, while its subspecies *terglouensis* Hacq. is in the neighborhood of *G. bavarica* L.. *G. terglouensis* Hacq. s.str. reaches from the far eastern end of the Alps to the border region between Italy, Austria and Switzerland, while the subspecies *schleicheri* Tutin colonizes the western part of the Alps and was also found in the Pyrenees (Müller 1982; Ho and Liu 2001) The morphological and ecological similarities lead often to confusions between the two taxa (Hess, Landolt et al. 1967). Differences can be found in the number and shape of leaves as well as mainly in the presence of a basal rosette in *G. terglouensis* Hacq. subsp. *schleicheri* Tutin, but also in the chromosome numbers of 2n=38 for *G. terglouensis* Hacq. s.str. and 2n=30 for *G. terglouensis* Hacq. subsp. *schleicheri* Tutin (Müller 1982). Our results show the need for a separation of these two taxa into independent subspecies, where *G. terglouensis* Hacq. s.str. is related to other non-*verna*-taxa and subsp. *schleicheri* Tutin becomes part of the *verna*-group.

G. bavarica L. is divided into the two subspecies *bavarica* and *subaccaulis* Müller, that are both located with *G. terglouensis* Hacq. s.str. as above within the same close subclade. Müller (1982) showed the difference in altitude of occurrence between the two subspecies, Hegi (1927) even mentions a series of intermediates and Hess (1967) explain them as ecological varieties. Pignatti (1997) mentions subsp. *subaccaulis* Müller also, but explains it as a synonym of *G. orbicularis* Schur. As consequence to the parsimony analysis the two subspecies would have to be separated into two species since the significant intermediate placement of *G. terglouensis* Hacq. s.str. would lead into a polyphyletic taxon *G. bavarica* L. In southeastern France *G. bavarica* L. s.str gets replaced in its ecology by *G. rostanii* Reuter. North of the line Briançon-Turin grows *G. bavarica* L., south we find *G. rostanii* Reuter. A occurrence of *G. rostanii* Reuter in the Savoian Alps (*G. bavarica*, Cormet de Roseland, France, "..au bord de petit lac, ca 2400 m", 18.7.1963 leg: A. Charpin, identified as *G. rostanii* by G. Müller [G]), found no proof. The both species *G. rostanii* Reuter and *G. bavarica* L. s.str. are clearly separated taxa although their ecological parallels (Aeschimann 2004, Ho and Liu 2001).

Western-*verna*-group

G. verna L. is split into a western and an eastern clade. A separation line can be found on the southeastern border of the Alpine chain as defined by Aeschimann et al. (2004). Two populations from the Slovenian Krn just northwest that line and the Italian Mt. Majur are member of the western clade, the populations from Učka on the Istrian Peninsula as well as the Slovenian Mt. Caven just east of the line are member of the eastern clade. All the plants included from the southern Carpathians are also members of the eastern clade, same as the Russian population from Altai Mountains. The plants from Ural turns out to be located within the western-*verna*-group.

The western-*verna*-group includes all populations of *G. verna* L. from the Cantabric Mountains and the Pyrenees. We found no difference as postulated based on different morphology by Montserrat, Villar (1975) and Romo (1988) as var. *willkommiana* Monts. & Vill. and var. *cantabrica* Romo. All western *G. verna* L. form a homogeneous group with few different restriction types that allow us no further classification. Therefore all *verna*-types of western Europe, Cantabric mountains, Pyrenees, Jura and Alps are representing one single taxon with a high amount of morphological variation (Hegi 1927). We estimate also plants from the Apennine Mountains and British Islands to be members of the same group. Also the Russian taxon from Ural provides no measures for a clear distinction from Alpine populations. Cherepanov (1995) identifies for Russia a *G. arctica* Grossh. occurring in the Ural region, treated by Komarov (1967) as synonym of *G. verna* L. We are able to show, that there populations from northern Russia are clearly to integrate within *G. verna* L.

As Bolòs (1984) proposed by postulating *G. verna* L. subsp. *schleicheri* Bolòs, all our material of that taxon *schleicheri* is fully integrated within our western-verna-group. Therefore it seems to be evident that, if the taxon schleicheri needs to be treated as subspecies, then it has to be a subspecies of *G. verna* L., not *G. terglouensis* Hacquet, well knowing ecological similarities and geographical vicariance (Ho and Liu 2001). The same conclusions could be taken for the taxon *delphinensis*. While Müller (1982) treated *G. delphinensis* Müller as independent species, Ho and Liu interpreted it as subspecies of *G. pumila* Jaquin. Our data shows clearly the loss of connections between taxon *delphinensis* and *G. pumila* Jaquin but its connection to the western-*verna*-group. Whether the two taxa s*chleicheri* and *delphinensis* should be fully integrated within *G. verna* L., placed *with G. verna* L. into a common aggregate or declared as its subspecies remains unclear and is subject of interpretation.

Eastern-*verna*-group

The eastern European mountains are populated by the eastern-*verna*-group, clearly different from the western one. That group comprises of the taxa *tergestina, balcanica, pontica*, as well as *G. verna* L. from the southern Carpathians.

G. tergestina Beck was described by Hegi (1927) and Müller (1982) as species independent from *G. verna* L.. Hegi mentioned the taxon for the Pyrenees, the Maritime Alps, Switzerland, Apennine as well as for the Eastern European mountains. Ho and Liu (2001) identified different Herbarium samples from the Swiss Jura (*Gentiana aestiva* Beck, Mt. Jorat, Vaud, Switzerland, 13.4.1882, leg: Ros. Masson, [BM]), the Maritime Alps (*Gentiana aestiva* Beck, Casterino, Piemonte, France, 13-15.6.1933, leg: ?, [BM]) and the Apennine (*Gentiana aestiva* R. & S., Mte Majella, Abruzzi, S. Italy, Aug. 1880, leg: ?, [BM]) as *Gentiana tergestina* Beck, while we were able to find several sheets of *G. verna* L. s.str. from the Dinaric Alps in the herbarium ZA/ZAHO as well as BM (*G. verna* L., Jahorina, Bosnia and Herzegovina, 7.6.1977, leg: Mennega & Driehuis,

[BM]), illustrating the variability in morphological forms within *G. verna* L. s.l..

The highly significant separation between the western and the eastern populations of *G. verna* L. s.l. as demonstrated by our parsimony analysis indicates a separation line between these two areas following the definition of the Alpine borderline as given by Aeschimann (2004). The occurrence of *G.verna* L. *subsp. tergestina* Hayek in Western Europe, as also indicated by Soltokovic (1901), seems to be doubtful. The deep gap between the western-*verna*-group and taxon *tergestina* is also reflected in its ecological preferences. *G. verna* L. s.str. grows in general on alpine to high alpine meadows with low vegetation, reaching to a high of about 5 cm, subsp. *tergestina* Hayek from the calcareous Dinaric Alps is often found in the middle of high vegetation on a altitudinal level of 300 to 1600 m above sea level, but also the missing intermediate forms as reported by Rogenhofer (1905). Same conclusion as above can be done for *G. verna* L. subsp. *balcanica*, originating from the Balcan Peninsula and Turkey. A clear separation between the two taxa *G. verna* L. subsp. *tergestina* Hayek and subsp. *balcanica* Pritch can't be done based on our results.

G. verna L. subsp. *pontica* Hayek is ccurring all over Turkish Pontic Alps and the southern Turkish ranges, the Caucasus till central Asia. We already included *G. uniflora* Georgi within the taxon *pontica* as indicated in Ho and Liu (2001), while (Cherepanov 1995) recognizes *G. krylowii* Grossh. as synonym to *G. uniflora* Georgi. The sample material from Turkey and the Altai Mountains we treated is also member of the eastern-verna-group without any doubt. The Turkish populations of that taxon (vp02 and vp03) are somehow isolated postion to the one from Russian Altai (vp01) while that one is part of an unresolved polytomie with other eastern taxt so that the integration of that later one within *G. verna* L. subsp. *pontica* Hayek can't be proofed upon our analysis

Nevertheless their morphology also the Southern Carpathian populations of *G. verna* L. (ve07 to ve10) are part of the eastern-*verna*-group with 17 to 29 different restrictions sites to the others within that group. Should they be separated from the western European *G. verna* L. included into a hypotetical new eastern taxon, a revision of Ho's determination key (2001) and the evaluation of new segregating morphological criteria would be necessary.

A phylogenetic analysis of *Gentiana* Sect. *Calathianae* Froel. (Gentianaceae) based on chloroplast maturase-K sequences

Abstract

Gentiana Sect. *Calathianae* Froel. is a small section that occurs in the mountains of Europe and Asia. Classifications within the section have been based on different morphological characters and cytological data. Here 55 accessions of maturase-K sequences representing 21 haplotypes are studied in regards to the currently recognized taxonomy. The results revealed that within this monophyletic section, two clades are present, one consisting of annual taxa, and one of perennial taxa. Perennials are further structured by the presence of a few sub-clades, one consisting of the western European subspecies of *G. verna* L. and related taxa, and the another composed of eastern European and Asian subspecies of *G. verna* L. Some rather western to central alpine non-verna-taxa are localized in another, the third small and distinct sub-clade, while a fourth comprises central alpine to Carpathian non-verna-taxa. The necessity of a taxonomic revision of several subspecies complexes is shown, such as *G. verna* L., but also *G. bavarica* L. and *G. brachyphylla* Vill.

Keywords

Phylogeny, *Gentiana*, chloroplast maturase K, Taxonomy sequencing

Introduction

Gentiana Sect. *Calathianae* Froel. is comprised of 15 taxa found mainly in alpine regions in Europe, less commonly in Asia, and one species each in North America and Africa. There are two annual taxa, and the remaining are perennials. The apomorphic characters of the section include sympodial stems, the flowering stems arising singly from stolons, solitary flowers, and a salver-shaped corolla subtended by an angled or winged calyx. The stigma lobes are expanded and connate or contiguous, forming a discoid structure. Seed coats are reticulate and winged on some side.

Kusnezow (1894) did an early revision of this section by placing it as an outstanding group distant from the other sections of *Gentiana* , neighboring nowadays *G.* Sect. *Ciminalis* Dum.. Halda (1995) used the presence of two different life-cycles to postulate a division this group into two sections. Müller (1982) proposed Sect. *Cyclostigma* Griseb. as the valid name for this section, nevertheless Tutin (1964) and later Ho & Liu (2001) reused the definition of Sect. *Calathianae* Froel.. Müller (1982) did a recent revision of the section by counting chromosome numbers for all taxa and predicted, descending from a common root providing n=5 each a solitary evolutionary path for the two annual taxa (n=7 for *G. nivalis* L., n=11 for *G. utriculosa L.*), a common one for *G. bavarica L.* and *G. rostanii* Reuter (n=15), colonizing as vicariant taxa almost the same ecology, further a cluster with common origin for the two vicariant *taxa G. terglouensis* Hacq. (n=19) and subsp. *schleicheri* Tutin (n=15) as well *as G. pumila* Jacq. (n=10). A last huge cluster starting at n=15 would provide *G. verna* L. and its subspecies, but also *G. brachyphylla* Vill. s.str. and in isolated positions *G. brachyphylla* Vill subsp. *favratii* Tutin and *G. sierrae* Briq., while Halda (1995) and as above Ho & Liu (2001) focused for that section mainly on morphological criteria. Ho & Liu postulated one section comprising of a large central species covering *G. verna* L. and its subspecies (Tutin 1964), a eastern and western alpine, disjunct species *G. pumila* Jacq. including subsp. *delphinensis* Fourn., a high alpine species covering the two vicariant taxa *G. terglouensis* Hacq. and subsp. *schleicheri* Tutin as well as a large taxon *G. brachyphylla* Vill. including the southern Spanish and northern African taxa, beside some smaller species. Halda (1995), some years before, proposed to separate the perennial taxa in an new *G.* Sect. *vernae* Halda and to arrange that one together with the remaining *G.* Sect. *Calathianae* Froel., hosting only the annual taxa, under the roof of *G.* subgen. *Calathaiane* (Froel.) Halda. Gielly & Taberlet (1996) derived a taxonomy on European Gentians from trnL (UAA) sequences, covering also some taxa of G. Sect. *Calathianae* Froel. They were able to separate the two taxa *G. nivalis* L. and *G. pumila* Jacq. from the others, but didn't get a clear phylogeny for the entire section. Yuan & Küpfer (1995) did the same by the use of ITS-sequences. The selection of taxa fitted perfectly to resolve the intersectional order but was not sufficient to give a closer insight within the section.

At this place we present results from a study based on the incomplete chloroplast maturase K sequences, comparing a large part of the section as described by Tutin (1964) and Ho & Liu (2002). We specially try to proof the division into two sections done by Halda (1995) and the repartition of taxa. Also do we ask the question how legitimous the recent rearrangements were as done by Tutin (1964) and Ho & Liu (2002). Specially were these: a) the subordination of taxon *delphinensis* under *G. pumila* Jacq. as described already in Fournier (1938), bit then also the subordination of taxon *schleicheri* under *G. terglouensis* Hacq. and those of *G. favratii* under *G. brachyphylla* Vill. Also is to mention the complete integration of *G. sierrae* Briq., the gentians from Spanish Sierra Nevada, into *G. brachyphylla* Vill..

Material and Method

As working basis we accept the taxonomy proposed by Tutin (1964) and Ho & Liu (2001). Our sampling comprises of individuals from the mountain ranges of Northern Spain and the Central Spain Sierra Nevada, the Central and Southern Pyrenees, the entire Alpine range as well as the Swiss Jura, Scandinavia and Island, the Dinaric Alps and the Balkan range, the southern Carpathians, the Northern and Southern Pyrenees, the Pindus and the Pontic Mountains as well as few material from the Ural and Altai Range. Not included is material from Northern Africa, the Apennine, the British and Irish Islands, Greenland or North America. On the taxonomic level we are missing *G. verna* L. subsp. *oschtenica* Halda, a *verna*-like taxa with lemon-yellowish corolla, growing endemic in the Caucasus and southwestern Russia (Komarov 1967; Halda 1995) and *G. penetii* Romo (Romo 1988), a *brachyphylla*-like taxon from the Higher Atlas. Ho & Liu (2001) treated *G. penetii* Romo as *G. brachyphylla* Vill. s.str.. We are also missing sample material clearly identified as taxon *G. angulosa* Bieb. from Eastern Turkey and the Caucasus synonymized under *G. verna* L. subsp. *pontica* Hayek by Ho & Liu (2001).

Sampling sites were selected by studies of the herbaria NEU, G, P, K, BM and ZA/ZAHO, but also the CRSZ-database for Switzerland (BÄUMLER 1999). The taxa and populations collected for this study are listed in the sample table (Annex). Leaf material was fresh dried on silica gel and approximately 20 mg of it was grounded using Tungsten Carbide beads and Qiagen Tissue Lyser (Qiagen, Basel). A DNA extraction was performed using DNEasy Plant Mini Kit (Qiagen, Basel), leading into about 50ng total DNA per µl extract. 50ng of extracted DNA was used for a PCR in combination with 1U Taq DNA Polymerase (Qiagen, Basel), 200µM of each dNTP, 200µM of each primer and 1x PCR buffer (Qiagen, Basel) 2mM $MgCl_2$ in a total volume of 25µl. The procedure was carried out in a Biometra T3000 thermocycler (Biometra, Göttingen) by performing a 3 min initial denaturation at 94°C, followed by 40 cycles: 30s denaturation at 94°C, 45s annealing at 55°C, 1 min extension at 72°C and completed by a final extension step of 10min at 72°C. Primer pairs used were maturase-K558F (5'-CAAAGAATACCAGCCTTGCTCT-3') / maturase-K1490R (5'-CACCAAAAAGGGGGAA-AGA-3') respectively maturase-K1442F (5'-TTTTTGCGGAGTT-CCTCG-3') / psbAR (5'CTTT-CGCGTCTCTCTAAAA-3'), while the two pairs overlap for about 50 bp providing the possibility to connect the two sequence fragments. Nomenclature of primers refers to the base pair-position relative to primers used by (Endress, Sennblad & al. 1996) for the 3 primers located within the maturase-K-sequence. PsbAR was created upon a consensus sequence of different Gentianaceae and is located within psbA-sequence neighboring maturase-K.

Amplification products were purified using Qiaquick PCR Purification Kit (Qiagen, Basel) following the manufacturers protocol on a microcentrifuge and re-eluted within 25µl of double-distilled H_2O. That way enriched and cleaned maturase-K-product eas the base for a cycle sequencing reaction in a total volume of 10µl liquid of following composition: 4µl of PCR purification product, 1.5ml Terminator Ready Reaction Mix (BigDye® Terminator v1.1

Cycle Sequencing Kit, Applied Biosystems Switzerland, Rotkreuz), 1x BigDye Terminator Sequencing Buffer and 200µM primer as indicated above. Primers used were maturase-K558F, maturase-K1490R and maturase-K1442F as described above. The cycle sequencing reaction was done on a Biometra T3000 thermocycler (Biometra, Göttingen) by applying an initial denaturation of 1min at 94°C and 30 cycles of 10s denaturation at 94°C, 5s annealing at 50°C and 4min extension at 60°C.

Resulting cycle sequencing products were precipitated with each 1µl of 125mM EDTA and 3M sodium acetate as well as 25µl of 100% ethanol for about 20min at room temperature and then on a Rotina 35R (Hettich, Tuttlingen/Germany) centrifuge for 30min at 4°C and 15000rpm (23900g). The supernatant liquid was removed and the precipitation washed with 75µ of 70% ethanol and centrifuged for further 10min at above conditions. The supernatant was removed again and the precipitated product dried in a Univapo 10H (Uniequip, Martinsried/Munich) under vacuum at about 50°C till dry.

The cleaned sequencing product got resuspended in 12µl HIDI formamide (Applied Biosystems Switzerland, Rotkreuz) and denatured for 2min at 95°C prior to immediate cooling on ice and scanning on a ABI Prism 310 Genetic Analyser (Applied Biosystems Switzerland, Rotkreuz). Raw sequences were worked out and revised upon scanning information in Sequencing Analysis 3.7 software (Applied Biosystems Switzerland, Rotkreuz).

Data Analysis

Sequences were aligned in Clustal X ver. 1.8 (Thompson, Gibson & al. 1997) and BioEdit ver. 7.053 (Hall 1999). Parsimony analysis (Fitch 1971) was done on PAUP* (Swofford 1998) using a heuristic search access with random sequence addition and 1000 sequence replicates via stepwise addition, the TBR branch-swapping-algorithm in effect. A bootstrap analysis was performed using the above settings with 1000 replicates (bootstrap supports indicated in fig. 1).

To evaluate optimal parameters for likelihood analysis Modeltest ver 3.7 (Posada & Crandall 1998) was used. maximum likelihood analysis was performed on PAUP* (Swofford 1998).

Results

Sequences were cropped from base pair 608 to base pair 1530 relative to *Nicotiana tabacum* L. maturaseK-sequence as reference (GenBank accession: NP_001879, Nadot, Bittar & al. 1995). We were able to identify sequences with a maximal length of 960 bp, whereof 836 were constant and 39 parsimony informative. Tree indels with each 6 base pairs length were detected. Excluding the outgroup taxa 23 polymorphisms in amino acid composition resulting from present sequences were discovered. The total set of 55 complete sequences was reduced to 21 different haplotypes representing one or several accessions. For further analyses the alignment was reduced to these haplotypes. Some haplotype covering a large number of individuals were detected as haplotype 1, hosting all G. *terglouensis* Hacq. s.str. and most *G. bavarica* L. s.str.. Haplotype 3 is home to all Carpathian *G. brachylpylla* Vill subsp. *favratii* Tutin and haplotype 4 of *G. verna* L. s.str. as much as *G. pumila* subsp. *delphinensis* Fourn. and *G. terglouensis* Vill subsp. *schleicheri* Tutin. The alpine *G. brachyphylla* subsp. *favratii* Tutin are united in haplotype 6. Haplotype 7 and 8 are home to *G. verna* L. subspecies from Southern Carpathian, Balkan and Dinaric Alps. Haplotype 16 covers the two taxa *G. brachyphylla* Vill. s.str. and *G. bavarica* L. subsp. *subacaulis* Müller, both from alpine populations. Haplotype 19 represents two accessions of *G. utriculosa* L. and 21 two accessions of *G. nivalis* L. Distant taxa were found in haplotype 1, 4, 8 and 16. Specially remarkable is also the separation of *G. terglouensis* s.str. and *subspecies* schleicheri Tutin in distant haplotypes (1 and 4), the same for *G. brachyphylla* s.str. and subsp. *favratii* Turin (3, 4, 5 and 6), the separation of *G. bavarica* L. s.str. and subspecies *subacaulis* in three haplotypes (1, 2, and 16) and finally the separation of *G. pumila* Jacq. s.str. and subspecies *delphinensis* in two haplotypes (4 and 18).

Parsimony analysis

Parsimony analysis resulted in 5 most parsimonious trees with scores of 142 (CI = 0.930, RI = 0.888). A 50% majority tree, rooted on outgroup species, was calculates based on these (see fig. 1). Identical in all trees are the positions for haplotypes 14 to 21 as much as the two outgroup types. Same can be said for the internal order of haplotype 5 to 13. Alternative arrangements are shown in fig. 2.

The maturase-K-sequences of G. Sect. *Calathianae* Froel. is divided into two clades; the first clade composed of all annual and one perennial species, and the second clade composed by all others. Between the two clades we identified 4 different base pairs in maturase-K sequence.

Compared to the second perennial clade, the annual clade provides a relatively high number of internal base pair differences. A first bifurcation in both analyses we find between *G. utriculosa* L. (17) and the other two taxa, with a bootstrap value of only 62%. *G. nivalis* L. splits into three different haplotypes: a fist representing individuals from the Italian Alps and from Iceland (21), and each one from German Alps (19) and Norway (20). The German and Norway samples differ by two different base pairs, from the third sequence type by one difference each. The closest relative to *G. nivalis* L. is the only perennial taxon within that annual subclade: *G. pumila* Jacq. s.str. (18). The bifurcation between *G. nivalis* L. and *G. pumila* Jacq. s.str. shows bootstrap support 91%.

The second clade is composed by a higher number close related sequences and therefore host to all differences between the 5 outcomes of parsimony analysis. The most distinct group within represents individuals of taxa *G. rostanii* Reuter in two haplotypes (14 and 15) and the Alpine populations of *G. brachyphylla* Vill. s.str. as well as *G. bavarica* L. subsp. *subacaulis* Schleicher in one common sequence type (16). The position of that group is highly supported with a bootstrap value of 98%.

The rest of that perennial clade provides only one further group with significant bootstrap support of 60% covering all populations of *G. verna* L. and its subspecies from eastern Europe, Turkey and the far eastern Altai Mountains (07 to 13) but also the Alpine populations of *G. brachyphylla* Vill. subsp. *favratii* Tutin (06) and the Sierra-Nevada-population of *G. brachyphylla* Vill. s.str. (05). Similar protein compositions can be found between the Alpine *G. brachyphylla* Vill. subsp. *favratii* Tutin (06) and the haplotype 08, composed by *G. verna* L. s.str. from the Southern Carpathians, *G. verna* L. subsp. *balcanica* Pritch. form Greece and *G. verna* L. subsp. *tergestina* Hayek from Slovenia, but also with the Carpathian *G. brachyphylla* Vill. subsp. *favratii* Tutin (03) located outside that group in sequence based trees. The Pyrenean population of that taxon has a common maturase-K-sequence with alpine *G. verna* L. (04).

That alpine *G. verna* L.-haplotype (04) mentioned above is composed by individuals of *G. verna* L. s.str. from Alpine, Pyrenean and one Ural population, by the southwestern Alpine *G. pumila* Jacq. subsp. *delphinensis* Fourn., *G. terglouensis* Hacq. subsp. *schleicheri* Tutin and as above by the Pyrenean population of *G. brachyphylla* Vill. subsp. *favratii* Tutin. Together with their neighbor-species these taxa form a group providing no bootstrap support. Closest relatives are the Carpathian and the Alpine *G. brachyphylla* Vill. subsp. *favratii* Tutin (03 and 06), the Sierra Nevada G. *brachyphylla* Vill. s.str. (05) but also *G. bavarica* L. s.str. and *G. terglouensis* Hacq. s.str.(01 and 02). The precise arrangement of *G. bavarica* L. s.str. haplotypes, Carpathian G. brachyphylla Vll subsp. favratii Tutin haplotypes and *G. verna* L s.str. haplotypes remains unclear due to differences between the 5 outcoming trees from that analysis. Always present is the proximity between baplotype 03 and 04. In question is the position of haplotypes 01 and 02 relative to these two types, but also to the group covering haplotype 05-13. Haplotypes 14-16 are basal to the ones mentioned above or then part of an unresolved polytomie including the above. Haplotype 01 can be sister to haplotype 02 or become basal to a group covering haplotypes 02-15, or in one case, together with haplotype 02 become basal to haplotypes 03-15. For details see fig. 2.

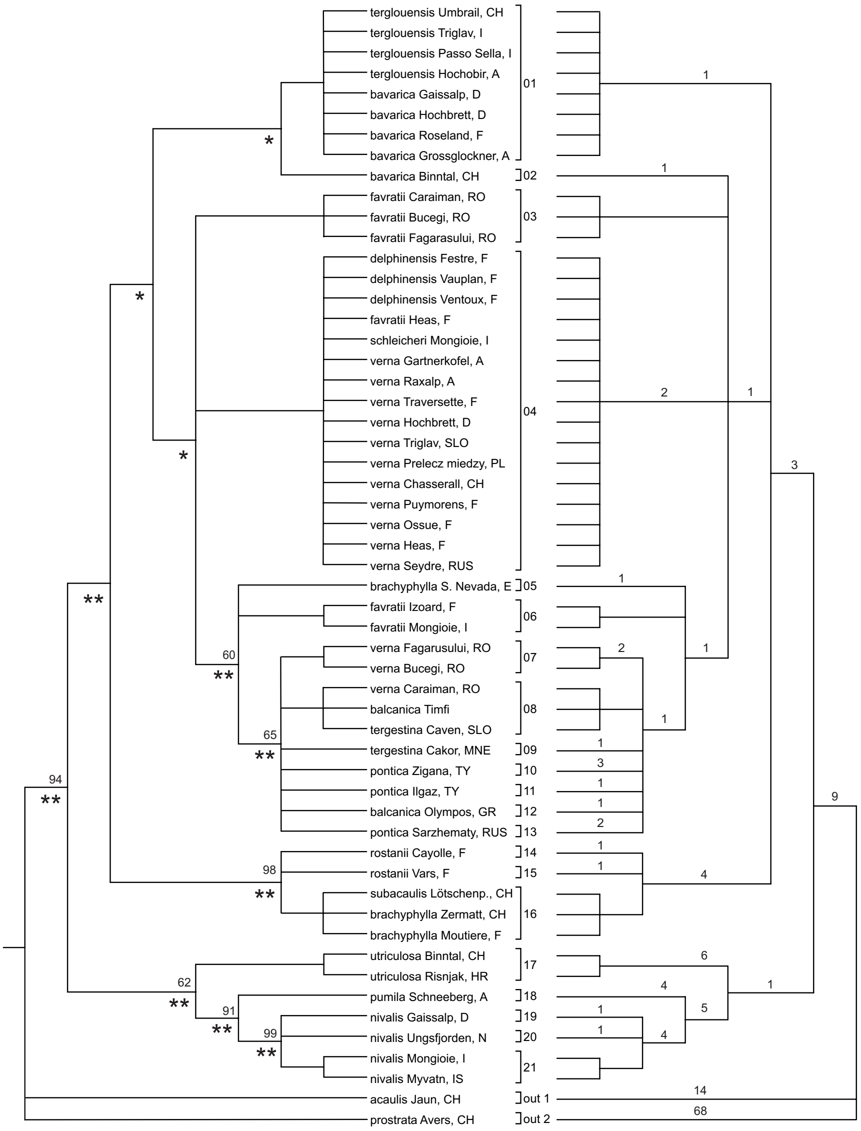


fig 1: left: 50% majority consensus out of 5 most parsimonious tress. Bootstrap supports for significant nodes are indicated. ** indicate supports through all 5 parsimony trees, * indicate supports through 3 parsimony trees.
right: maximum likelihood tree. Base pair differences are indicated.

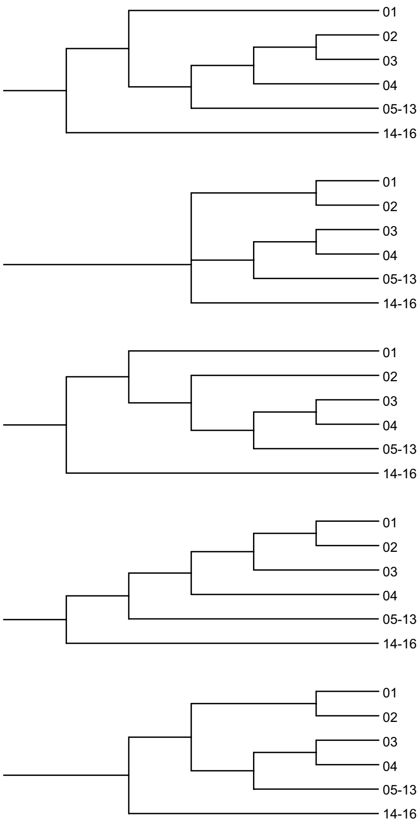


fig 2: Alternative trees 1 to 5 from parsimony analysis (top to bot.). Shown is only the perennial haplotypes 01-16. Braches hosting haplotypes 05-13 and 14-16 are reduced.

Maximum likelihood analysis

Modeltest ver 3.7 (Posada & Crandall 1998) suggested the HKY85 (Hasegawa, Kishino & al. 1985) model for the matK sequence data, with the Likelihood parameters as followed; base frequencies A=0.2984, C=0.1823, G=0.1639, a gamma value of 0.4825 and as transition/transversion ratio 1.3207. Likelihood analysis applying the suggested model and parameters generated a best tree with a likelihood score of 2117.46706. For each bifurcation the number of different base pairs between the subsequent paths was calculated (see fig. 1)

The tree resulting from likelihood analysis in large parts identical to the one obtained from parsimony analysis. Within the first, annual subclade 11 distinct base pairs difference of *G. utriculosa* L. to the other taxa were discovered. *G. pumila* Jacq. s.str. has 4 private base pairs, while *G. nivalis* L. does the same for its own. The 3 haplotypes of *G. nivalis* L. (19-21) differ by each one base pair. At least 10 base pairs are in between the annual and the perennial subclade.

The perennial subclade hosts some unresolved polyomies. We can identify the same groups as in parsimony analysis. Separated in an early bifurcation there is haplotype 01, sister to 02-13 and to 14-16 in a first polytomie. That placement represents tree 2 from parsimony analysis and places the central Alpine *G. bavarica* L. s.str. from Binntal (02) in a position distinct from the other individuals from that taxon, separated by 2 different base pairs.

The group covering haplotypes 02 to 13 hosts the next unresolved polytomie due to a low amount of internally distinct base pairs. As such, the above mentioned haplotype 02 differs from the Carpathian *G. brachyphylla* Vill. subsp. *favratii* Tutin (03) by only one base, from *G. verna* L. (04) by only 3 bases and from haplotypes 05-13 by 3 to 6 bases. Close is also the relatedness of the Carpathian *G. brachyphylla* Vill subsp. *favratii* Tutin (03) to the western Alpine populations of that taxon (06) and to *G. verna* L (04).

In the same way supported as I parsimony analysis is the group covering haplotypes 05-13. Also that group is home to two unresolved polytomies with haplotype 05, 06 representing the Sierra Nevada population of *G. brachyphylla* Vill. s.str. as well as the Alpine *G. brachyphylla* Vill subsp. *favratii* Tutin, and 07-13 hosting eastern *G. verna* L. being sister to each other and a level lower within haplotypes 07-13 each type being sister to each other. As in the cases above

The distance in base pair differences is low between the haplotypes involved. Based on their one common base pair separating them from haplotypes 05 and 06, the eastern *G. verna* L. and subspecies in haplotypes 07-13 form a closer group also supported in parsimony analysis.

The group hosting haplotypes 14-17 is home to the southwestern Alpine *G. rostanii* Reuter and the central Alpine *G. bavarica* L. subsp. *subacaulis* Müller with each one base pair distance in between and a long distance to the other taxa, specially 5 resp. 6 base pairs haplotypes 02 and 01 hosting *G. bavarica* L. s.str.

Discussion

Halda (1995) proposed the revision of *G.* Sect. *Calathianae* Froel. by excluding all perennial species into a new *G.* Sect. *Vernae* Halda. The remaining *G.* Sect. *Calathianae* would then be composed of nothing more than the two annual species *G. nivalis* L. and *G. utriculosa* L. Tzevelev (1987) delivers a parallel definition but by creating a *G.* Sect. *Vernae* composed by only *G. verna* L. Our result does conform to the above mentioned rearrangements when reducing life form to a secondary criterion, allowing the integration of *G. pumila* Jacq. s.str. within one section together with the two annual taxa. With a chromosome number of 2n=20, shown by Müller (1982), *G. pumila* Jacq. s.str. is far separated from other perennial taxa, all providing numbers from 2n=28 to 2n=38, but close to *G. nivalis* L. (2n=14) respectively *G. utriculosa* L. (2n=22). In his interpretations, Müller (1982) proposes an evolution both of *G. utriculosa* L. and *G. pumila* Jacq. s.str. from an common ancestor, both via individual hypothetical intermediate with n=10 chromosomes to the final state, by indicating an own pathway for *G. utriculosa* L., while *G. pumila* Jacq. s.str. would have evolved together with other perennial taxa. Based on our data, we can clearly show the distance to all other perennial taxa of *G.* Sect. *Calathianae* Froel. specially to the taxon *delphinensis*, integrated within *G. pumila* Jacq by Fournier (1938) due to morphological criteria. Ho & Liu (2001) indicates an occurrence also of *G. pumila* Jacq. s.str. in France. This could be a consequence of that fusion with the French taxon *delphinensis* and of confusions with that later one.

Compared to the perennial cluster, the annual one hosts a high number of different base pairs between its taxa, possibly indicate an early separation between the taxa, while the intra-specific radiation took place possibly late. Both individuals of *G. utriculosa* L. provide the same sequence type, while one is located in the central Alps, the other at the northern border of the Dinaric Alps, 350 miles away. An even much more extreme situation we find between the two populations of *G. nivalis* L. from the Ligurian Alps and Iceland, identical in their sequence but 1700 miles geographical distance in between, while the two samples from Norway and the German Alps have an over all genetic distance of 2 base pairs.

In parsimony analysis the perennial cluster is composed of 5 different subclades which have no clear hierarchical order supported by bootstrap values. One of these represents all populations of *G. verna* L. s.str. from Alps, Pyrenees, Cantabric Mountains and also from the Russian Ural, *G. pumila* Jacq. subsp. *delphinensis* Fourn. and *G. terglouensis* Hacq. subsp. *schleicheri* Tutin. Based on maturase-K no sufficient measures were detected to distinguish these taxa in an significant, respecting the knowledge of their different morphology and ecology. Our data would even allow the reintegration of the taxon *schleicheri* within *G. verna* L. as it was proposed by Vaccari (1911) and Bolòs & Vigo (1984) respectively of taxon *delphinensis* within *G. verna* L. as done by Kunz (1963). Evident is the missing connection between taxon *delphinensis* and *G. pumila* Jacq s.str. (Fournier 1938) as mentioned above, but also the link between taxon *schleicheri* and *G. terglouensis* Hacq. s.str. (Heywood, Tutin & al. 1971) has no prove. The somehow comparable morphology could therefore be an effect of convergence due to parallels in ecology (Müller 1982; Ho & Liu 2001; Aeschimann & Lauber 2004).

Same as taxa above, we have no indications to separate *G. terglouensis* Hacq. s.str. from *G. bavarica* L. s.str. based on maturase-K. Both species show exactly the same sequence while providing distant morphologies. While we can declare *G. bavarica* L. s.str. and G. *terglouensis* Hacq s.str. as close related taxa, *G. bavarica* L. subsp. *subacaulis* Müller has to be excluded from *G. bavarica* L. s.str., differing by 5 base pairs. The taxon *subacaulis* has its closest relatives in *G. rostanii* Reuter and *G. brachyphylla* Vill. s.str., indicating also the position of *G. rostanii* Reuter distant from *G. bavarica* L. s.str. Both species, *G. bavarica* L. s.str. and *G. rostanii* Reuter populate about the same ecological environments: wet meadows and damp places from 1500 to 2600 m (Müller 1982), in case of *G. rostanii* Reuter sometimes also lower (Ho & Liu 2001). Literature, indicated above, herbaria specimen but also our own observations indicate distinct distribution area for these taxa: *G. bavarica* L. s.str occurs all along the alpine chain from Austria and Germany south- and westward to the region of the both cities Turin (Italy) and Briançon (France). South of that line it gets replaced entirely by *G. rostanii* Reuter. The taxon *subacaulis*, in maturase-K sequence close to *G. rostanii* Reuter overlaps with the distribution of *G. bavarica* L. s.str.

Same as above is the case for *G. brachyphylla* Vill. s.str.. Ho & Liu (2001) indicates for that taxon a distribution area all over Europe including also Southern Spain and northern Africa, treated separately in that study, while Müller (1982) mentions Alps and Pyrenees. The occurrence within the Pyrenees is reported to be reduced to few populations represented by some herbarium sheets (France, Pic du Midi de Bigorre: *Merxmüller & Zollitsch 27107* [M]; France, Port de Vénasque, *Vetter 18.7.1833* [LAU]). We found by our own one population from that region, but identified as *G. brachyphylla* Vill. subsp. *favratii* Tutin (NEU 398312) that provides the same sequence as *G. verna* L. s.str. From the northern Spanish Cantabric Mountains *G. brachyphylla* Vill. s.str. is indicated by Rivas-Martinez, Izco & al. (1991) but also in that case we could possibly deal with *G. verna* L. (Müller 1982) since no clear herbarium sheets were found. We doubt therefore in proofs of occurrence on other mountain chains other than the Alps and estimate *G. brachyphylla* Vill. s.str. therefore to be an Alpine taxon.

The close related complex of *G. brachyphylla* Vill s.str., *G. rostanii* Reuter and the taxon *subacaulis* populate different ecological environments within the alps: *G. rostanii* Reuter colonises damp meadows and gets replaced by *G. bavarica* L. in northern and eastern Alps, *G. brachyphylla* Vill. s.str. occurs all over the Alps on deep soils with low nutrition level (Landoldt 1977), while the taxon *subacaulis* could be found in high altitudes on sandy soils on crystalline underground (Müller 1982).

G. brachyphylla Vill. subsp. *favratii* Tutin is clearly different and distant form *G. brachyphylla* Vill. s.str. The two distribution Areas from the Alps and the Carpathians are closely related with just one base pair difference in maturase-K sequence. Both analysis, parsimony and likelihood place them in different subclades but the given bootstrap support is low. We estimate both types to be close related and conclude to accept them as a single taxon. The distance to *G. brachyphylla* Vill. s.str. shows the evidence for a separation from that second one. Therefore we are able to show connections between taxon *favratii* and populations of *G. brachyphylla* Vill. from Spanish Sierra Nevada and to Eastern European and Asian types of *G. verna* L. and subspecies. These two taxa have an intermediate position between the western European *G. verna* L. on one side, and slightly closer on the other side to the eastern European and Asian subspecies of *G. verna* L. discussed later. It is evident that the complete integration of the Sierra Nevada populations of *G. brachyphylla* Vill., also described by Briquet (1931) as *G. sierrae* finds no support in maturase-K sequences. We could perhaps discuss an interpretation as subspecies of *G. verna* L. as proposed by Rivas-Martinez, Asensi & al. (1991) but even more consequent would be to return to the interpretation as independent species. Same is the case for the taxon *favratii*, described by Rittener (1887) for the Alps as *G. favratii* and by Schur (1852, 1866), for the Carpathians as *G. orbicularis*. We have no useful measures for a clear distinction so we conclude in accepting these two taxa as identical.

The last group within the perennial taxa is built by all subspecies of *G. verna* L. from the Dinaric Alps, the Balkan Mountains, Turkey and the Altai Mountains such as *G. verna* L. subsp. *tergestina* Hayek, *G. verna* L. subsp. *balcanica* Pritch. and *G. verna* L. subsp. *pontica* Hayek, but also the populations of *G. verna* L. s.str. from the southern Carpathians. The group provides at its base a significant but not strong bootstrap support and a strong internal differentiation. Its closest relatives are the taxa *favratii* and the Sierra Nevada populations of *G. brachyphylla* Vill.. Its distinct position to *G. verna* L. illustrates the necessity for a separation from that species, whether as independent taxa or as subspecies of another species. The oldest description is given therefore by Beck (1887) that defines *G. tergestina* by mentioning this taxon as different from *G. aestiva* Schult. that represents *G. verna* L. For clear conclusions about internal differentiation of that group we miss a sufficient amount of samples, but we can separate them from alpine *G. verna* L. and related taxa. Also unclear is the position of *G. verna* L. subsp. *oschtenica* Halda from the eastern Black Sea Region as well as *G. angulosa* Bieb. from the same area. We suppose to find them also within that cluster of eastern *verna* subspecies, but we miss any kind of sample material for examination.

Phylogenetic relationships within *Gentiana* Sect. *Calathianae* Froel. (*Gentianaceae*) as revealed by the nuclear coded chloroplast expressed glutamine synthetase (ncpGS) sequence

Abstract

Gentiana Sect. *Calathianae* Froel. is a small section that occurs in the mountains of Europe and Asia. In this paper, 36 nucleotide sequences of the ncpGS gene are studied in regards to the currently recognized taxonomy. The annual taxa *G. nivalis* L. *and G. utriculosa* L. as well as the perennial *G. pumila* Jacq. s. str. form one group. A second is composed of the remaining perennial taxa. Close relatedness can be found between the populations of *G. verna* L. and *G. pumila* Jacq. subsp. *delphinensis* Fourn. in the Alps., while the eastern European and Asian subspecies of *G. verna* L. form another group. Related to the *verna*-taxa are *G. brachyphylla* Vill. s.str. and *G. terglouensis* Jacq. subsp. *schleicheri* Tutin, while *G. terglouensis* Jacq. s.str. is a representative of a group of perennial non-*verna*-taxa distant o the *verna*-taxa. This group also comprises *G. bavarica* L. s.str. that splits between a western and an eastern group, *G. bavarica* L. subsp. *subacaulis* Fourn. as most closely related to *G. brachyphylla* Vill. subsp. *favratii* Tutin, and *G. rostanii* Reuter, linked to the western *G. bavarica* L. s.str.

Keywords

Phylogeny, Taxonomy, *Gentiana*, chloroplast expressed glutamine Synthetase (ncpGS)

Introduction

Gentiana Sect. *Calathianae* Froel. is comprised of about 15 taxa found mainly in alpine regions in Europe, less commonly in Asia, with one species each in North America and Africa. While there are two annual taxa, all others are perennials. The synapomorphic characters of this section include the sympodial stems, the flowering stems arising singly from stolons, solitary flowers, salver-shaped corolla subtended by an angled or winged calyx, the stigmata lobes that are expanded and connate or contiguous forming a discoid structure, and reticulate and winged seed coats. Halda (1995) used the presence of two different life-cycles to postulate a division of this group into two sections. The presence of common criteria, mainly the same type of stigmata, speaks much more for only one section. Müller (1982) proposed Sect. *Cyclostigma* Griseb. as the valid name for this section, nevertheless Tutin (1964) and later Ho and Liu (2001) reused the definition of Sect. *Calathianae* Froel. Müller (1982) did a recent revision of the section by counting chromosome numbers for all taxa and supported with his interpretations the placement for *G.* Sect. *Calathianae* proposed already by Kusnezow (1894). Müller (1982) predicted, descending from a common root providing n=5 each a solitary evolutionary path for the two annual taxa (n=7 for *G. nivalis* L., n=11 for *G. utriculosa* L.), a common one for *G. bavarica L.* and *G. rostanii* Reuter (n=15), colonizing as vicariant taxa almost the same ecology, further a cluster with common origin for the two vicariant *taxa G. terglouensis* Hacq. (n=19) and subsp. *schleicheri* Tutin (n=15) as well *as G. pumila* Jacq. (n=10). A last huge cluster starting at n=15 harbors *G. verna* L. and its subspecies, but also *G. brachyphylla* Vill. s.str. and in isolated positions *G. brachyphylla* Vill subsp. *favratii* Tutin and *G. sierrae* Briq., while Halda (1995) and as above, Ho and Liu (2001) focused for that section mainly on morphological criteria. They proposed mainly one section comprising of a large central species covering *G. verna* L. and its subspecies (Tutin 1964), a eastern and western alpine, disjunct species *G. pumila* Jacq. including subsp. *delphinensis* Fourn., a high alpine species covering the two vicariant taxa *G. terglouensis* Hacq. and subsp. *schleicheri* Tutin as well as a large taxon *G. brachyphylla* Vill. including the southern Spanish and northern African taxa, beside some smaller species. Gielly and Taberlet (1996) studied on European gentians by using the trnL (UAA) sequences, covering also some taxa of G. Sect. *Calathianae* Froel. They were able to separate the two taxa *G. nivalis* L. and *G. pumila* Jacq. from the others, but didn’t get a clear phylogeny for the entire section. Yuan and Küpfer (1995) did the same by the use of ITS-sequences. The selection of taxa fit perfectly to resolve the intersectional order but was not sufficient to give a closer insight within the section. Based on chloroplast PCR-RFLP (Haemmerli and Küpfer in prep. e) as much as from chloroplast maturase K sequence (Haemmerli and Küpfer in prep. a) phylogenies were deduced separating the section within two parts basically *G. nivalis* L. and *G. utriculosa* L. on one side and all other taxa except *G. pumila* Jacq. s.str. on the other. The western and eastern subspecies if *G. verna* L. were located each time on two separated branches. While chloroplast PCR-RFLP resulted into well supported trees, maturase K suffered a lack of significance. Here we revise the systematic relationships within this section by the use of the nuclear encoded and chloroplast expressed glutamine synthetase sequences of a sample set covering a large part of known taxa and geographical distributions. Our aim is to provide phylogenetic relationships of this section in a larger scale to identify the clusters of closely linked taxa and in a fine scale to resolve intraspecific relationships. We specially try to resolve a) the placement of annual and perennial taxa within the phylogeny of *G.* Sect. *Calathianae* Froel.; b) the phylogeny of the controversial taxa: *G. pumila* Jacq. including subsp. *delphinensis* Fourn.; *G. brachyphylla* Vill including subsp. *favratii* Tutin and the Spanish and northern African taxa; c) the phylogeny of the large complex *G. verna* L. and its subspecies.

Material and Method

Sampling

As a working base we followed the taxonomy proposed by Tutin (1964) and Ho and Liu (2001). Our sampling comprises individuals from the mountain ranges of Northern Spain and the Central Spain Sierra Nevada, the Central and Southern Pyrenees, the entire Alpine range as well as the Swiss Jura, Scandinavia and Island, the Dinaric Alps and the Balkan range, the southern Carpathians , the Pindus and the Pontic Mountains as well as few material from the Ural and Altai Range. Not included is Material from Northern Africa, the Apennine, the British and Irish Islands, Greenland or North America. On the taxonomic level we are missing *G. verna* L. subsp. *oschtenica* Halda, a *verna*-like taxa with lemon-yellowish corolla endemic to the Caucasus and southwestern Russia (Komarov 1967, Halda 1995) and *G. penetii* Romo (Romo 1988), a *brachyphylla*-like taxon from the Higher Atlas. Ho and Liu (2001) treated *G. penetii* Romo as *G. brachyphylla* Vill. s.str.. We are also missing sample material identified as typically *G. angulosa* Bieb. from Eastern Turkey and the Caucasus, a taxon integrated into *G. verna* L. subsp. *pontica* Hayek by Ho and Liu (2001). Sampling sites were selected by studying the collections of the herbaria NEU, G, P, K, BM and ZA/ZAHO, and the CRSZ-database for Switzerland (Bäumler 1999).

To root our phylogeny we selected *G. acaulis* L out of *G.* Sect *Ciminalis* Dum. as outgroup species due to the close relatedness between the two sections (Ho and Liu 2002, Kusnezow 1894). *G. acaulis* L. was the only species out of a range of different outgroup Gentians that provided satisfying results in ncpGS-sequencing. The t{Barrett, 1998 #224}axa and populations collected for this study are listed in the sample table (Annex).

Molecular methods

Leaf material was freshly dried with silica gel. Approximately 20 mg of leaf material was grounded using Tungsten Carbide beads with a Qiagen TissueLyser (Qiagen, Basel). DNA extraction was performed using DNEasy Plant Mini Kit (Qiagen, Basel), following the protocol recommended by the manufacturer.

About 50ng of DNA was used for a PCR in combination with 1U Taq DNA Polymerase (Qiagen, Basel), 200µM of each dNTP, 200µM of each primer ncpGS737F and ncpGS940R and 1x PCR buffer (Qiagen, Basel) with a final Mg^{2+} concentration of 2mM in a total volume of 25µl on a Biometra T3000 thermocycler (Biometra, Göttingen) by performing a 3min initial denaturizing at 94°C, followed by 40 cycles: 30s denaturizing at 94°C, 45s annealing at 55°C, 1min elongation at 72°C and completed by a final elongation step of 10min at 72°C. Primer nomenclature corresponds to base pair positions relative to Emshwiller and Doyle (2002). For details on primers used see Tab. 1.

The amplification product was purified using Qiaquick PCR Purification Kit (Qiagen, Basel) following the manufacturers protocol on a micro centrifuge. The final product was re-eluted within 25µl of double-distilled H_2O. cleaned PCR product was used for cycle sequencing reactions performed in a total volume of 10µl liquid of following composition: 4µl of PCR purification product, 1.5ml Terminator Ready Reaction Mix (BigDye® Terminator v1.1

Cycle Sequencing Kit, Applied Biosystems Switzerland, Rotkreuz), 1x BigDye Terminator Sequencing Buffer and 200µM primer (ncpGS737F, ncpGSintF or ncpGSintF2 for forward sequences, and ncpGS940R for reverse direction). Primers were designed upon a study on *Oxalis* done by Emshwiller and Doyle (1999). For details see Tab. 1. The cycle sequencing reaction was performed on a Biometra T3000 thermocycler (Biometra, Göttingen) by applying an initial denaturizing of 1min at 94°C and 30 cycles of 10s denaturizing at 94°C, 5s annealing at 50°C and 4min elongation at 60°C.

Resulting cycle sequencing products were precipitated with each 1µl of 125mM EDTA and 3M sodium acetate as well as 25µl of 100% ethanol for about 20min at room temperature and then centrifuged with a Rotina 35R (Hettich, Tuttlingen/Germany) for 30min at 4°C and 15000rpm (23900g). The precipitation washed with 75µ of 70% ethanol and centrifuged for further 10min at above conditions. The precipitated product was dried in a Univapo 10H (Uniequip, Martinsried/Munich) under vacuum at about 50°C till dry, then resuspended in 12µl HIDI formamide (Applied Biosystems Switzerland, Rotkreuz) and denatured for 2min at 95°C prior to immediate cooling on ice, and loaded on a ABI Prism 310 Genetic Analyser (Applied Biosystems Switzerland, Rotkreuz). Raw sequences were worked out and revised upon scanning information in Sequencing Analysis 3.7 software (Applied Biosystems Switzerland, Rotkreuz).

ncpGS737F	5’-GGCCAGGTGCTTATTCCCTT-3’
ncpGS940R	5’- AAGCCTCCGTCTTCCCTCAT-3’
ncpGSintF	5’- GATCACGTCTGGTGTGCTAGA-3’
ncpGSintF2	5’- TTCTCACATTAGATCCAAAACCAA-3’

Table 1: Primer sequences used in present study.

Data Analysis

Sequences were aligned with the software ClustalX ver. 1.8 (Thompson, Gibson et al. 1997) and BioEdit ver. 7.053 (Hall 1999) and compared to sequences of *Oxalis* as done by Emshwiller and Doyle (1999). The flanking primer ncpGS737F is located within intron 7, while the reverse primer ncpGS940R anneals just after intron 10 resulting into a sequence leading from intron 7 to 10. The internal primer ncpGSintF is located between intron 7 and 8, ncpGSintF2 between intron 8 and 9. All of the introns are longer than the ones described by Emshwiller and Doyle (2002), especially intron 10 with several hundreds base pairs in some taxa and hosting a large indel region, exons are of the same size except the one between intron 8 and 9, which is 9 base pairs shorter than that proposed for Oxalis. The exon sequences cover 178 base pairs, whereof 165 are constant and 8 are parsimonious informative. Preliminary parsimony and likelihood analysis done only on these coding regions provided no good resolution and significant supports. Further treatments were therefore carried out on the entire sequences. These totally 46 sequences represented 37 different haplotypes. Further analyses were done only on these haplotypes, each representing one or several samples. Indels were coded using the SeqState Program (Müller 2005) using modified complex indel coding MCIC (Müller 2006). We were able to identify 1517 characters, whereof 1148 were constant and 245 parsimony informative.

Parsimony analysis was done by using PAUP*4.0 (Swofford 1998) using heuristic searches with random sequence addition and 1000 sequence replicates via stepwise addition, the TBR branch-swapping-algorithm in effect. A bootstrap analysis was performed using the above settings with 1000 repetitions.

Modeltest ver 3.7 (Posada and Crandall 1998) was used to obtain optimal models and parameters for likelihood analysis. Based on the selected models and parameters, a likelihood analysis was performed by using PAUP* 4.0 (Swofford 1998).

Results

The aligned sequences provided 1498 characters, whereof 1148 were constant and 245 parsimoniously informative. Base frequencies were calculated as A=0.2604, C=0.2233, G=0.2049. A region of uncertainty in alignment between position 599 and 618 had to be excluded from analysis by coding as missing information due to alignment ambiguity. Also missing was the initial 129 bps of *G. acaulis* L due to incomplete sequence.

50 different indels were coded. A very long indel with a maximal size of 710 base pairs was found in *G. nivalis* L. as well as in *G. pumila* Jacq. s.str. and *G. terglouensis* Hacq. s.str. The second annual species *G. utriculosa* L. did not show that indel, located within hypothetic intron 10 as described above, but was also present in the outgroup species *G. acaulis* L. All indels cover potentially non coding sequence (Emshwiller and Doyle 1999). In coding sequence part, 13 polymorphic sites were found resulting into codons for 4 different amino acids. Not enough resolution was obtained to produce a resolved phylogeny on only these coding characters.

<u>Results from parsimony analysis</u>

Parsimony analysis resulted in 2 most parsimonious trees with a length of 651 steps (CI = 0.708, RI = 0.829). The strict consensus with bootstrap support values are shown in Fig. 1. These 2 trees form parsimony analysis are basically identical. The only difference can be found within the group of eastern European *G. verna* L. subsp. *tergestina* Hayek (sequences 28 to 30). The three populations represent either an unresolved polytomy or alternatively the sequences 29-Cakor Pass and 30-Risnjak are sister to each other.

An early bifurcation separates the taxa *G. utriculosa* L., *G. nivalis* L. and G. *pumila* Jaquin s.str. into an annual cluster. Bootstrap support indicates a strong structure also within that first group by placing *G. pumila* Jacq. into an intermediate position between the two others. In case of *G. nivalis* L. we are able to identify two different haplotypes: one representing sequences from Mt. Mongioie (Ligurian Alps, Italy, sequence 36) and Umbrail Pass (Alps, Switzerland), the other hosting all other sequences examined of that species (Alps, Jura, Carpathians, Norway and Island, sequence 35), differing in two independent base pairs.

The remaining taxa forming the perennial cluster with a weak bootstrap support (54%). Within that group, a first division splits of the taxon *G. terglouensis* Hacquet s.str. from eastern to central alps and in a next step the western alpine populations of *G. bavarica* L. and the southwestern alpine *G. rostanii* Reuter. The two populations of western alpine *G. bavarica* L. differ in 2 base pairs, same between the two populations of *G. rostanii* Reuter. In-between *G. bavarica* L. and *G. rostanii* Reuter we identify a difference of 34 base pairs. Within the 3 examined populations of eastern alpine *G. bavarica* L. we found 23 to 38 different base pairs, between the two groups of *G. bavarica* L. s.str. 73 to 81 base pairs distance. But still highly significant with 99% is the support of that second eastern group.

A third group with maximal bootstrap support at its base is built by *G. bavarica* L. subsp. *subacaulis* Müller and *G. brachyphylla* Vill. subsp. *favratii* Tutin. While the two populations of *G. bavarica* L. subsp. *subacaulis* Müller differ in only 2 base pairs and provide a clear separation from *G. brachyphylla* Vill. subsp. *favratii* Tutin (71 to 82 different base pairs), the second one is structured into an alpine group (7 different base pairs in between) and an outstanding Carpathian population with 19 to 20 differing base pairs to the alpine populations. The internal order of *G. brachyphylla* Vill. subsp. *favratii* Tutin finds less bootstrap support than the basal division from . *bavarica* L. subsp. *subacaulis* Müller.

A large third group can be identified providing weak bootstrap support (54%). That group covers a larger variety of taxa than all others. Within we find: a) *G. brachyphylla* Vill. s.str. is strictly separated from its subspecies *G. brachyphylla* Vill. subsp. *favratii* Tutin by 57 to 70 different base pairs. With an internal distance of 21 base pairs the two populations of *G. brachyphylla* Vill. s.str. from Central to western Alps are high compared to the taxa mentioned above, with a bootstrap support of 84%. b) *G. terglouensis* Hacq. subsp. *schleicheri* Tutin with 7 base pairs difference

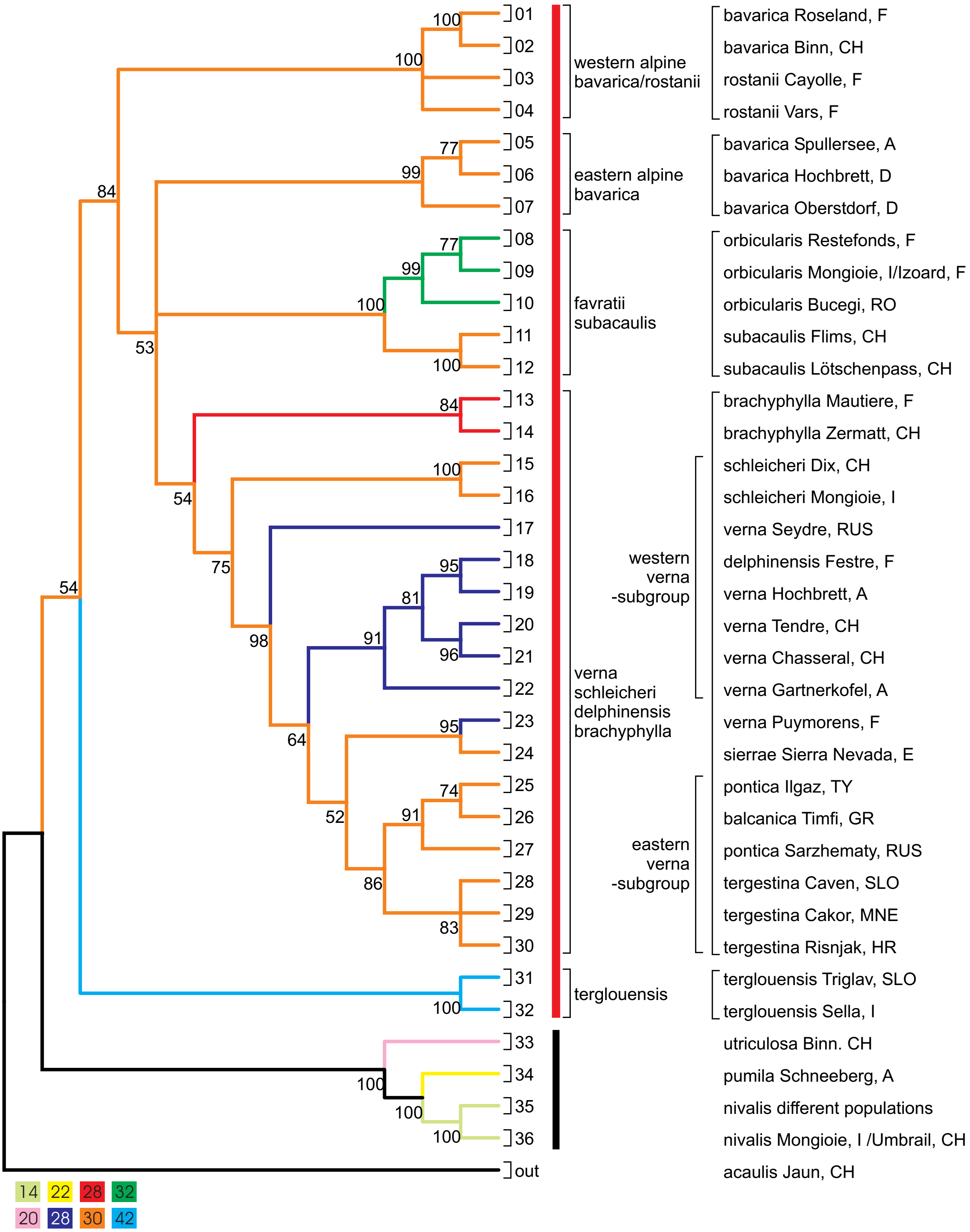


Figure 1: consensus tree from 2 most parsimonious phylogenies. Bootstrap supports are indicated. Numbers from 01 to 36 respectively out refer to haplotypes (see sample table, Annex). Solid black vertical bar marks the annual cluster (haplotype 33-36), solid red vertical bar the perennial one (haplotype 1-32). Groups within the perennial cluster are indicated, same as the subgroups within the verna-taxa (for detail see discussion). Colors code for chromosome numbers as proposed by Müller (1982). For diploid numbers see color legend.

between the two individuals and 46 to 54 respectively 48 to 51 base pairs to its closest neighbors *G. brachyphylla* Vill. s.str. respectively the Russian *G. verna* L. s.str. (verna-Seydre); c) a close related cluster of *G. verna* L and *G. pumila* Jacq. subsp. *delphinensis* Fourn. from western European Mountains with a bootstrap support of 91%; d) an eastern cluster covering the subspecies of *G. verna* L. also supported significantly (86%) and split into sequences 25 to

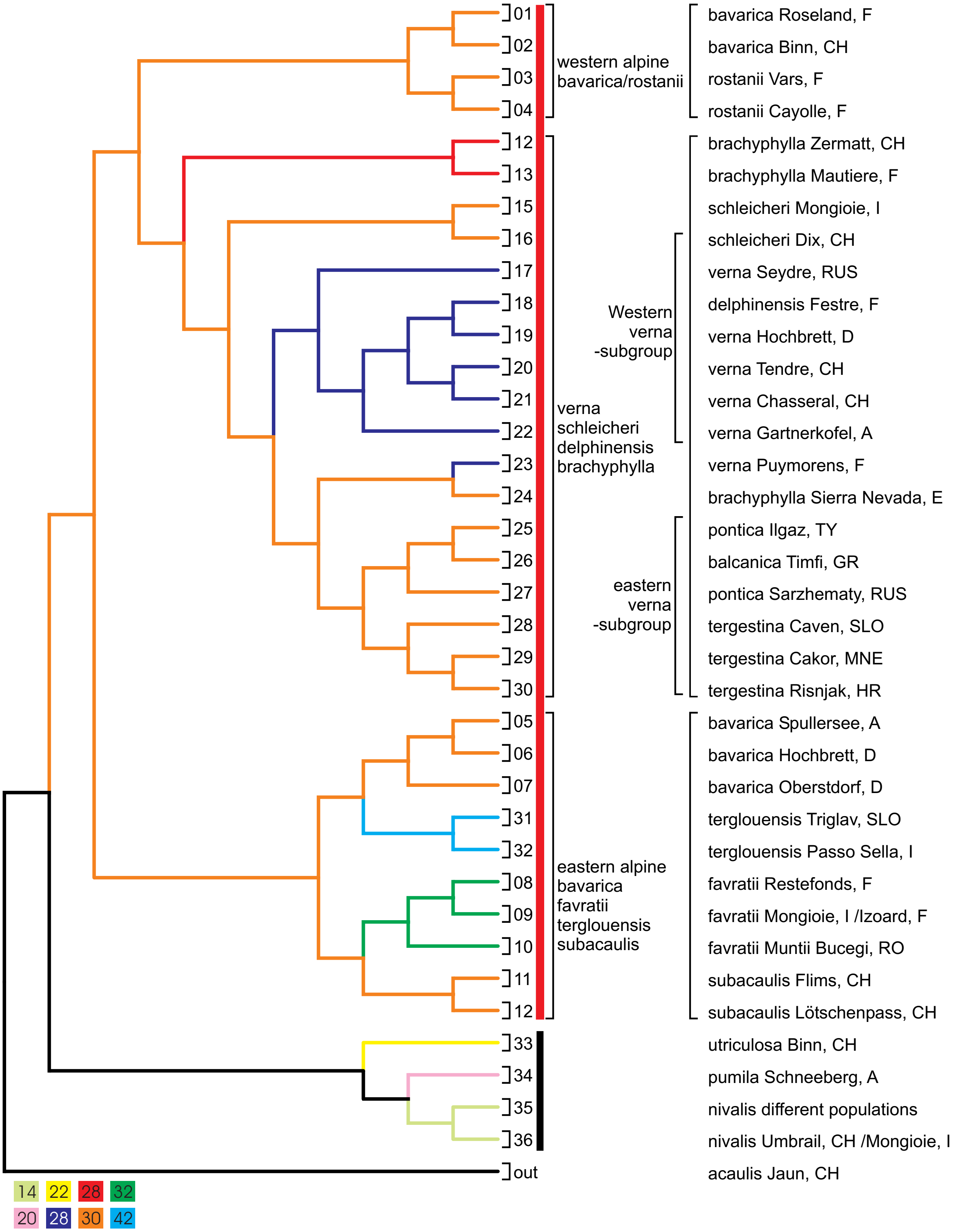


Figure 2: tree from maximum likelihood analysis Numbers from 01 to 36 respectively out refer to haplotypes (see sample table, Annex). Solid black vertical bar marks the annual cluster (haplotype 33-36), solid red vertical bar the perennial one (haplotype 1-32). Groups within the perennial cluster are indicated, same as the subgroups within the verna-taxa (for detail see discussion). Colors code for chromosome numbers as proposed by Müller (1982). For diploid numbers see color legend.

27 from Balkan mountains and Pontic Alps and sequences 28 to 30 from Dinaric Alps; e) an intermediate to the two previous subgroups composed of one sequence of *G. verna* L. from southern Pyrenees (23-Col de Puymorens) and one of *G. brachyphylla* Vill. from southern Spain (24-Sierra Nevada) with 19 base pairs in between.

Results from likelihood analysis

Maximum likelihood analysis was performed under the HKY85+I+G (Hasegawa, Kishino et al. 1985) model as proposed by Modeltest 3.7 (Posada and Crandall 1998) pretending an unequal base frequency, unequal substitution rates, and invariable sites as well as gamma-distributes of substitution rates. Base frequencies for likelihood analysis was A=0.2604, C=0.2233, G=0.2049, gamma was 0.8912 and the transition/transversion ratio as 1.5913.

The analysis resulted into a best tree with a likelihood score of 5567.0035 as shown in Fig. 2. Elements identical to the topology of the parsimony tree are the position and internal order of the annual cluster covering species *G. nivalis* L., *G. utriculosa* L. and *G. pumila* Jacq. s.str., as well as the position and the internal order of the *verna-schleicheri-delphinensis-brachyphylla*-group. While parsimony analysis resolves the polytomy between sequences 28-Caven, 29-Cakor and 30-Risnjak only partially, maximum likelihood demonstrates the closer link between 29-Cakor and 30-Risnjak.

The differences to parsimony trees include: a) the position and the internal resolution of the western-alpine-*bavarica-rostanii*-group. A clear internal order can be identified by grouping the both sequences of *G. bavarica* L. as sister to each other as well as another pair of both sequences of *G. rostanii* Reuter. This group becomes sister to the *verna-schleicheri-delphinensis-brachyphylla*-group. b) a new group of eastern-alpine-*bavarica-favratii-terglouensis-subacaulis*, sister to all other perennials is recovered. That group hosts sequences out of parsimony's two groups *favratii-subacaulis* and eastern-alpine-*bavarica* plus the outstanding *terglouensis*. Internally we can identify one cluster covering eastern *G. bavarica* L. and *G. terglouensis* Hacq. s.str. and a second one comprising of high alpine *G. bavarica* L. subsp. *subacaulis* Müller as well as the alpine/Carpathian *G. brachyphylla* Vill. subsp. *favratii* Tutin

As identical in both analyses can be outlined the position of *G. verna* L. and its subspecies including the *G. brachyphylla* Vill. s.str. from Sierra Nevada, *G. terglouensis* Hacq. subsp. *schleicheri* Tutin and *G. pumila* Jacq. subsp. *delphinensis* Fourn. All these taxa provide a common root, although its bootstrap support is somewhat weak. A second consistent group will be built by the two annual taxa *G. nivalis* L. and *G. utriculosa* L. plus the eastern alpine perennial *G. pumila* Jacq. s.str., in this case with maximum support from bootstrap analysis. An affinity to each other demonstrate also *G. bavarica* L. from the western Alps and *G. rostanii* Reuter from the same region, but an uncertainty in position relative to the other groups remains unresolved between the two groups. The tree groups *terglouensis, favratii-subacaulis* and eastern-alpine-*bavarica,* inserted in different positions in parsimony analysis (the second two due to a lack in bootstrap support) become fused into one group in maximum likelihood analysis

Discussion

Gentiana Sect. *Calathianae* Froel. hosts annual and perennial taxa. Several authors have pointed toward a separation of the first ones from the perennial species (Rouy, Foucaud et al. 1893; Hegi 1927; Müller 1982; Zuev 1985; Tzevelev 1987; Halda 1995; Halda and Haldovâa 1996) supported by chromosome counts (Knaben 1950; Favarger 1965; Favarger 1969a; Favarger 1969b; Müller 1982), proposing for *G. nivalis* L. 2n=14, for *G. utriculosa* L. 2n=22 and for the only perennial taxon close to these two: *G. pumila* Jacq. s.str. 2n=20. The annual group represents plants without a basal leaf rosette and branched stems with usually many flowers, while the perennials provide unbranched stems with few flowers and in many cases basal leaf rosettes. The perennial taxa except *G. pumila* Jacq. s.str. provide chromosome numbers of 2n=28, 30, 32, 38 and 40. Our data suggest a basal separation of taxa *G. nivalis* L., *G. utriculosa* L. and *G. pumila* Jacq. s.str. from the others and a placement in a well defined clade of its own. The maximal internal bootstrap values at each bifurcation within that group indicate the clear structure within that group *G. pumila* Jacq. s.str. as perennial taxon limited to a small distribution area at the eastern border of the Alps and representing a somehow intermediate between the two annual species. Obvious is the need for a separation of the two subspecies of *G. pumila* Jacq. While subspecies *pumila* takes its position between the two annual taxa in a subclade distinct from all other perennial taxa, subspecies *delphinensis* Fourn. growing on the western border of the alpine chain and the eastern Pyrenees is placed within a cluster of perennial *G. verna* L. as discussed later and needs to be excluded from *G. pumila* Jacq. despite the morphological similarities between these two subspecies (Ho and Liu 2001).

G. nivalis L. with a distribution from the east coast of Canada through Greenland, Island, Scandinavia and the European Mountains, eastern Turkey and Caucasus shows a remarkable low amount of variation. In our sample material covering an area from Island till the Carpathians we were able to identify just two haplotypes, differing in just 2 base pairs. The surprising internal structure of that first subclade is also supported by the large 710 bp indel, where the deletion was found in the annual *G. nivalis* L. and the perennial *G. pumila* Jacq. s.str., but not so in the annual *G. utriculosa* L. Müller (1982) proposed for these tree species each time independent evolution descending from n=5 over n=10 to the final state of n=11 in *G. utriculosa* L. and n=10 in *G. pumila* Jacq. s.str., while *G. nivalis* L. passed directly to n=7. Our results would propose much more a chromosomal evolution via a common state n=10 for all tree species and then a gain of one chromosome for *G. utriculosa* L. while later on *G. nivalis* L. lost 3 chromosomes and *G. pumila* Jacq. s.str. stayed at n=10.

We group the remaining perennial taxa into two groups, hereafter *verna* and non-*verna* taxa.

Verna-taxa

G. verna L. as widest distributed taxon beside *G. nivalis* L. shows much more variation in ncpGS sequence than the later one. We included 16 different haplotypes within a *verna*-group with a bootstrap support of 75% in parsimony analysis. That group covers the taxa *G. verna* L. s.str., *G. verna* L. subsp. *tergestina* Hayek, *G. verna* L. subsp. *balcanica* Pritch., *G. verna* L. subsp. *pontica* Heyek, *G. pumila* Jacq. subsp. *delphinensis* Fourn, *G. brachyphylla* Vill. s.str. from Sierra Nevada and *G. terglouensis* Vill. subsp. *schleicheri* Tutin. At its base we find also the alpine populations of *G. brachyphylla* Vill. s.str. indicating somehow its weak relatedness to the *verna*-taxa.

The haplotypes in the *verna-schleicheri-delphinensis-brachyphylla*-group are basically arranged within two subgroups: one covering western European Alps and Jura Mountains (western *verna*-subgroup), the second one from eastern Europe and the Asian mountain (eastern *verna*-subgroup). Additionally there in an intermediate covering one haplotype from southern Pyrenees and one from Sierra Nevada, as well as the two taxa *G. brachyphylla* Vill. s.str and *G. terglouensis* Jacq. subsp. *schleicheri* Tutin inserted close to the base of that group.

G. verna L. s.str. haplotypes from the Alpine chain and the Jura Mountains show a strong link to *G. pumila* Jacq. subsp. *delphinensis* Fourn., as already indicated by Kunz (1972) in his definition that subspecies. Hegi (1927) reported a high number of variation within *G. verna* L. We do not obtain enough information in that study to define a clear structure within that western *verna*-subgroup to answer the question what the precise rank of taxon *delphinensis* could be: weather it should be integrated fully within *G. verna* L. or interpreted as a independent subspecies.

Eastern European and Asian populations form eastern *verna*-subgroup covering individuals from Dinaric Alps, the Balkan Mountains, Pontic Alps, Caucasus and Altai Mountains. The base of that group is clearly significant in parsimony analysis. *G. verna* L. subsp. *tergestina* Hayek growing on Dinaric Alps is represented by 3 close linked haplotypes, the both taxa *G. verna* L. subsp. *balcanica* Pritch. and subsp. *pontica* Hayek from Balkan Mountains and Asia provide another 3 close linked sequences with strong basal support. That eastern *verna*-subgroup represents therefore an assembly of taxa clearly separated from the western *verna*-subgroup, but sharing a common root with the later ones. Intermediate to these two groups is *G. verna* L. s.str. from the southern Pyrenees and the Sierra Nevada population of *G. brachyphylla* Vill. s.str.. The integration of that populations into *G. brachyphylla* Vill. can't be justified by our data. We support therefore a definition as *G. verna* L. subsp. *sierrae* Rivas Mart. (Rivas-Martinez, Asensi et al. 1991) to reflect also the morphological distance between that taxon and *G. verna* L. s.str. (Briquet 1931; Ho and Liu 2001). The middle position of these two far western populations between the Alpine and the eastern *G. verna* L. points toward an early time of immigration into the Pyrenees and the southern Spanish mountains, relative to the establishment of morphologically distinct subspecies in the eastern European mountains. That fact could also explain the existence of morphologically distinct *G. verna* L. var. *willkommiana* Monts. & Vill. and var. *cantabrica* Romo (Montserrat and Villar 1975; Romo 1988) in the Northern Spanish Cantabric Mountains.

G. verna L. s.str. from the Ural Mountains has a basal position to the taxa mentioned above. Cherepanov (1995) identifies for Russia a *G. arctica* Grossh. occurring in the Ural region, treated by Komarov (1967) as synonym of *G. verna* L. We are able to show, that there, populations from northern Russia are close to *G. verna* L. s.str.. If it should remain an independent taxon as proposed by (Grossheim 1947), become a subspecies of *G. verna* L. (Sergienko 1986) or gets fully integrated within *G. verna* L. as proposed by Ho and Liu (2001) and Tzevelev (1987).

Another member of that *verna*-group is represented by *G. terglouensis* Hacq. subsp. *schleicheri* Fourn., taking a basal position to all other taxa. As Bolòs and Vigo (1984) proposed by postulating *G. verna* L. subsp. *schleicheri*, our two individuals of *G. terglouensis* Hacq. subsp. *schleicheri* Fourn., are closely linked to *G. verna* L., but not so to *G. terglouensis* Hacq. s.str.. Therefore it seems to be evident that, if the taxon *schleicheri* needs to be treated as independent species or as subspecies of *G. verna* L. as proposed above, despite ecological similarities and geographical vicariance (Ho and Liu 2001).

A last taxon close to *G. verna* L. is the above already mentioned Alpine *G. brachyphylla* Vill. s.str.. Müller (1982) counted 2n=30 chromosomes in the Sierra Nevada, while he found 2n=28 in the Alpine *G. brachyphylla* Vill. s.str. and treated the populations as different taxa, in opposite to Ho and Liu (2001), that recognized them as synonyms. As Müller (1982) indicates a link between *G. verna* L. and *G. brachyphylla* Vill. we place *G. brachyphylla* Vill. close to *G. verna* L., building a root to these species. Remarkable is also the ecological proximity of these two taxa: *G. brachyphylla* Vill. s.str. grows in the highest altitudes also suitable to *G. verna* L., generally on deep soils with low nutrition level (Landoldt 1977) with a neutral to siliceous composition (Aeschimann and Lauber 2004), flowering in the middle of the summer, while *G. verna* L. is much more variable, occupying rather calcareous soil and flowering earlier, but both taxa highly overlapping in their distribution area.

Non-*verna*-taxa

Our two analyses do not completely agree in the placement for several taxa out of that group. Unclear remains how close related the taxa *G. bavarica* L., *G. brachyphylla* Vill subsp. *favratii* Tutin and *G. terglouensis* Hacq. s.str. are. A clear relationship can be found between the taxa *G. bavarica* L. subsp. *subacaulis* Müller and *G. brachyphylla* Vill. subsp. *favratii* Tutin. The first one representing plants growing in higher altitude relative to other taxa examined, on sandy soils on crystalline rockbed (Müller 1982), while the second one prefers rather calcareous, sometimes moving underground, but as above a very open vegetation. *G. brachyphylla* Vill. subsp. *subacaulis* Müller turns out to be clearly distinct from *G. bavarica* L. according to the definition as independent species given by (Schleicher 1821). Müller (1982) showed the difference in altitude of occurrence between the two subspecies of *G. bavarica* L., Hegi (1927) even mentions a series of intermediates and Hess, Landolt et al. (1967) explain them as ecological varieties. Pignatti (1997) mentions subsp. *subacaulis* also, but explains it as a synonym of *G. orbicularis* Schur. *G. orbicularis* Schur itself got described in the Carpathians and represents a synonym to *G. brachyphylla* Vill. subsp. *favratii* Tutin (Müller 1982; Ho and Liu 2001), while Halda and Haldovâa (1996) threat them as independent taxa and Aeschimann and Lauber (2004) uses entirely the definition of Schur (1852, 1866). Our individuals from the Alps and the Carpathians deliver no clear measures for separation and represent a single taxon. Due to the lack of material we can not answer the question, if populations indicated for south-eastern Turkey would also meet that group (Davis, Cullen et al. 1965). With a common placement in both analysis and a maximal bootstrap support in parsimony analysis we can propose *G. brachyphylla* Vill. subsp. *favratii* Tutin and *G. bavarica* L. subsp. *subacaulis* Müller as closely liked taxa, separated from their relatives *G. bavarica* L. s.str. and *G. brachyphylla* Vill. s.str.

G. bavarica L. s.str. is divided into two distinct types: a first one covering the north eastern Alps (individuals from Austria and Germany, eastern alpine bavarica) linked in likelihood analysis to *G. terglouensis* Hacq. s.str, and the second one from the western Alps (material from Switzerland and France, western alüine bavarica / rostanii) in proximity to southwestern Alpine *G. rostanii* Reuter. The precise order of these mentioned species is not uniform between parsimony and likelihood. The western group of *G. bavarica* L. and *G. rostanii* Reuter is placed as sister to the *verna*-taxa and to the cluster of the taxa *subacaulis* and *favratii*. Whether the neighbourhood to one or the other group dominates remains unclear since the both analysis give controversial results with low supports specially in parsimony. Both taxa populate about the same ecological environments: wet meadows and damp places from 1500 to 2600 m (Müller 1982), in case of *G. rostanii* Reuter sometimes also lower (Ho and Liu 2001). Out observations in field and the above indicated herbaria allow us to postulate to distinct distribution area for these taxa: *G. bavarica* L. s.str. occurs all along the Alpine chain from Austria and Germany south- and westward till to the region of the both cities Turin (Italy) and Briançon (France). South of that line it gets replaced entirely by *G. rostanii* Reuter.

The second type composed of eastern *G. bavarica* L. and *G. terglouensis* Hacq. s.str. reported only from likelihood analysis, while parsimony puts *G. terglouensis* Hacq. s.str into a distinct position, basal to all other *verna*- and non-*verna*-taxa. We identify a weak indication that the two taxa could be sisters to the taxa *subacaulis* and *favratii* and therefore sister or root to the *verna*-taxa. The basal position of *G. terglouensis* Hacq. s.str. in parsimony analysis would support the hypothesis of the establishment of a *terglouensis*-like type and afterwards the separation into the clade hosting *verna*-taxa and the one origin to today's *G. terglouensis* Hacq. s.str., *G. bavarica* L. s.str., the taxa *subacaulis* and *favratii*. *G. rostanii* Reuter and the western *G. bavarica* L. s.str. would originate therefore at the interface between eastern G. *bavarica* L., the taxa *subacaulis* and *favratii* to the *verna*-taxa.

(Müller 1982) proposes an evolution of *G. bavarica* L. together with *G. rostanii* Reuter but roots these two taxa directly at the base of his clade, while *G. terglouensis* Hacq. s.str. represents another basal group together with *G. pumila* Jacq.. The relatedness of *G. pumila* Jacq. s.str. and *G. terglouensis* Hacq. s.str., is supported by our data.

Conclusions

Following conclusions can be made upon present study:

a) *G.* Sect. *Calathianae* Froel. can be divided into two Subsections: a first one covering the annual taxa *G. nivalis* L. and *G. utriculosa* L. as well as the perennial *G. pumila* Jacq. s.str., the second one comprises of all other taxa

b) The large *G. verna* L. complex is divided into an eastern part covering *G. verna* L. subsp. *tergestina* Hayek, *G. verna* L. subsp. *balcanica* Pritchard and *G. verna* L. subsp. *pontica* Hayek. The western one comprises of *G. verna* L. s.str. and *G. pumi*la Jacq. subsp. *delphinensis* Fourn.. Also part of the western part is *G. verna* L. from the Russian Ural Mountains.

c) *G. terglouensis* Hacq. s.str. and its subspecies *schleicheri* Tutin have a clearly different position in phylogeny. *G. terglouensis* Hacq. subsp. *schleicheri* Tutin is closely related to *G. verna* L.-clusters.

d) *G. brachyphylla* Vill s.str. and *G. brachyphylla* Vill subsp. *favratii* Tutin have a clearly different position in phylogeny *G. brachyphylla* Vill subsp. *favratii* Tutin is closest to *G. bavarica* L. subsp. *subacaulis* Müller.

e) The Sierra Nevada population of *G. brachyphylla* Vill s.str. shows no relation to the rest of that taxon and demonstrates proximity to geographically close haplotypes if *G. verna* L. s.str.

Conclusions on the systematics of *Gentiana* Sect. *Calathianae* Froel. (*Gentianaceae*) derived from 5S-NTS polymorphisms

Abstract

Gentiana Sect. *Calathianae* Froel. is a small species group that occurs in the mountains of Europe and Asia with about 15 taxa differentiated by few morphological characters. Previous studies based on morphology and cytological examinations have not elucidated the phylogenetic relationships. Here the results of a comparison of 29 5S-NTS haplotypes of a wide range of taxa are presented.Two subsections are identified: the first composed of the annual *G. nivalis* and the two eastern alpine perennials *G. pumila* s.str and *G. terglouensis* s.str. The second comprises three groups: a widely *G. verna* group including all subspecies of that taxon, a second with the high alpine taxa *G. terglouensis* subsp. *schleicheri* and *G. brachyphylla* subsp. *favratii*, and the third is of *G. bavarica*, *G. rostanii* and *G. brachyphylla* s.str.

Keywords

Phylogeny, Gentiana, 5S-NTS, PCR, Restriction, taxonomy

Introduction

Gentiana Sect. *Calathianae* Froel. has about 15 taxa found mainly in alpine regions in Europe, less common in Asia, and with one species each in North America and Africa. While there are two annual taxa, all others are perennials. Common characters include the sympodial stems, the flowering stems arising singly from stolons, solitary flowers, and a salver-shaped corolla subtended by an angled or winged calyx. The stigma lobes are expanded and connate or contiguous, forming a discoid structure. Seed coats are reticulate and winged on some side.

Kusnezow (1894) did an early revision of the section by placing it as distant from the other sections of *Gentiana*, nowadays sister to *G.* Sect. *Ciminalis* Dum. Halda (1995) used the presence of two different life-cycles to postulate a division this group into two sections. The presence of common criteria, mainly the same type of stigmata speaks much more for only one section. Müller (1982) proposed Sect. *Cyclostigma* Griseb. as the valid name for this section, nevertheless Tutin (1964) and later Ho and Liu (2001) reused the definition of Sect. *Calathianae* Froel. Müller (1982) did a recent revision of the section by providing chromosome numbers for all taxa, and predicted an origin descending from a common root providing n=5 each a solitary evolutionary path for the two annual taxa (n=7 for *G. nivalis* L., n=11 for *G. utriculosa* L.), a common one for *G. bavarica L.* and *G. rostanii* Reuter (n=15), colonizing as vicariate taxa almost the same ecology, further a cluster with common origin for the two vicariate taxa *G. terglouensis* Hacq. (n=19) and subsp. *schleicheri* Tutin (n=15) as well *as G. pumila* Jacq. (n=10). A last huge cluster starting at n=15 would provide *G. verna* L. and its subspecies, but also *G. brachyphylla* Vill. s.str. and in isolated positions *G. brachyphylla* Vill subsp. *favratii* Tutin and *G. sierrae* Briq, while Halda (1995) and as above Ho and Liu (2001) focused for that section mainly on morphological criteria. They proposed mainly one section comprising of a large central species covering *G. verna* and its subspecies (Tutin 1964), an eastern and western alpine, scattered species *G. pumila* including subsp. *delphinensis* Fourn., a high alpine species covering the two vicariate taxa *G. terglouensis* Hacq. and subsp. *schleicheri* Tutin as well as a large taxon *G. brachyphylla* including the southern Spanish and northern African taxa, beside some smaller species. Halda (1995), some years before, proposed to separate the perennial taxa in a new *G.* Sect. *Vernae* Halda and to arrange that one together with the remaining *G.* Sect. *Calathianae*, hosting only the annual taxa, under *G.* subgen. *Calathianae* (Froel.) Halda. Gielly and Taberlet (1996) derived a taxonomy on European gentians from trnL (UAA) sequences, covering also some taxa of G. Sect*. Calathianae* They were able to separate the two taxa *G. nivalis* L. and *G. pumila* from the others, but didn't get a clear phylogeny for the entire section. Yuan and Küpfer (1995) did the same by the use of ITS-sequences. The selection of taxa fitted perfectly to resolve the intersectional order but was not sufficient to give a closer insight within the section.

The present study is designed to enlighten the intrasectional arrangement of taxa based on a nuclear non-coding sequence. I specially ask the question whether the division of present *G.* Sect. *Calathianae* as done by Halda (1995) is legitimate how the taxa should be affected. Also we try to justify the taxonomic rearrangements done respectively accepted by Tutin (1964) and Ho and Liu (2001) and some authors before, specially the subordination of taxa *delphinensi*s under *G. pumila*, the subordination of taxon *favratii* under *G. brachyphylla* and the subordination of taxon *schleicheri* under G. *terglouensis* Hacq. I also wonder if *G. sierrae* from the Sierra Nevada (Spain) should be integrated within *G. brachyphylla* as previously suggested by Tutin (1964) or within G. verna as proposed by Rivas-Martinez et al. (1991).

Material and Methods

Sampling sites were selected by the study of specimens in various European herbaria (NEU, G, P, K, BM and ZA/ZAHO), as well as the CRSZ-database for Switzerland (Bäumler 1999). The taxa and populations collected for this study are listed in sample table (Annex III). Leaf material of sam-pled specimens was dried with silica gel. Approximately 20 mg of dried leaves were ground using Tungsten Carbide beads and Qiagen TissueLyser (Qiagen, Basel). DNA extraction was performed using DNEasy Plant Mini Kit (Qiagen, Basel), leading to about 50ng total DNA per µl extract. Based on approximately 50ng DNA, a PCR was performed, using 1U Taq DNA Polymerase (New England Biolabs, Ipswich MA), 200µM of each dNTP, 0.2µM of each primer PI and PII (Cox, Bennett et al. 1992), 12.5µg of BSA, 1x PCR buffer (New England Biolabs, Ipswich MA) and additional 0.5mM $MgCl_2$ in a total volume of 25µl. The procedure was carried out on a Biometra T3000 thermocycler (Biometra, Göttingen) by performing a initial denaturizing of 3 min at 95°C, 36 cycles: 1 min at 94°C, 1 min annealing at 53°C and 1 min at 72°C for extension, followed by final 10 min at 72 °C. The products of amplification were separated using gel electrophoresis in 0.5 TBE buffer (Maniatis, Fritsch et al. 1982) and 1% agarose. The amplified fragments of interest were extracted using the Qiaquick Gel Extraction Kit (Qiagen, Basel) by applying the manufacturer's manual and eluting the extraction product in 20µl of double-distilled H_2O. Amplification products were purified using Qiaquick PCR Purification Kit (Qiagen, Basel) following the manufacturers protocol on a micro centrifuge and re-eluted within 25µl of double-distilled H_2O.

A cycle sequencing reaction in 10µl liquid of liquid was done on these purified amplification products: 4µl of PCR purification product, 1.5ml Terminator Ready Reaction Mix (BigDye® Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems Switzerland, Rotkreuz), 1x BigDye Terminator Sequencing Buffer and 200µM PII primer as indicated above. The cycle sequencing reaction was done on a Biometra T3000 thermocycler (Biometra, Göttingen) by applying an initial denaturizing of 1 min at 94°C and 30 cycles of 10s denaturizing at 94°C, 5s annealing at 50°C and 4 min extensions at 60°C.

Resulting cycle sequencing products were precipitated with each 1µl of 125mM EDTA and 3M sodium acetate as well as 25µl of 100% ethanol for about 20 min at room temperature and then on a Rotina 35R (Hettich, Tuttlingen/Germany) centrifuge for 30 min at 4°C and 15000rpm (23900g). The supernatant liquid was removed and the precipitation washed with 75µ of 70% ethanol and centrifuged for further 10 min at above conditions. The supernatant was removed again and the precipitated product dried in a Univapo 10H (Uniequip, Martinsried/Munich) under vacuum at about 50°C till dry.

The cleaned sequencing product got re-suspended in 12µl HIDI formamide (Applied Biosystems Switzerland, Rotkreuz) and denatured for 2 min at 95°C prior to immediate cooling on ice and scanning on a ABI Prism 310 Genetic Analyser (Applied Biosystems Switzerland, Rotkreuz). Raw sequences were worked out and revised upon scanning information in Sequencing Analysis 3.7 software (Applied Biosystems Switzerland, Rotkreuz).

Data Analysis

Sequences were aligned in ClustalX ver. 1.8 (Thompson, Gibson et al. 1997) and BioEdit ver. 7.053 (Hall 1999). Of the 41 sequenced samples plus one outgroup, 30 different haplotypes were identified. As outgroup, *G. acaulis* L. of *G.* Sect. *Ciminalis* Dum. was selected due to its proposed relatedness to *G.* Sect. *Calathianae* (Kusnezow 1894, Müller 1982, Yuan and Küpfer 1995, Gielly and Taberlet 1996). The outgroup sequence showed only poor alignment to the other samples. To obtain a root for further analyses the sequences were cropped to a 190 base pair long alignment of maximal fit. Indels were coded using the SeqState Program (Müller 2005) using modified complex indel coding MCIC (Müller 2006) and analyzed in PAUP* (Swofford 1998) by performing a parsimony analysis using the heuristic search option with random sequence addition and 1000 repeats. The position of the outgroup species in the one obtained most parsimonious tree of that preliminary analysis was used as root for further analyses.

Analysis was performed on the 29 complete and aligned sequences without outgroup a parsimony analysis (Fitch 1971) on PAUP* (Swofford 1998) using a heuristic search access with random sequence addition and 100000 sequence replicates via stepwise addition, with the TBR branch-swapping-algorithm in effect. Bootstrap and jack-knife supports were calculated each upon 1000 repeats of each 1000 heuristic searches with similar parameters as above.

To evaluate optimal parameters for likelihood analysis Modeltest ver 3.7 (Posada and Crandall 1998) was used. Based on these values a likelihood analysis was performed on PAUP* (Swofford 1998) under a GTR+G model (Lanave, Preparata et al. 1984; Rodriguez, Oliver et al. 1990). A heuristic search with random sequence addition and 1000 repeats resulted into two best trees.

A distance analysis was also performed on PAUP* (Swofford 1998). Bootstrap support of the optimal tree was evaluated by running a bootstrap analysis with 1000 repetitions.

Results

The original sequence alignment with a length of 570 base pairs was cropped by removing the initial 173 base pairs only present in *G. nivalis* The remaining 397 base pairs were indel coded as above by resulting into 411 characters. 14 indel hosting loci were identified with overall 21 different indels with a length from 1 to 40 base pairs

Parsimony analysis resulted into one optimal tree with length of 364 steps, consistency index CI=0.791, retention index RI=.938 (see Fig. 1).

The parsimony tree is divided at its base into a cluster covering the annual *G. nivalis* and the perennial *G. pumila* s.str. and a second one home to all other taxa. The separation between the two clusters has maximal support both in bootstrap and jack-knife analysis. Within the first cluster covering *G. nivalis* and *G. pumila* s.str., the separation between the two species is also maximal.

Within the second cluster a first bifurcation with 100% bootstrap and jack-knife support separates a group covering *G. terglouensis* s.str. from the rest. The remaining taxa are divided a) into a *brachyphylla-bavarica-rostanii*-group, b) a *verna*-group and c) a *favratii-schleicheri*-group.

The *brachyphylla-bavarica-rostanii*-group hosts each one single sequence for all alpine *G. brachyphylla* s.str. (25), for all *G. bavarica* s.str. (26) and for all *G. rostanii* (29) as much as two independent sequences for *G. bavarica* subsp. *subacaulis* (27 and 28). G. brachyphylla s.str. is sister to all other haplotypes, *G. rostanii* sister to the two subspecies of *G. bavarica.*

With 11 haplotypes from different taxa the *verna*-group is much larger than the other two within the second sub-clade. Taxa represented are G. verna and its eastern subspecies *tergestina*, *balcanica* and *pontica*, *G. pumila* subsp. *delphinensis*, as well as each one population of *G. brachyphylla* subsp. *favratii* and *G. brachyphylla* s.str.

Two subgroups can be identified inside the *verna*-group: one covering the haplotypes 21 to 24 comprising of the eastern subspecies of *G. verna* as well as the Sierra Nevada population of *G. brachyphylla* S.str. (21). The second subgroup covers *G. verna* from the Alpine chain, the Pyrenees and Jura Mountains as well as the Russian Ural Mountains. The south-western Alpine *G. pumila* subsp. *delphinensis* (14) has its closest relatives in *G. verna* from Gartnerkofel (15, Austria) and Chasseral (16, Switzerland. Also included within that subgroup are the Ural (Russia) population of *G. verna* with a sequence identical to an individual from Mt. Tendre (Switzerland, both 20), *G. verna* from the southern Pyrenees (Col de Puymorens, France, 18). *G. verna* subsp. *tergestina* Hayek from the Slovenian Mt. Caven (19) and *G. brachyphylla* subsp. favratii from the Pyrenees (17).

G. brachyphylla subsp. favratii and *G. terglouensis* subsp. *schleicheri* are located within the *favratii*-schleicheri-group providing maximal bootstrap and jack-knife support upon parsimony analysis at its base. *G. brachyphylla* subsp. favratii was collected from the Alps providing two different haplotypes with 5 base pair distance in between, as well as from the Carpathians returning a single haplotype with 2 to 6 base pair distance to the Alpine populations. That taxon is clearly separated from the other within that group: *G. terglouensis* subsp. *schleicheri*. The two examined populations from the French and the Swiss Alps provide 5 base pairs distance in between and 17 to 24 to the taxon mentioned above.

Likelihood analysis

Optimal parameters for likelihood analysis proposed by Modeltest ver 3.7 (Posada and Crandall 1998) was +G model (Lanave, Preparata et al. 1984; Rodriguez, Oliver et al. 1990). Following parameters were used for analysis: base frequencies: A=0.2210, C=0.3064, G=0.1620 and T=0.3106; Gamma = 2.754, substitution frequencies were: AC=1.0000, AG=3.42070, AT=1.0000, CG=1.0000, CT=1.962800, GT=1.0000. The heuristic search returned one optimal tree with a score of 2398.95625. The topology of a consensus tree was similar to the one from parsimony analysis as shown in Fig 1.

Discussion

Gentiana Sect. *Calathianae* has both annual and perennial species. Several authors have suggested a separation of annual species from the perennial species (Rouy, Foucaud et al. 1893; Hegi 1927; Müller 1982; Zuev 1985; Tzevelev 1987; Halda 1995; Halda and Haldovâa 1996). This was supported by chromosome counts (Knaben 1950; Favarger 1965; Favarger 1969a; Favarger 1969b; Müller 1982), proposing for *G. nivalis* 2n=14, for *G. utriculosa* 2n=22 and for the only perennial taxon close to these two, *G. pumila* s.str. 2n=20. The perennial taxa except *G. pumila* s.str. have chromosome numbers of 2n=28, 30, 32. Only G. terglouensis s.str. differs with a higher number of 2n=38 and 40. The two subclades fits within that image: the first covers all taxa with chromosome numbers different from 2n=28 to 2n=32, the second one hosts the taxa complying with that criterion.

First clade

Information from both analyses demonstrates the distinct position of *G. nivalis*, itself providing a surprisingly large amount of homogeneity over a large distribution area. The taxon provides morphological characters separating them from all other taxa. These annual plants grow without a basal leaf rosette and branched stems with usually many flowers, while specially perennial taxa arrange their leaves in rosettes, stems are unbranched porting just one flower. The only further annual taxon of *G. sect Calathianae*, *G. utriculosa* shares its morphological characters with *G. nivalis* but was not included in that study.

The separated position of *G. pumila* s.str. is also indicated by Müller (1982), who provided a chromosome number of 2n=20, different from all other perennial taxa and also from its subspecies *G. pumila* subsp. *delphinensis*

(Tutin 1964; Ho and Liu 2001). While that second one grows on the western border of the alpine chain and the eastern Pyrenees, *G. pumila* s.str is restricted to the most eastern part of the alps in Austria, Slovenia and Italy. Since we were neither able to find such herbarium specimen nor to find plants in nature, we doubt into the presence of *G. pumila* s.str more westward. *G. pumila* s.str. is estimated to be a strict eastern alpine taxon. Indications elsewhere are possible confusions especially with the narrow leafed forms of *G. verna* (Müller 1982) and the taxon *G. pumila*

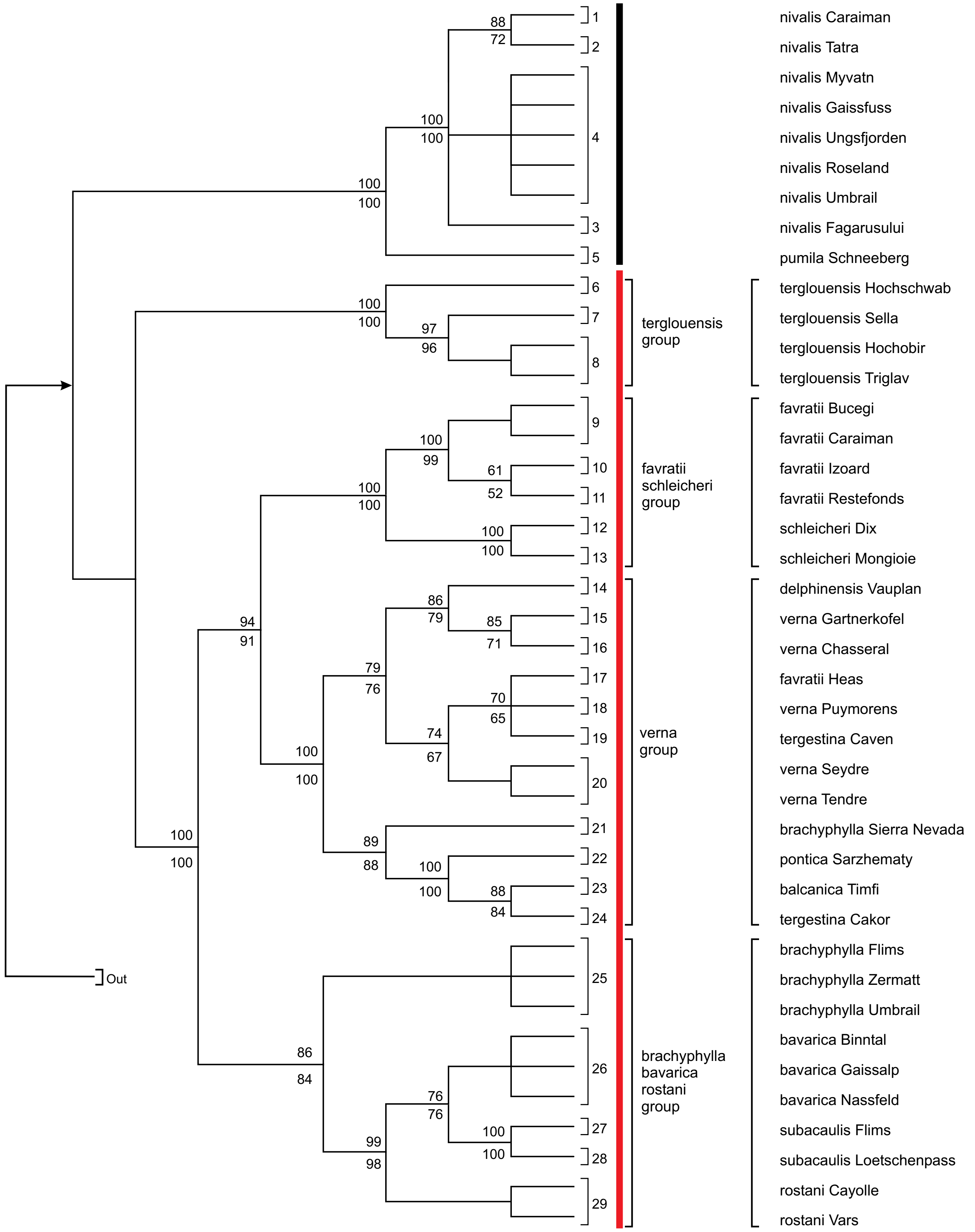


Figure 1: Strict consensus of 2 most parsimonious trees. Above the lines: bootstrap values from 1000 bootstrap replicates, below jackknife values from 1000 jackknife replicates. Haplotype numbers refer to sample table (Annex III)

Parsimony analysis

subsp. *delphinensis*, which will be discussed later. The two subspecies of *G. pumila* Represent clearly two distant taxa. *G. pumila* s.str. is outstanding the other perennial species and close to the annual *G. nivalis*

<u>Second clade</u>

G. brachyphylla s.str. is a polyphyletic taxon with one haplotype representing all Alpine populations and another one for an individual from Sierra Nevada. While that later one is placed in the middle of *G. verna* the first type has a distinct position as sister to *G. bavarica* s.l. and *G. rostanii*. Müller (1982) counted 2n=30 chromosomes for the *G. brachyphylla* for Sierra Nevada and found 2n=28 for those from the Alps. Rivas-Martinez, Asensi et al. (1991) treated the Sierra Nevada populations of *G. brachyphylla* as *G. verna* subsp. *sierrae* by excluding them from *G. brachyphylla* Our data support such a definition resulting into a monophyletic *G. brachyphylla* s.str. with a distribution through the Alps and a taxon *sierrae* in proximity of *G. verna* for Sierra Nevada. A monophyletic species is also without any doubt is *G. rostanii* from the southern French and Italian Alps, not overlapping its distribution area with *G. bavarica* s.str. but occupying about the same ecological environment (Müller 1982; Ho and Liu 2001; Aeschimann and Lauber 2004).

G. bavarica s.str. and subsp. *subacaulis* result in being neighbor taxa separated by a bifurcation both significant but not maximal supported. These both taxa have about the same distribution (Aeschimann and Lauber 2004) but as Müller (1982) have: they tend to grow on different altitude levels: while *G. bavarica* s.str. occurs from 1800 to 1600 m., *G. bavarica* subsp. *subacaulis* can be found generally in higher altitudes from 2100 to 3200 m growing on generally drier soil than the taxon above.

G. brachyphylla subsp. favratii is represented by Carpathian and Alpine haplotypes with a common root and few differences in sequence composition. *G. brachyphylla* subsp. favratii is clearly separated from *G. brachyphylla* s.str. The taxon *favratii* could therefore be estimated as independent taxon as *G. favratii* (Rittener 1887) or *G. orbicularis* (Schur 1866). The existence of two independent taxa *G. favratii* Rittener for the Alps and *G. orbicularis* Schur for the Carpathians as postulated by (Halda and Haldovâa 1996) finds no proof in our analysis. Between the haplotypes of *G. verna* we find also one type representing a population of taxon favratii from the Central Pyrenees. Although the clear morphological criteria pointing toward *G. brachyphylla* subsp. favratii, the existence of that taxon in the Pyrenees can't be proofed basing on our data. The above mentioned population has to be moved to *G. verna* therefore and *G. brachyphylla* subsp. *favratii* remains restricted to the Alps and the Carpathians.

G. terglouensis Subsp. *schleicheri* becomes sister to *G. brachyphylla* subsp. favratii, and together with that later one they are neighboring the *verna*-group. The subordination of *G. schleicheri* H. Kunz under *G. terglouensis* as done by Tutin can't be further supported therefore since it stands in conflict to our outcome. Not so the definition of *G. verna* subsp. *schleicheri* as done by Vaccari (1911) and Bolòs and Vigo (1984) or even the definition as independent taxon as above (Kunz 1939). Evident is the distance in ecological preferences between *G. verna* and *G. terglouensis* subsp. *schleicheri*: *G. verna* prefers fresh soil while the second one grows on stony slopes and moraines, often on places exposed to strong wind (Hess, Landolt et al. 1967). Sister to the two taxa above is large group composed of *G. verna* and its subspecies as well as *G. pumila* subsp. *delphinensis*, divided into two subgroups as described: one rather hosting western European taxa, the other eastern European and Asian taxa.

G. pumila subsp. *delphinensis* 5S-NTS-sequences are close related to Alpine *G. verna* s.str. The western *verna*-group covers all individuals from the Alps and the Jura Mountains as much as the Pyrenees by including also the above mentioned *G. pumila* subsp. *delphinensis*, is touching the northern part of the Dinaric Alps with one individual from Slovenia (19) and hosts also an individual from the Russian Ural Mts. (20). The eastern *verna*-group covers beside the already described Sierra Nevada population of *G. brachyphylla* also *G. verna* subsp. *tergestina* Hayek, *G. verna* subsp. *balcanica* Pritchard and *G. verna* subsp. *pontica* Hayek. The outcome of a not monophyletic species *G. verna* s.l. illustrates the need for a redefinition by either including all taxa of that *verna*-group within *G. verna* of the creation of two different species for the eastern and the western part. Such integration has already been proposed for the Sierra Nevada population of *G. brachyphylla* as *G. verna* subsp. *sierrae* by Rivas-Martinez, Asensi et al. (1991) following the estimation of Hegi (1927) who described *G. verna* as taxon hosting large variability.

Conclusions

Following conclusions can be made:

a) *G.* Sect. *Calathianae* is deeply divided into a unit hosting *G. nivalis* and *G. pumila* s.str. and a second unit comprising *G. verna* and subspecies, *G. pumila* subsp. *delphinensis*, *G. bavarica*, *G. rostanii*, *G. brachyphylla* and *G. terglouensis*.

b) *G. pumila* subsp. *delphinensis* has to be separated from *G. pumila* s.str.

c) *G. terglouensis* s.str. has a basal and isolated position and needs to be separated from *G. terglouensis* subsp. *schleicheri*.

d) *G. terglouensis* subsp. *schleicheri* demonstrates relatedness to *G. brachyphylla* subsp. favratii which has to be separated from *G. brachyphylla* s.str.

e) *G. brachyphylla* s.str., *G. rostanii* and *G. bavarica* s.l. form a related group.

f) The Sierra Nevada population of *G. brachyphylla* and *G. pumila* subsp. *delphinensis* from southern French Alps are embedded within *G. verna.*

Genetic diversity and phylogeography of *Gentiana verna* L. (*Gentianaceae*) in European Mountain ranges inferred from AFLP

Abstract

Gentiana verna L. s.l. (spring gentian) has colonized most European mountain ranges and neighboring Asia. Based on an AFLP-study including four primer-combinations, a system of four genotypes was elaborated: a) the first having mostly Alpine and western Carpathian populations of *G. verna* L. s.str. and some southwestern Alpine populations of *G. pumila* Jacq. subsp. *delphinensis* Fourn.; b) a small genotype restricted only to the southwestern French and Italian Alps hosting *G. verna* L. s.str. and the above *G. pumila* Jacq. subsp. *delphinensis* Fourn.; c) a genotype restricted to the Cantabric Mountains of northern Spain influencing the Pyrenees, and d) a largely distributed cluster of diverse genotypes reaching from Dinaric Alps eastward through the Southern Carpathians and Balkan to the Turkish Pontic Alps home to *G. verna* L. s.str. as much as the outgroup taxa *G. verna* L. subsp. *tergestina* Hayek, *G. verna* L. subsp. *balcanica* Pritch. and *G. verna* L. subsp. *pontica* Hayek.

The distance between the Alpine genotypes including populations from Tatra Mountains and the diverse Eastern European and Asian outgroup genotypes illustrates the different origin of western and eastern spring gentians in Europe in large scale and the different colonization pathways within the Carpathians in small scale. While the western Carpathians are presenting also a western European genotype, the Southern Carpathians are hosting an Eastern genotype.

Pyrenees and Cantabric Mountains host a genotype different from the Alpine one indicating a certain distance, while northern Alps and Tatra Mountains (western Carpathians) get colonized by almost only one uniform genotype pointing toward a rapid colonization leading from a glacial refuge area. Only one small genotype in the southwestern Alps overlapping with taxon *G. pumila* Jacq. subsp. *delphinensis* Fourn. indicates a second and independent refuge area in that region.

Keywords
Gentiana, AFLP, genotype, population, Europe, ice-age, isolation

Introduction

Gentiana verna L. is a broadly distributed alpine species with a scattered area of occurrence from the Spanish Cantabric Mountains and Pyrenees, the British Islands to the central European Mountains like the Alps and Jura, but also the Apennines, the eastern European Dinaric Alps and Balkan Mountains, the Peloponnesus, and the Carpathians. In Asia the species is present in the Ural mountains, the Pontic Alps and the Caucasus as well as the western Altai Mountains.

G. verna L. is a 2 to 20 cm high plant with basal leaves rosettes and sterile stolons. Leaves are 8-20 mm in length and 3-8 mm in width, ovate, elliptic or lanceolate. Flowers are solitary, corolla 15-25 mm, dark blue with a whitish center and a tabuliform stigma closing the corolla tube. Corolla lobes are spreading 8-10 mm in length, ovate-orbicular to elliptic with small plicae of 1-2.5 mm. The angled and more or less winged calyx has 12-22 mm in length. Individuals from distant regions of the distribution area differ in overall plant size, length of corolla lobes, size of calyx wings and leaf shape. A large number of synonyms are known for that species imaging their long history in taxonomy. The populations from Dinaric Alps got identified by Soltokovic (1901) as *G. tergestina* respectively as *G. pontica* for population from Pontic Mountains, replaced later as *G. verna* L. subsp. *tergestina* respectively *G. verna* L. subsp. *pontica* by Hayek (1930). Pritchard (1977) identified *a G. verna* L. subsp. *balcanica* for plants from Balkan mountains. Plants from Dinaric Alps are generally taller and provide long, lanceolate leaves and a strongly winged calyx, while individuals from Pontic Mountains differ from the Alpine ones only by their broader leaves. There while types from Balkan Mountains provide strong winged calyxes compared to the Alpine.

The southwestern Alps is host to a type with generally smaller plants equipped with pointed lanceolate leaves treated as *G. verna* L. subsp. *delphinensis* by Kunz (1972) respectively as *G. pumila* subsp. *delphinensis* (Fournier 1938). For the Cantabric Mountains *G. verna* L. var. *willkommiana* Monts. & Vill. and *G. verna* L. var. *cantabrica* Romo (Montserrat and Villar 1975; Romo 1988) got postulated.

Our aim is it to enlighten the population history of the morphologically close related western European, Alpine and Carpathian plants and to show the relations to the eastern European and Asian populations.

nr	taxon	country	range	locality	coordinates/altitude	leg	herb. voucher	H_{sh}	evenness	A_f	P_f	F_p	F_r	R_M
1	v	E	CC	Peñas de Hachero	N 43°10’3.49’’, w 5°12’1.9’’, Alt: 1797 m	MH, MLC	NEU 398322	5.67	0.82	317	0.30	3	31.33	
2	v	E	CC	Rosada de Valedon	N 43°10’19.49’’, w 5°4’1.39’’, Alt: 1642 m	MH & MLC	NEU 398320	5.60	0.81	297	0.28	5	31.00	0.0426
3	v	F	P	Col de Puymorens	N 42°33’39.49’’, E 1°48’36.90’’, Alt: 1923 m	MH & MLC	NEU 398321	5.66	0.81	315	0.30	3	22.00	
4	v	F	P	H. de Héas	N 42°45’18.0’’, w 0°6’35.99’’, Alt: 2400 m	RD, MH	NEU 398297	5.70	0.82	329	0.32	5	24.67	
5	v	F	P	H. d’ Ossue	N 42°45’52.68’’, w 1°53’8.4’’, Alt: 2436 m	RD, MH	NEU 398293	5.63	0.81	311	0.30	8	26.67	
6	d	F	A	Col de Champs	N 44°10’14.22’’, E 6°42’1.44’’, Alt: 2096 m	MH	NEU 399292	5.63	0.81	303	0.29	0	18.67	0.0435
7	d	F	A	Vauplan	N 43°52’54.24’’, E 6°38’30.35’’, Alt: 1685 m	MH	NEU 398325	5.66	0.81	312	0.30	5	25.00	0.0393
8	v	F	A	Col de Restefonds	N 44°20’18.71’’, E 6°51’2.9’’, Alt: 2421 m	MH	NEU 399313	5.71	0.82	332	0.32	14	50.33	
9	d	F	A	Mtgne de Lachens	N 43°44’44.70’’, E 6°39’8.16’’, Alt: 1654 m	MH	NEU 399291	5.55	0.80	273	0.26	0	22.00	0.0647
10	d	F	A	Col de Festre	N 44°40’10.38’’, E 5°50’43.73’’, Alt: 1538 m	AL, MH	NEU 398317	5.84	0.84	383	0.37	4	31.33	
11	d	F	A	Mt. Ventoux	N 44°10’53.15’’, E 5°15’19.2’’, Alt: 1411 m	MH, AL	NEU 399301	5.67	0.82	319	0.31	1	14.67	
12	d	F	A	Mtgne de Lure	N 44°7’8.76’’, E 5°48’59.51’’, Alt: 1680 m	AL, MH	NEU 398316	5.72	0.82	338	0.32	1	17.33	
13	v	F	A	Col de Larche	N 44°25’21.53’’, E 6°53’29.87’’, Alt: 2013 m	MH	NEU 398405	5.68	0.82	321	0.31	3	21.00	0.0325
14	v	F	A	Col du Galibier	N 45°3’46.49’’, E 6°24’40.32’’, Alt: 2525 m	MH	NEU 399293	5.62	0.81	301	0.29	0	11.67	0.0386
15	v	F	A	Mt. Gondran	N 44°53’31.61’’, E 6°43’19.38’’, Alt: 2360 m	MH	NEU 398298	5.65	0.81	316	0.30	0	18.67	
16	v	F	A	Pt St.-Bernard	N 45°40’39.72’’, E 6°52’42.6’’, Alt: 2177 m	MH	NEU 398300	5.62	0.81	299	0.29	0	8.67	0.0372
17	v	F	A	Cormet de Roseland	N 45°40’35.76’’, E 6°40’28.13’’, Alt: 2237 m	MH	NEU 399311	5.57	0.80	282	0.27	0	10.67	0.0502
18	v	F	A	La Monta	N 44°45’0.0’’, E 7°0’0.0’’, Alt: 1500 m	MH	NEU 399305	5.71	0.82	336	0.32	1	18.00	
19	v	I	A	Mt. Mongioie	N 44°10’21.84’’, E 7°47’28.74’’, Alt: 2320 m	PK, MH	NEU 398295	5.64	0.81	306	0.29	2	17.67	0.0351
20	v	CH	J	Mont Tendre	N 46°36’0.55’’, E 6°19’8.98’’, Alt: 1620 m	MH	NEU 079440	5.66	0.81	314	0.30	1	15.00	
21	v	CH	J	Chasseral	N 47°7’28.83’’, E 7°23’42.53’’, Alt: 1560 m	MH		5.54	0.80	277	0.27	0	5.67	0.0426
22	v	CH	J	Weissenstein	N 47°9’59.99’’, E 7°31’59.99’’, Alt: 1280 m	MH		5.56	0.80	282	0.27	1	7.33	0.0398
23	v	CH	A	Gurnigel	N 46°42’39.25’’, E 7°27’23.82’’, Alt: 1720 m	MH	NEU 079803	5.56	0.80	285	0.27	0	8.00	0.0377
24	v	CH	A	Gasterental	N 46°27’39.67’’, E 7°40’50.73’’, Alt: 1370 m	MH	NEU 079800	5.58	0.80	290	0.28	1	7.33	0.0413
25	v	CH	A	Grindelwald	N 46°35’7.55’’, E 7°59’23.60’’, Alt: 2060 m	MH	NEU 079801	5.65	0.81	313	0.30	1	13.67	
26	v	CH	A	Zermatt	N 45°59’56.4’’, E 7°43’6.26’’, Alt: 2360 m	MH	NEU 079445	5.80	0.83	368	0.35	3	22.33	
27	v	CH	A	Binntal	N 46°22’45.48’’, E 8°16’7.98’’, Alt: 1960 m	MH	NEU 079791	5.53	0.80	272	0.26	0	16.00	0.0498
28	v	CH	A	Alp Languard	N 46°29’10.93’’, E 9°55’9.68’’, Alt: 2200 m	MH		5.59	0.80	288	0.28	0	11.67	0.0461
29	v	CH	A	Ofenpass	N 46°38’22.3’’, E 10°17’58.12’’, Alt: 2079 m	MH	NEU 079799	5.71	0.82	334	0.32	3	17.00	
30	v	I	A	Passo Sella	N 46°33’48.65’’, E 11°43’55.32’’, Alt: 2330 m	MH	NEU 398406	5.58	0.80	287	0.28	2	19.33	0.0364
31	v	A	A	Spullersee	N 47°9’21.9’’, E 10°4’12.29’’, Alt: 1841 m	MH		5.57	0.80	287	0.28	2	9.00	0.0386
32	v	D	A	Gaissalp	N 47°25’14.19’’, E 10°19’44.9’’, Alt: 1873 m	MH	NEU 399314	5.61	0.81	298	0.29	3	9.67	0.0348
33	v	D	A	Hochbrett	N 47°35’2.90’’, E 13°2’53.40’’, Alt: 2317 m	MH	NEU 398301	5.63	0.81	307	0.29	2	12.33	
34	v	A	A	Hochschwab	N 47°36’58.70’’, E 15°10’44.89’’, Alt: 1621 m	MH	NEU 398302	5.59	0.81	294	0.28	1	14.67	0.0371
35	v	A	A	Gartnerkofel	N 46°34’19.40’’, E 13°17’49.9’’, Alt: 1864 m	MH	NEU 398323	5.64	0.81	312	0.30	0	12.67	
36	v	A	A	Dobratsch	N 46°35’43.9’’, E 13°43’13.9’’, Alt: 1617 m	MH	NEU 399309	5.63	0.81	309	0.30	1	10.00	
37	v	A	A	Raxalp	N 47°41’15.49’’, E 15°42’53.49’’, Alt: 1346 m	GK, MH	NEU 398296	5.63	0.81	309	0.30	2	15.00	
38	v	SLO	A	Triglav Mts.	N 46°22’8.20’’, E 13°52’7.30’’, Alt: 1574 m	MH	NEU 398324	5.54	0.80	279	0.27	0	13.00	0.0420
39	v	SLO	A	Mangart	N 46°26’1.89’’, E 13°38’16.59’’, Alt: 2108 m	MH	NEU 399308	5.66	0.81	315	0.30	1	14.67	
40	t	SLO	D	Mt. Čaven	N 45°55’48.24’’, E 13°51’31.73’’, Alt: 1253.00 m	AL, MH	NEU 398271	5.58	0.80	283	0.27	3	20.00	
41	v	I	A	Mt. Majur	N 46°12’43.80’’, E 13°31’45.60’’, Alt: 1638 m	AL, MH	NEU 398272	5.54	0.80	280	0.27	1	17.00	
42	t	MNE	D	Cakor Pass	N 42°39’43.19’’, E 19°59’31.0’’, Alt: 1611 m	MH	NEU 398276	5.63	0.81	305	0.29	0	23.67	0.0408
43	t	HR	D	Risnjak	N 45°28’17.39’’, E 14°37’1.90’’, Alt: 975 m	MH		5.47	0.79	263	0.25	1	21.67	
44	b	GR	B	Timfi Mts.	N 39°57’11.20’’, E 20°51’23.20’’, Alt: 2025 m	MH	NEU 398267	5.70	0.82	326	0.31	3	23.67	
45	b	GR	B	Olympos Mt.	N 40°4’37.20’’, E 22°22’14.60’’, Alt: 2256 m	MH	NEU 398268	5.60	0.81	294	0.28	5	33.00	0.0511
46	v	PL	C	Western Tatra Mts.	N 49°15’49.99’’, E 19°56’32.99’’, Alt: 1300 m	M&AR		5.66	0.81	317	0.30	1	14.67	
47	v	PL	C	Tatra Mts.	N 49°14’0.0’’, E 19°59’0.0’’, Alt: 1952 m	ZM		5.66	0.81	316	0.30	4	18.67	
48	v	RO	C	Munții Bucegi	N 45°26’7.99’’, E 25°27’14.99’’, Alt: 2407 m	M&AR		5.74	0.83	345	0.33	2	29.00	
49	v	RO	C	Munții Latoriței	N 45°24’45.0’’, E 23°47’45.0’’, Alt: 1870 m	M&AR		5.56	0.80	282	0.27	1	31.33	0.0453
50	v	RO	C	Munții Fagaraşului	N 45°35’0.0’’, E 24°45’0.0’’, Alt: 2200 m	LN		5.80	0.84	365	0.35	2	31.33	
51	v	RO	C	Munt Caraiman	N 45°25’59.99’’, E 25°27’59.99’’, Alt: 2250 m	LN		5.52	0.80	275	0.26	5	22.00	
52	p	TR	P	Ilaz Dağ	N 41°4’4.80’’, E 33°46’56.49’’, Alt: 2084 m	MH	NEU 398318	5.50	0.79	261	0.25	3	22.00	0.0584
53	p	TR	P	Zigana Pass	N 40°38’45.29’’, E 39°23’41.99’’, Alt: 2156 m	WI, MH	NEU 398319	5.61	0.81	300	0.29	1	26.67	0.0368

Table 1: Populations included. Taxon: d = *G. pumila* Jacq. subsp. *delphinensis* Fourn., v = *G. verna* L. s.str., t = *G. verna* L. subsp. *tergestina* Hayek, b = *G. verna* L. subsp. *balcanica* Pritch., p = *G. verna* L. subsp. *pontica* Hayek.. Country: E = Spain, F = France, I = Italy, CH = Switzerland, D = Germany, A = Austria, SLO = Slovenia, MNE = Montenegro, HR = Croatia, GR = Greece, PL = Poland, RO = Romania, TR = Turkey. Range: CC = Cantabric Mountains, P = Pyrenees, A = Alps, J = Jura, D = Dinaric Alps, B = Balkan, C = Carpathians, P = Pontic Alps. Collectors were: AL: A. Lendel, GK: G. Karrer, LN: L. Nussbaum, MH: M. Haemmerli, M&AR: M. & A. Ronikier, MLC: M.-L. Cheung, PK: P. Küpfer, RD: R. Deutschle, WI: W. Imhof. H_{SH}: Shannon diversity, A_f = total number of fragments present; P_f = proportion of fragments polymorphic within the population; F_p = number of fragments private to that population; F_r = mean number of rare fragments (rare = common to 10% or less of individuals); R_M = Mantel correlation ($P < 0.05$).

Materials and Methods

Fresh leaf material was collected by respecting a minimal distance of 1 m between two samples of the same population to prevent from sampling twice on the same individual. From each population 5 to 10 individuals were collected and dried on silica gel. Of 3 samples per population randomly chosen 20 mg of it was grounded using Tungsten Carbide beads and Qiagen TissueLyser (Qiagen, Basel). A DNA extraction was performed using DNEasy Plant Mini Kit (Qiagen, Basel), leading into about 50ng total DNA per µl extract. 40ng of extracted DNA was double digested with 2U MseI and 8U EcoRI (New England Biolabs, Ipswich MA) in a total volume of 20µl 1x NEB2 buffer for 3 hours at 37°C, followed by an inactivation step of 20min at 68°C. 5pmol of both EcoRI- and MseI-adapter as described by (Vos and Zabeau 1995) were linked to the digestion product using 0.5U T4 DNA Ligase (Promega, Madison) in 25µl of liquid containing 1x T4 Ligase buffer for 3 hours at room temperature. A preselectif PCR was performed based on 1µl of the above ligation product with 0.25U GoTaq (Promega, Madison) in 1x GoTaq buffer with additional $MgCl_2$ to a total concentration of 2mM, 200µM of each dNTP, 200µM of each dNTP, 200µM of each primer EcoRI-A and MseI-C as described by Vos and Zabeau (1995) in a total volume of 10µl. The procedure was carried out in a Biometra T3000 thermocycler (Biometra, Göttingen) by performing a 2min initial denaturizing at 94°C, followed by 28 cycles: 45s denaturizing at 94°C, 45s annealing at 56°C, 1min extension at 72°C and completed by a final extension step of 10min at 72°C. The amplification product was diluted 20 times in water.

Selective PCR were performed with 2.5µl diluted amplification product, together with 0.25U GoTaq (Promega, Madison), 1x GoTaq buffer with additional $MgCl_2$ to a total concentration of 2mM, 250µM of each dNTP, 250µM of EcoRI-ANN- and 300µM of MseI-CNN-primer (Vos and Zabeau 1995) in a total volume of 10µl of liquid. PCR-conditions were 2min initial denaturizing at 94°C, followed by 13 cycles: 30s denaturizing at 94°C, 30s annealing at 65°C, 1min extension at 72°C with a decrease in annealing temperature of 0.7°C per cycle, followed by 23 cycles: 30s denaturizing at 94°C, 30s annealing at 56°C, 1min extension at 72°C of and completed by a final extension step of 5min at 72°C in a Biometra T3000 thermocycler (Biometra, Göttingen). Primer pairs used were EcoRI-ACA/MseI-CAT, EcoRI-ACT/MseI-CTT, EcoRI-AGA/MseI-CAT, and EcoRI-AGT/MseI-CAC. EcoRI-ANN-primers were marked with FAM at their 5'-end (Microsynth, Balgach Switzerland).

The GeneScan application was carried out on ABI Prism 310 and 3100 Genetic Analyser machines (Applied Biosystems Switzerland, Rotkreuz). Therefore 0.5µl of product from selective amplification was diluted in 12µl HIDI formamide (Applied Biosystems Switzerland, Rotkreuz) and additional 0.5µl of GS500 ROX size standard (Applied Biosystems Switzerland, Rotkreuz), heated for 2min at 94°C and immediately cooled down on ice. Raw data was treated in GeneScan 3.7 software (Applied Biosystems Switzerland, Rotkreuz) and transformed into a binary presence/absence matrix. Therefore all peaks in electropherograms from ABI 310 and 3100 scanning procedure were standardized with the proportion of surface under that peak to the total of surface under all peaks in one sample. That way a virtual uniformity of all samples in regard of their overall fluorescence intensity was achieved. All signals of one only primer combination each were ranked according their fragment length. Resulting table was scanned for non overlapping intervals with a length of in general not more than 2 bp (maximum length 3.29 bp, 97.1% quantile 1.99 bp, mean length 0.58 bp, SE = 0.57 bp) as base for AFLP markers. To separate between background noise and real signals a threshold for a minimal peak high of 1.45 units was defined. All signals below that limit weren't taken in account, same as all peaks below 75 and above 350 bp. The threshold of 1.45 units as optimal parameter was established in a simulation by calculating the quotient of minimal evolution (ME) score of a NJ tree divided by the sum of Jaccard distances of the underlying data matrices filtered with different thresholds respective. The most optimal NJ tree represented by the lowest quotient was found by applying the above value. From standardized and filtered signals as above a presence/absence matrix was designed for AFLP markers as described.

Data Analysis

Shannon diversity was calculated for each population as $H_{SH} = -\Sigma(p_i*\ln(p_i))$ where p_i was the relative frequency of the i-th AFLP-Fragment. Evenness was determined as $J = H_{SH}/\ln(q)$ where q represented the overall number of different fragments (Legendre and Legendre 1998; Clote and Backofen 2000). As another diversity measure, the number and distribution of private fragments F_P per population (fragments confined to a single population), as well as rare fragments F_R (fragments confined to 10% or less of the individuals in the entire data set) were counted. In addition we counted the total numbers of different fragments present A_F in each population, and the proportion of polymorphic fragments P_F. To access the relationship of H_{Sh}, F_R, P_F and A_F to the altitude of the populations, Pearson correlations were calculated on R package (R 2006).

Principal component analysis (PCA) (Gower 1966) based on the Jaccard distances among individuals was conducted on the R package (Becker, Chambers et al. 1988; Chambers and Hastie 1992; R 2006).

Mantel tests (Mantel 1967) were done to quantify the correlation based on Jaccard distances between groups of individuals as function of an inter-individual distance. For all Mantel tests, the normalized Mantel statistic R_M was calculated. Analysis were done a) to access the goodness-of-fit to the clusters as derived from PCA and AMOVA analysis and b) population-wise by comparing genetic by comparing all 3 samples of one population (Stehlik, Schneller et al. 2001. All mantel correlations were calculated on R package (Becker, Chambers et al. 1988; Chambers and Hastie 1992; R 2006)

An analysis to detect ancestral patterns was performed on Structure 2.0 (Pritchard, Stephens et al. 2000) by adapting an evolutionary model assuming admixture between populations, based on diploid individuals. 20'000 MCMC repetitions following 5'000 posterior burnin repeats were simulated, each in 100 replicates for 2, 3, or 4 ancestral genotypes.

A genetic distance between populations was calculated according Nei (1972) on R package. The matrix of population distances was analyzed by neighbor joining (NJ, Saitou and Nei 1987) in PAUP (Swofford 1998).

Results

Genetic diversity

1040 clear fragments were derived from 4 AFLP primer combinations within the scanning range of 75 to 350 bp. Each 3 samples were from one of totally 53 populations. Due to the high polymorphism each sample represents an independent genotype.

Shannon index D and evenness H are comparable in all populations. More resolution is produced by the total number of fragments present A_F and the proportion of Fragments polymorphic within the population P_F. A_F varies from 261 to 383 (mean A_F = 306, SE = 25.64) different fragments and shows no obvious correlation to geographical distribution. P_F covers a range from 0.250 to 0.367 (mean P_F = 0.293, SE = 0.0247), where the highest numbers represent mostly eastern and south-western populations (P_F >= 0.35 in: 26-Zermatt, 10-Col de Festre, 50-Munții Fagaraşului). The number of fragments private to a single population F_P reaches from 0 to 14 (mean F_P=2.09, SE = 2.436, central F_P = 1). Populations with 4 or more private fragments (4 ore more in: 47-Tatra Mts., 10-Col de Festre, 51-Munt Caraiman, 45-Olympos Mt., 2-Rosada de Valedon, 7-Vauplan, 4-Hourquette de Héas, 5-H. d' Ossue, 8-Col de Restefonds) are illustrating a concentration within eastern outgroup populations and the western European Cantabric/ Pyrenean resp. southwestern Alpine populations. The mean number of rare fragments (fragments common to 10% of the individuals or less) F_R within a range of 5.67 to 50.33 (mean F_R = 19.06, SE = 8.459, central F_R = 18.00) illustrates a almost equivalent distribution as above with values above the mean for populations from far western Europe and the southwestern Alps as well as for the Dinaric Alps, Balkan Mountains, Southeaster Carpathians and the Asian Mountain ranges except relatively high value also in the Alpine population 26-Zermatt. No significant correlation between D (respectively A_F P_F, F_R) and elevation as well as geographic position (Alps vs. outside the alps, alps and westward vs. eastern mountains, alps and Jura vs. far western and eastern mountains) except rare fragments F_R to the third geographical distribution set (r = -0.524, P < 0.001).

Variation and Cluster analysis

The overall mantel analysis based on pearson correlation of Jaccard distances between individuals versus their geographic distance points toward a correlation between these two measures (R_M = 0.4399, P < 0.001).

Two dimensional PCA (26.83% of total variation along axis 1, 13.61% of total variation along axis 2) of all individuals derived segregation into 4 different main groups as shown in fig. 1. All populations were clearly integrated within one unique group. Group A and B are distant from the others two (C and outgroup). Group A hosts beside the western Carpathian populations (46-western Tatra Mts and 47-Tatra Mts.) all Alpine populations except 6-Col de Champs, 7-Vauplan, 8-Col de Restefonds, 13-Col de Larche, 18-La Monta and 19-Mongioie. Population 8-Col de Restefonds is placed together with outgroup taxa, all others excluded from group A are member of the southwestern Alpine group B. Group C comprising of all populations from Cantabric Mountains and Pyrenees is closely linked to a large cluster of outgroup taxa. That last one covers beside all plants from Asia, the Balkan Mountains and the Dinaric Alps also all populations from the Southeastern Carpathians (48-Munții Bucegi, 49- Munții Latoriței, 50- Munții Fagaraşului and 51.Munt Caraiman), but also the above mentioned 8-Col de Restefonds.

Mantel analysis of groups as above derives R_M as given in tab. 2. Group A and B provide a significant positive internal correlation (A: R_M=0.658, B: R_M=0.658, P < 0.001). In group C only plants from Cantabric Mountains correlate weakly positive to their self (R_M=0.061, P<0.005). Outgroup taxa are significantly negative correlated to their self (R_M=-0.164, P<0.001). Alpine group A also correlates to all other groups in a strong positive way (to Group C only if plants from Cantabric Mountains and Pyrenees become separated, maximum R_M=0.0.709, minimum R_M=0.578). Correlation between group A and outgroup taxa is weaker, but still significantly positive (R_M=0.173, P<0.001). Same as group A does southwestern Alpine group B (no correlation to Pyrenees and negative correlation to outgroup taxa) but R_M are much lower than above(B-B: R_M=0.134, B-$C_{\text{Cantabric Mts}}$ R_M=0.091, P<0.001 rsp. P<0.005). Correlation of B to outgroup taxa is significantly negative (R_M=-0.194, P<0.001). Group C draws the image of a relative diverse cluster with no self correlation overall, but a significant but weak correlation between plants from Cantabric Mountains (R_M=0.061, P<0.005) as well as significant negative correlations to outgroup taxa (R_M=-0.205 to -0.255, P<0.001) for all populations as also for both individual subgroups.

A separate PCA was done for all outgroup taxa except the outstanding population 8-Col de Restefonds. Fig. 2 illustrates the first 3 dimensions explaining 18.84%, 14.67% and 13.01% of overall variation within that group by resulting into widely dispersed populations and no strong structure. Mantel analysis was done by separating following geographic subgroups: I: Dinaric Alps (40- Mt. Čaven, 42-Cakor Pass and 43-Risnjak), II: Balkan Mountains (44-Timfi Mts. and 45-Olympos Mt.), III: Southern Carpathians (48- Munții Bucegi, 49- Munții Latoriței, 50-Munții Fagarașului and 51-Munt Caraiman) and IV: Asian Mountains (52- Ilaz Dağ and 53-Zigana Pass). The only significant Mantel value received was a self-correlation in subgroup IV (R_M=0.3958, P<0.001).

AMOVA analysis was performed a) for all groups as defined above including also outgroup taxa and b) only for populations within group A, B and C as shown in fig. 1. The overall analysis (a) proposes 15.92% of total variation explained through the order between groups while the analysis excluding outgroup taxa (b) provides for the same measure a higher value of 16.96%. Overall analysis derives 21.46% of total variation explained through the order between populations and the remaining 62.62% through the order within populations. The analysis without outgroup taxa explains more variation through the order within populations (63.98%) while the order between populations has a lower impact (19.07%) as above. AMOVA analysis was leading into significant variation percentages with P < 0.001 for all values.

<u>Structure analysis</u>

Structure analysis was performed spring gentians excluding outgroup taxa (Southern Carpathians, Dinaric Alps, Balkan Mountains and Turkey) as well as the outstanding population 8-Col de Roseland.

In a preliminary analysis the evolution under the existence of 1 to 5 ancestral genotypes were simulated proposing a minimal posterior probability for 1 ancestral and a maximal posterior probability for 5 ancestral populations reflecting the ability of the applied clustering method to split even closely related ancestral pattern into parts basing on smallest differences (Pritchard, Stephens et al. 2000). A model assuming 5 genotypes was resulting each in series of different outcomes with no clear dominance of a particular distribution pattern. A structure model assuming 2 distinct ancestral genotypes ended in 100 identical outcomes out of 100 runs proposing genotypes for i) Cantabric Southwestern/Pyrenees/southwestern Alps and ii) Alps, providing an average Ln Likelihood =-64803.3 (σ^2=4.070, N=100). A structure model assuming 3 distinct ancestral

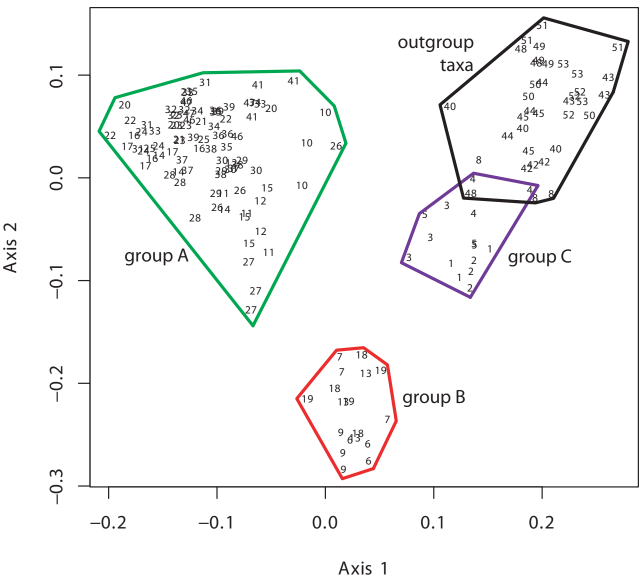


Figure 1: Results from PCA analysis upon Jaccard distance between individuals. Axis 1 represents 26.83%, Axis 2 13.61% of total variation. Numbers represent population identifiers as indicated in tab. 1

Group	A	B	C	C, only Cantabric Mts.	C only Pyrenees	outgroup taxa
A	0.658	0.709	---	0.613	0.578	0.173
B	0.709	0.134	---	0.091*	---	-0.194
C	---	---	---	---	---	-0.255
C, only Cantabric Mts.	0.613	0.091*	---	0.061*	---	-0.203
C, only Pyrenees	0.578	---	---	---	---	-0.225
outgroup	0.173	-0.194	-0.255	-0.203	-0.225	-0.164

Table 2: Mantel correlations R_M (P<0.001, P<0.005 if marked with *) for groups as defines in fig. 1. Only values with significant support are shown, dashed fields show no significant correlation.

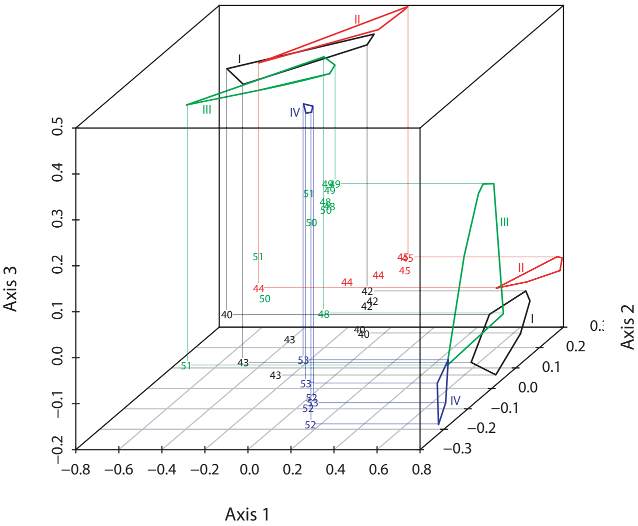


Figure 2: Results from PCA analysis upon Jaccard distance between individuals from outgroup taxa and *G. verna* L. from Southern Carpathians. Arab numbers represent populations as indicated in tab. 1, latin numbers represent groups as described.

N of groups	Source of variation	df	MS	absolute variance	percentage of variance
Groups A, B, C and outgroup					
4	among groups	3	1928.74	16.02	15.92
	among populations	49	6263.84	21.60	21.46
	among individuals	106	6680.67	63.03	62.62
Groups A, B, C without outgroup					
3	among groups	2	1086.10	16.22	16.96
	among populations	38	4403.98	18.24	19.07
	among individuals	82	5017.33	61.19	63.98

Table 3: Results from AMOVA analysis upon groups as defined in fig. 1.

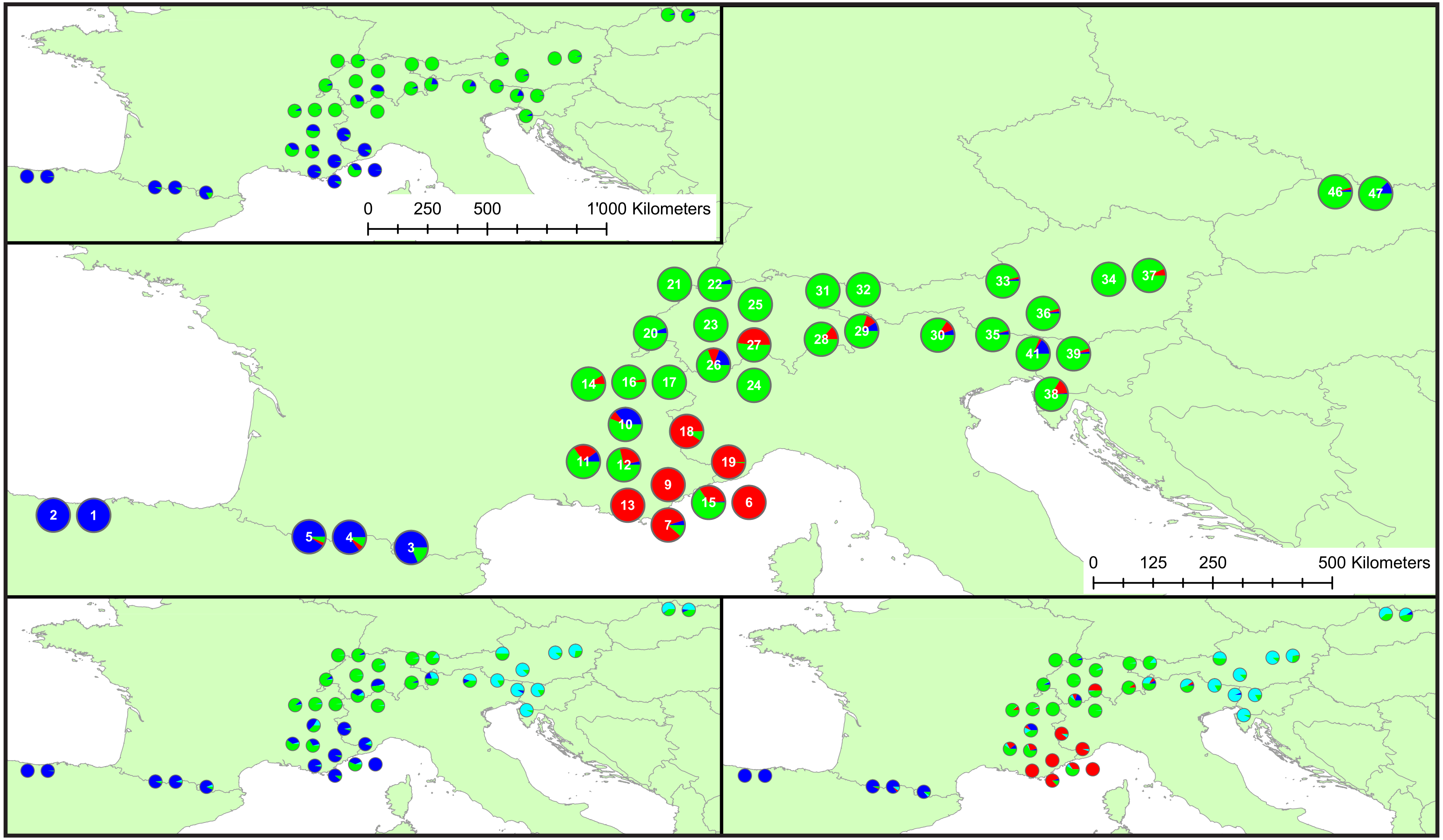


Figure 3: Genotype dispersal as calculated through structure analysis. Outgroup taxa as well as outstanding population 8-Col de Restefonds and *G. verna* L. from Southern Carpathians were not considered. Upper left: model 2 assuming 2 ancestral genotypes; center: model 3a assuming 3 ancestral genotypes; lower left: model assuming 3 alternative ancestral genotypes; lower right: model 4 assuming 4 ancestral genotypes. Models 2 and 4 base on each 100 simulations, 3a on 70 simulations, 3b on 30 simulations. Colors are corresponding to colors used as in fig. 1 and 4. Numbers within the central map refer to population numbers as given in tab. 1.

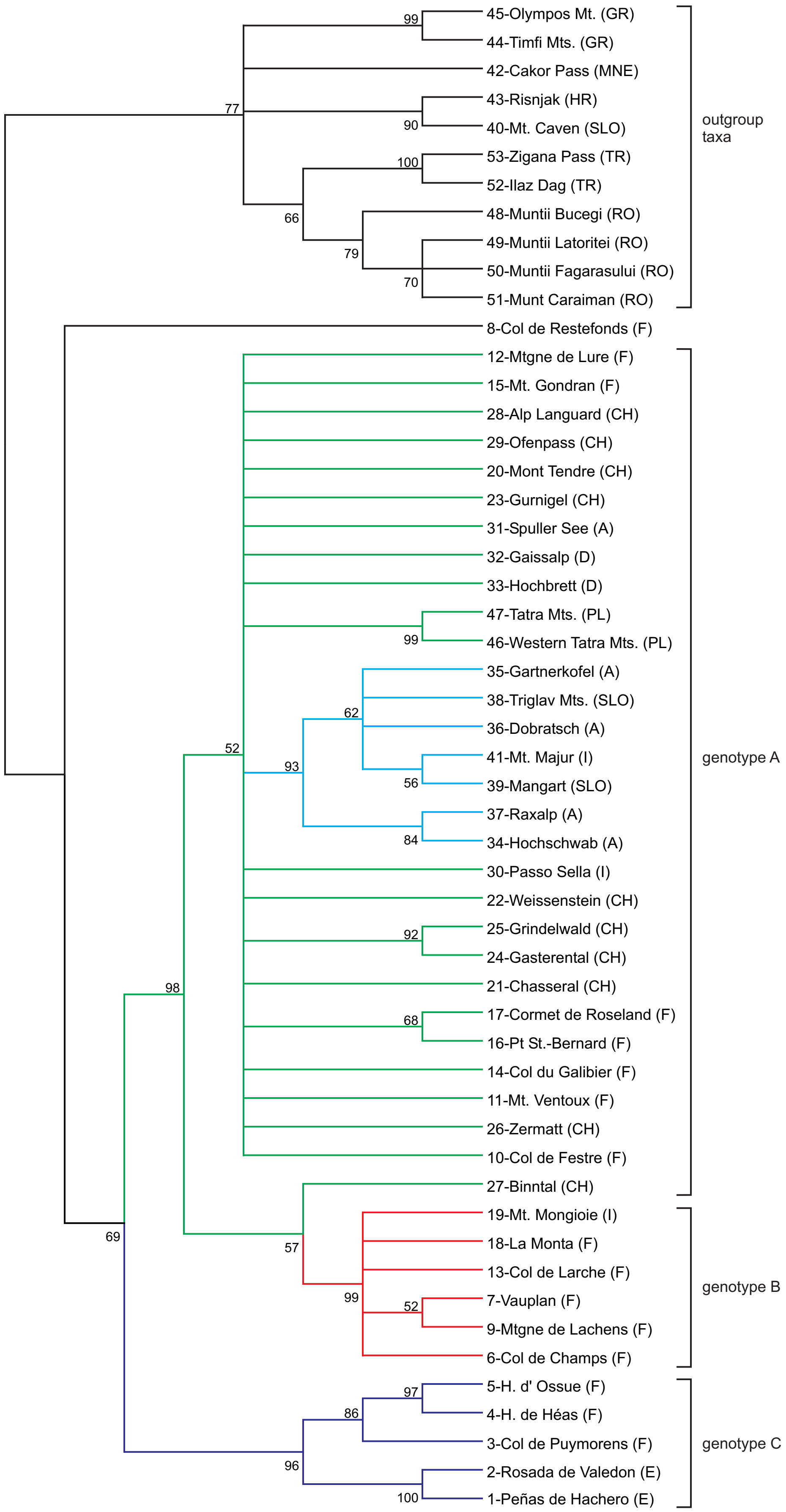


Figure 4: NJ tree upon Nei population distances (Nei 1972). Clusters are named corresponding to outcomes of PCA analysis (fig. 2). Bootstrap supports basing on 1000 replicates are indicated.

genotypes resulted in a) 70 identical outcomes out of 100 runs proposing genotypes for i) Cantabric Mountains/ Pyrenees, ii) Alps and iii) southwestern Alps by providing an average Ln Likelihood =-61718.0 (σ^2=4.666, N=70) and b) 30 identical outcomes out of 100 runs proposing genotypes for i) Cantabric Mountains/Pyrenees/southwestern Alps, ii) Northwestern Alps and iii) Eastern Alps while providing an average Ln Likelihood =-62586.7 (σ^2=3.626, N=30). A structure model assuming 4 distinct ancestral genotypes resulted in identical outcomes, providing an average Ln Likelihood =-59527.6 (σ^2=6.627, N=100) and into genotypes as followed: i) Cantabric Mountains/Pyrenees, ii) Northern/Eastern Alps, iii) Pyrenees/southwestern Alps and iv) southwestern/Eastern Alps. An alternative but rare model (11 outcomes within 200 runs) with Ln likelihood =-60146.2 (σ^2=10.876, N=11) separated between i) Cantabric Mountains, ii) Pyrenees, iii) southwestern Alps and iv) Alps. This last model seems to be less favorable due to its low likelihood score and is therefore not further treated, but it supports a split between Cantabric Mountains and Pyrenees as shown also in Mantel analysis. Model 3a is therefore derived from model 2 (dividing between Cantabric/Pyrenean/western alpine and alpine populations) by inserting an additional split to separate Cantabric/ Pyrenean from western Alpine populations. Only one population (27-Binntal) is not clearly dominated by one only genotype. In model 3b such an additional separation is done between Central and Eastern Alps. The southwestern Alpine Genotype in model 3a is identical to Group B from PCA analysis meanwhile the Eastern Alpine genotype from Model 3b represents a subgroup of group A with significant bootstrap support in NJ tree reconstruction. Model 3b seems to be less favorable compared to 3a due to its lower likelihood score. Model 4 is a fusion of both model 3a and 3b by proposing genotypes by proposing a number of populations show no clear dominance of one only genotype. One genotype within model 4 has no dominance in a particular population but is represented as minority compound within the southwestern and eastern Alps.

Assuming 3 potential initial genotypes and a distribution as proposed by model 3a, all individuals showed a proportional ancestry fitting to outcomes of PCA analysis and NJ tree reconstruction by indicating a dominance of a most western genotype C for populations from Cantabric Mountains (1-Peñas de Hachero and 2- Rosada de Valedon) as well as for the Pyrenees (3-Col de Puymorens, 4-H. de Héas and 5-H. d' Ossue). The Pyrenean populations are consisting also minor influences of Alpine genotypes A and B. Within southwestern Alps also structure analysis locates a distinct genotype B within populations 6-Col de Champs, 7-Vauplan, 9-Mtgne de Lure, 13-Col de Larche, 18-La Monta and 19-Mt. Mongioie, identical to PCA analysis and NJ tree reconstruction.

Distance analysis

NJ clustering on population level and bootstrap analysis result in some branches with significant bootstrap support. The NJ tree supports the groups already proposed by PCA analysis. Both group B and C are monophyletic with significant support at their base (bootstrap values of 99% rsp. 96%). Group C covers therefore populations from the northern Spanish Cantabric Mountains and the Pyrenees. Group B from NJ analysis corresponds to the one from PCA and covers some of the southwestern European populations. Group A hosting the Alpine populations of *G.* verna L. as in PCA is also home of group B which is inserted on a branch of that first one. Samples of group A and B have a common root with a bootstrap support of 98%. Closest to group B stands population 27-Binntal from the Central Swiss Alps. Population 8-Col de Restefonds is clearly separated from all other *G. verna* L.-populations.

Outgroup taxa miss a significantly supported common root. Regional grouping if proposed for plants from Balkan Mountains (44-Timfi Mts. And 45-Olympos Mts.) and for Turkish populations (52-Ilgaz Dag and 53-Zigana Pass). Populations from Dinaric Alps as well as for Southern Carpathians provide no further regional structuration with significant support in bootstrap analysis.

Discussion

Genetic patterns within the Alpine chain

The Alpine populations of *G. verna* L. represent two distinct genotypes. These are 1) an Alpine and northern Carpathian genotype called A before and 2) a southwestern Alpine genotype called B. While the first genotype A with the western Carpathians also an extra Alpine range, genotype B is fully restricted to the Alpine chain. The central to southern populations 26-Zermatt and 27-Binntal are implicating the existence of further genotypes within the southern Alpine Chain due to their position in NJ tree reconstruction and the outcome of structure analysis.

Individuals of genotype A are strongly correlated to each other on a significant level, in contrast to other studies that propose a higher number of genotypes for that region as Füchter (2000) for Draba aizoides, Stehlik, Schneller et al. (2001) for *Eritrichium nanum* or Mátyás and Sperisen (2001) for Quercus spp.. From all three applied approaches we get evidence of a closer connection between eastern Alpine and western Carpathian populations. His south-western limit reaches genotype A on the separation line between Alps and Dinaric Alps as defined in Aeschimann and Lauber (2004). Southern French and the western Italian Alps genotype A overlap with the other two genotypes present.

Genotype B includes populations from the French population 18-La Monta south wards to the Mediterranean Sea and from 9-Mtgne de Lachens in the west till 10-Mt. Mongioie in the east. The genotype is well defined as much in PCA analysis with significant internal mantel correlation as also in distance analysis with significant bootstrap support.

The third and fourth genotypes C and outgroup are only represented as minor compounds in some western and eastern populations as shown in structure analysis. PCA as well as also distance analysis support only the existence of the two major genotypes A and B within the Alps as described. Nj tree reconstruction upon Nei population distance gives a certain indication for a closer related Eastern Alpine subtype within the Alpine Genotype with significant basal support, while further resolution is missing within the rest of the Alps. Population 8-Col de Restefonds is somehow outstanding in all approaches if even included. We decide to do no further considerations on these plants since a possible confusion with a distinct taxon or a contamination can't be excluded

Genetic pattern within western European ranges

The populations of Pyrenees and Cantabric mountains are part of genotype C. Mantel analysis only proposes a correlation between populations from Cantabric Mountains, but no overall correlation within that genotype. While Structure analysis turns out an almost 100% presence of genotype A within populations from Cantabric Mountains, the Pyrenees possess some introgressions of Alpine genotypes. The intermediate geographic positions of the Pyrenees in between the Cantabric Mountains and the Alps supports this effect and leads to the hypothesis of the Cantabric Mountains being origin or refugial zone for genotype C.

Genetic pattern within eastern mountain ranges

In Eastern Europe and Turkey outgroup taxa are present except within the western Carpathians were genotype A was found as described above. Despite the problems of missing correlation within the outgroup taxa, PCA analysis supports the existence of different distinct types within these populations. Clearly recognized is a largely distributed genotype covering the two Turkish populations 52-Ilgaz Dag and 53-Zigana Pass. Other genotypes within the outgroup taxa are resulting from PCA analysis but are missing significant positive autocorrelation in Mantel analysis.

Isolated within the eastern European mountain ranges are the western Carpathians representing a link to the Alpine chain as mentioned above. While the rest of the eastern mountains host a genotype different from the one in the Alp, that later one is also present in Tatra Mountains. Comparable patterns have been found by Konnert and Bergmann (1995) for *Abies alba* Mill. or by Dumolin-Lapegue, Pemonge et al. (1997) for *Quercus spp*. in opposite to Schonswetter, Paun et al. (2003) who proposed a genetic isolation of western Carpathian populations of *Rancunculus glacialis* L. from the Alpine ones.

General considerations

A general survey of genetic variability in context of geographic distribution over Europe reflects the post ice-age re-colonization patterns and hybridization zones as proposed by Hewitt (2000) for tree animal species. Beside the 2 distinguishable genotypes identified within the Alps as mentioned above, we are able to separate for *G. verna* L. in Europe another western genotype restricted to the Pyrenees and the Cantabric Mountains as explained in detail above. While the Alps and the Carpathians provide more than one distinct genotype, the Pyrenees and Carpathians, but also the Dinaric Alps and the mountains eastward host only one genotype (in case of structure analysis: dominant genotype). The genetic isolation of the far western mountains is in relation to their topographic isolation, separated from the Alps a basin of comparable low altitude. The presence of *G. verna* L. in the Auvergne in between the Alps and the Pyrenees as indicated in Fournier (1938) could point toward a link in the past between the Alps and the far western Mountains, although we don't find an evidence therefore in our data. The French Central Massif between the Alpine chain and the Pyrenees where the Auvergne is part of it, is of moderate altitude with low limit forest. Its isolated high peaks harbor considerable relict alpine flora that dates from a pre-glacial colonization (Braun-Blanquet 1923).

The relations as showed between far western and far eastern genotypes complies to Schonswetter, Paun et al. (2003) who proposed for populations of *Rancunculus glacialis* L.: a relatedness between far eastern, far western and northern types while some types out of the Alps represent a distinct type. The two poles of distribution reflect potential refuges as postulated by Taberlet, Fumagalli et al. (1998). Respecting the genetic distance between such refuges, the postulation of two distinct genotypes for the far western and the far eastern populations is preferred against just one common type flanking the Alpine one on both sides. Beside these two extra alpine refuges, within the Alps two genotypes, one covering the northern and central Alps as much as the Jura Massif and the second one restricted to the south-western Alps, were discovered.

Genotype A hosts *G. verna* L. s.str. and in two populations also *G. pumila* Jacq. subsp. *delphinensis* Fourn., the other individuals that later one built the central part of Genotype B. In Genotype A the populations identified as *G. pumila* Jacq. subsp. *delphinensis* Fourn. are grouped with southern Alpine *G. verna* L. s.str. specimen in distance analysis. The definition of two distinct genotypes for the alpine chain has therefore a reflection in taxonomic and morphological considerations. The South-western Alps host plants rather smaller in all parts and providing very narrow leaves (Müller 1982; Halda and Haldovâa 1996; Ho and Liu 2001) in the opposite to the broad leaved and tall spring gentians from the northern Alps and the Jura, overlapping with the patterns represented by genotype B and A.

Genotype B is isolated within a small area in the southwestern French and Italian Alps, comprising of individuals from taxa *G. verna* L. s.str. and *G. pumila* Jacq. subsp. *delphinensis* Fourn. Müller (1982) treated the second taxon as subspecies of the first. Rouy, Foucaud et al. (1893) declared a *G. verna* L. var. *villarsiana* and Villars (1787) even used the name *G. pumila* for that second taxon. As *G. pumila* Jacq. subsp. *delphinensis* Fourn. is also present in the neighboring populations member of genotype A, it is perfectly mixed with *G. verna* L. s.str. and limited to the southwestern French Alps, representing a morphological variation of *G. verna* L. that can't be separated from that taxon using AFLP markers.

Outgroup taxa are home to south eastern Europe including as much as to Asia (represented by Turkey), including also the southern Carpathians. Within that region we deal with *G. verna* L s.str. as much as with *G. verna* L. subsp. *tergestina* Hayek, *G. verna* L. subsp. *balcanica* Pritch and *G. verna* L. subsp. *pontica* Hayek. Our result turn out a heterogeneous cluster hosting different distinct genotypes where we miss enough sample material to provide more resolution. Genotype C is closest related to the cluster of outgroup taxa and together they form a bracket around the Alps hosting much more distinct genotypes. That last genotype C from Pyrenees and Cantabric Mountains covergi *G. verna* L. s.str. as much as the Pyrenean *G. verna* L. var. *willkommiana* Monts. et Villar and the Cantabric *G. verna* L. var. *cantabrica* Romo. *G. verna* L. var. *willkommiana* Monts. et Villar (Montserrat and Villar 1975) gets described as plant with shorter leaves than the typical form, providing a shape somehow similar to *G. brachyphylla* Vill. s.str.. Same is the situation for *G. verna* L. var. *cantabrica* Romo.

G. verna L. and its subspecies including *G. pumila* Jacq. subsp. *delphinensis* Fourn. demonstrate a complex dispersal on the European continent. Beside the populations on the British Islands, the Apennine Mountains not included in that study we deal with a large eastern European and Asian taxa distinct from *G. verna* L. s.str. covering the Pontic Alps and westward finally including the Dinaric Alps and northward the southern Carpathians. Their closest neighboring genotype based on results from PCA analysis occupies the Cantabric Mountains and the Pyrenees forming large distance east west dispersal. A recent migration over the intermediate Alpine chain without leaving traces that mountain chain seems to be fairly unlikely. That east-west-dispersal seems to be the remaining patterns of an initial colonization of these mountain ranges with the taxa presented and later interrupted within central Europe by extinction and isolation mechanisms somehow parallel to models described by Merxmüller (1952) for *Saxifraga incrustata* Vest. or *Anthyllis montana* L. with occurrence both on the eastern mountains, the Alps and Pyrenees. If such an isolation took place in only one or few different refugial areas remains unclear since the genetic diversity within the southern Alps is not sufficiently resolved. North of the Alps only one genotype is dominates all populations except in the southwestern Alps where a local genotypes is also present. Our study doesn't deliver a clear indication where the origin of that species complex could have been, but since between the Caucasus respectively the Iranian Mountain ranges and Spanish Mountains these taxa are present in all ecologically suitable mountains with a high variety of taxa, we can assume the origin somewhere in that region. Further eastwards only few taxa common also to Pontic Alps is reported from the Russian and Chinese Altai pointing much more toward a recent expansion. The calcicole *G. verna* L. s.l. had the possibility to migrate through the eastern European Mountains and via the calcareous Dinaric Alps into the also calcareous Alps and westward via the partially calcareous central French massifs into the Pyrenees and the Cantabric Mountains adjacent in the north.

During the Weichsel-glazial from 20000 to 8000 BP the Alpine chain in its central part was under the cover of an ice shield (Denton and Hughes 1981; Lang 1994), not so the southwestern border and the far eastern and south-eastern part. Only local glaciers were recorded for the Dinaric Alps and the Balkan mountains as much as the Carpathians, the Pyrenees and the Cantabric Mountains. The Jura massif north of the Alps also host to *G. verna* L. s.str. was located at the northern end of the ice shield, overtopping that one with its highest summits.

The dispersal of genotypes within the Alps proposes a widespread extinction of these taxa within the glaciated area and their conservation in two refuges. A first refuge represented by the small genotype B was limited to the south-western French and Italian Alps and reached after the retreat of the ice only a limited expansion. The expansion of that genotype westward was limited by the lack of ecological niches, eastward a zone of acid rockbed (Asch 2005) not favored by these taxa, and northward by the ice shields. The second refuge home to genotype A is not clearly to localize. The presence of only one genotype in central Alps points against the nunatak theory as proposed by Holderegger, Stehlik et al. (2002) for *Saxifraga oppositifolia* L. or by Stehlik, Schneller et al. (2001) for *Eritrichium nanum* L. assuming a survival of these taxa in different refuge within the Alps. Much more probable is the presence of only one refuge or few outside the chalky area as the Jura Mountains or the far eastern border of the Alpine chain, the western Carpathian Mountains or even as introduced by Stewart and Lister (2001) in a cryptic northern refuge, and perhaps also in case f undiscovered southern Alpine genotypes in refugial regions on the southern border of the Alpine chain.

Revision of *Gentiana* Sect. *Calathianae* based on molecular phylogenies

Abstract

Based on results from six different molecular studies and cytology, a taxonomic revision of *Gentiana* Sect. *Calathianae* Froel. is proposed. The revised section comprises two subsections: Subsect. *Calathianae* with three taxa having relatively low chromosome numbers, *G. nivalis* L. and *G. utriculosa* L. (annuals), and *G. pumila* Jacq. (perennial); and Subsect. *Vernae* (Tzevelev) Haemmerli stat. nov. with nine species and seven subspecies including *G. verna* L. (with two subspecies, *G. verna* subsp. verna, and *G. verna* subsp. *delphinensis* Kunz), *G. tergestina* Beck (with three subspecies, *G. tergestina* subsp. tergestina, *G. tergestina* subsp. *balcanica* Haemmerli, and *G. tergestina* subsp. *pontica* Haemmerli), *G. sierrae* Briquet, *G. schleicheri* Kunz, *G. rostanii* Reuter, *G. bavarica* L. (with two subspecies, *G. bavarica* subsp*. bavarica* , and *G. bavarica* L. subsp. *subacaulis* Müller*), G. terglouensis* Hacq., *G. brachyphylla* Vill., and *G. orbicularis* Schur . These results provide indications into the biogeography of the section. While several taxa such as *G. pumila* Hacq., *G. bavarica* L. subsp. *subacaulis* Müller, *G. rostanii* Reuter or *G. verna* L. subsp. *delphinensis* Kunz, *G. terglouensis* Hacq. or *G. schleicheri* Kunz are restricted to the Alpine chain, others occur on different European Mountain ranges (*G. utriculosa* L., *G. sierrae* Briq. and *G. brachyphylla* Vill.), but only few taxa also grow outside Europe (*G. nivalis* L., *G. verna* L, *G. tergestina* Beck and *G. penetii* Romo). Two species are not represented at all within the Alps: *G. penetii* Romo from Atlas Mts. and *G. tergestina* Beck including its subspecies from Dinaric Alps eastward, throughout minor Asia to the Altai Mts. Most taxa are limited to only a limited distribution area or to only one or few mountain ranges. Widespread taxa are the annual *G. nivalis* L. and the perennials *G. verna* L. s.str. and *G. tergestina* Beck s.l.

Keywords

Gentiana, Sect. *Calathianae*, 5S-NTS, trnL, maturase K, nuclear encoded chloroplast expressed Glutamine Synthetase (ncpGS), chloroplast PCR-FRLP, AFLP, chromosome number, phylogeny, taxonomy, biogeography

Introduction

Gentiana Sect. *Calathianae* Froel. has been subject to many arrangements. As noted by Müller (1982) the description of Froelich (1796) is somehow vague. The description accepted here is similar to that of Tutin (1964) and Ho and Liu (2001) and in opposition to Müller (1982) who preferred *G.* Sect. *Cyclostigma* of Grisebach (1839). *Gentiana* Sect. *Calathianae* is circumscribed by a series of common characters, such as flower morphology with a slender cylindrical tube within an angled to winged calyx. The flower tube is almost closed at its top by discoid stigmata, hiding the free anthers. The 5 corolla lobes are spreading salver form and generally colored in a typical azure while the stigmata remains white. Between the corolla lobes small, 2-clef plicae are localized. Flowers are every time terminal on branched (in case of annual plants) or unbranched, monopodial stems. Leaves are of different shape, from narrow lanceolate to rhomboid or broadly elliptic, sometimes arranged in a basal rosette but some taxa only arranged in the lower stem region without forming a real rosette. Perennial plants provide sterile rosettes arising from stolons.

Sect. *Calathianae* Froel. is dispersed over the main European Mountains as the Alps, Pyrenees, Cantabric Mountains, the southern Spanish Sierra Nevada Range, Apennine, Jura Mountains, Auvergne, Dinaric Alps, Balkan, Pindus and Rhodope Mountains, Carpathians and the Ural. The section is also present in Scandinavia, the British Islands, on Ireland and in the western Russian Arctic Region. Outside Europe fewer taxa are also reported from the north-western Canadian Coastline (Newfoundland, New England, Labrador), the southern coast of Greenland, and Iceland. Further in northern Africa on the High Atlas Mountains and in Asia from the Pontic Alps and the Taurus, the Caucasus and the Lesser Caucasus Mountains, Elbours Range, Pamir, Tienschan and finally most eastern the Altai Mountains. Most of the taxa are restricted to Europe, a large number even to the Alps or only reported from few places outside the Alps.

Taxa of the section grow mainly in different alpine environment, preferring often chalky soil or rock bed but show a certain radiation in their ecological parameters: from taxa able to grow in high vegetation in the relatively low Dinaric Alps up to other taxa only occurring on high summits or other places of extreme weather conditions and radiation. While some prefer loose slate or dolomite, others grow on decalcified soil with long snow cover or even in wet damp. Only few taxa can be found outside classical mountain ranges: in the arctic flatlands or along rivers as the Rhine.

While first taxa were equipped with descriptions by Linnaeus (1753) which are still in use today, others were subject to a large number or rearrangements made by numerous of authors over time. The high number of synonyms reflects on the one hand the interest of many botanists, also the low amount of useful characters for separation based on classical morphology, but also a possible tendency towards a variable morphology, depending much on ecological factors, but also on season (Hegi 1927). Also more recent work didn’t overcome the confusions in that section. A series of authors delivered sometimes highly divergent chromosome numbers. The broadest study there was probably done by Müller (1974, 1982) providing first but not in each case useful criteria for an intrasectional order. Also pollen micromorphology (Nilsson, Hellbom et al. 2002) or chemotaxonomy (Rivaille and Raulais 1969; Mpondo and Garcia 1989; Mpondo, Garcia et al. 1989; Mpondo and Garcia 1990; Mpondo and Garcia 1990; Jensen and Schripsema 2002) does not provide useful criteria respectively enough resolution for intrasectional classification.

Method and Data analysis

Information from above studies was evaluated in two manners: a) a consensus was elaborated between parsimony trees from 5S-NTS, maturase-K, ncpGS and trnL sequencing including also chloroplast PCR-RFLP; b) a combined analysis of 5S-NTS, maturase-K, ncpGS and trnL sequences.

In a preliminary analysis groups of closest related accessions were worked out that were the base for the afterwards used taxonomical units.

Based on results from different independent studies using molecular markers we tried to overcome the problem of missing clear morphological criteria by doing a synthesis in a widely supported and to revise the taxonomy in regard of these new results. That new order should not be in conflict neither to morphological nor to cytological criteria and should be explainable also in regard of species distribution.

As working taxonomy we accepted meanwhile the terminology proposed by Tutin (1964) and Ho and Liu (2001), except the one taxon added by Müller (1982): *G. bavarica* L. subsp. *subacaulis* Müller, which was neglected by the other two.

Consensus between parsimony tree topology

Parsimony trees from studies above were first reduced to branches having at least a support of 50% in bootstrap analysis. Afterwards each node dividing different taxonomical units was numbered and each connected branch became a character starting from 1. For each of these numbered nodes a marker was defined. For the taxonomic connected to that node the character of their branch was provided, for all others the character “-“. Classical

taxon	provenience	maturase K						ncpGS																			
		1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
G. acaulis		2	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G. bavarica s.str.		1	1	-	-	-	-	1	1	1	?	?	?	?	-	-	-	-	-	-	-	-	-	-	-	-	-
G. bavarica subsp. subacaulis		1	5	-	-	-	2	1	1	1	2	-	2	1	-	-	-	-	-	-	-	-	-	-	-	-	-
G. brachyphylla s.str.	Alps	1	5	-	-	-	-	1	1	1	2	-	3	-	-	1	-	-	-	-	-	-	-	-	-	-	-
G. brachyphylla s.str	Sierra Nevada	1	4	1	-	-	-	1	1	1	2	-	3	-	-	2	2	2	2	-	1	2	-	-	-	-	-
G. brachyphylla subsp. favratii	Alps	1	4	2	-	-	-	1	1	1	2	-	2	2	1	-	-	-	-	-	-	-	-	-	-	-	-
G. brachyphylla subsp. favratii	Carpathians	1	2	-	-	-	-	1	1	1	2	-	2	2	2	-	-	-	-	-	-	-	-	-	-	-	-
G. nivalis		1	6	-	2	2	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2
G. pumila s.str.		1	6	-	2	1	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	1
G. pumila subsp. delphinensis		1	3	-	-	-	-	1	1	1	2	-	3	-	-	2	2	2	1	1	-	-	-	-	-	-	-
G. rostanii		1	5	-	-	-	1	1	1	1	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G. terglouensis s.str.		1	1	-	-	-	-	1	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G. terglouensis subsp. schleicheri		1	3	-	-	-	-	1	1	1	2	-	3	-	-	2	1	-	-	-	-	-	-	-	-	-	-
G. utriculosa		1	6	-	1	-	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
G. verna s.str.	Alps, Jura	1	3	-	-	-	-	1	1	1	2	-	3	-	-	2	2	2	1	2	-	-	-	-	-	-	-
G. verna s.str.	Pyrenees	1	3	-	-	-	-	1	1	1	2	-	3	-	-	2	2	2	2	-	1	1	-	-	-	-	-
G. verna s.str.	Ural	1	3	-	-	-	-	1	1	1	2	-	3	-	-	2	2	1	-	-	-	-	-	-	-	-	-
G. verna subsp. balcanica		1	4	3	-	-	-	1	1	1	2	-	3	-	-	2	2	2	2	-	2	-	1	1	2	-	-
G. verna subsp. pontica		1	4	3	-	-	-	1	1	1	2	-	3	-	-	2	2	2	2	-	2	-	1	?	-	-	-
G. verna subsp. tergestina		1	4	3	-	-	-	1	1	1	2	-	3	-	-	2	2	2	2	-	2	-	2	-	-	-	-

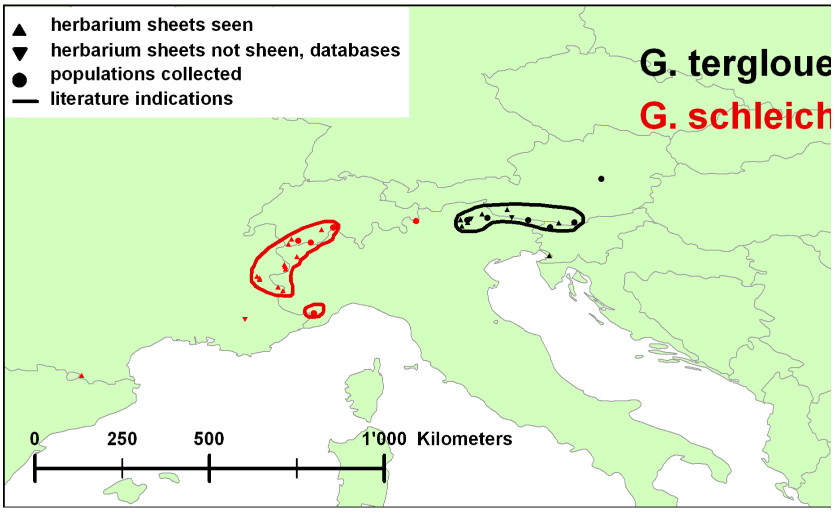
taxon	provenience	5S-NTS																	cloroplat PCR-RFLP													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	1	2	3	4	5	6	7	8	9	10	11	12	13	14
G. acaulis		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
G. bavarica s.str.		1	3	-	2	-	-	-	-	-	-	-	-	-	-	2	1	1	2	2	2	-	1	2	2	1	1	2	2	1	-	-
G. bavarica subsp. subacaulis		1	3	-	2	-	-	-	-	-	-	-	-	-	-	2	1	2	2	2	2	-	1	2	2	1	1	1	-	-	-	-
G. brachyphylla s.str.	Alps	1	3	-	2	-	-	-	-	-	-	-	-	-	-	1	-	-	2	2	2	-	1	2	2	1	2	-	-	-	2	-
G. brachyphylla s.str	Sierra Nevada	1	3	-	1	2	-	-	2	-	-	-	1	-	-	-	-	-	2	2	2	-	1	1	-	-	-	-	-	-	-	-
G. brachyphylla subsp. favratii	Alps	1	3	-	1	1	1	2	-	-	-	-	-	-	-	-	-	-	2	2	2	-	1	2	2	2	-	-	-	-	-	-
G. brachyphylla subsp. favratii	Carpathians	1	3	-	1	1	1	1	-	-	-	-	-	-	-	-	-	-	2	2	2	-	1	2	1	-	-	-	-	-	-	-
G. nivalis		1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	1	-	1	-	-	-	-	-	-	-	-	-	-
G. pumila s.str.		1	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2	1	-	-	-	-	-	-	-	-	-	-	-
G. pumila subsp. delphinensis		1	3	-	1	2	-	-	1	1	1	-	-	-	-	-	-	-	2	2	2	-	1	2	3	-	-	-	-	-	-	-
G. rostanii		1	3	-	2	-	-	-	-	-	-	-	-	-	-	2	2	-	2	2	2	-	1	2	2	1	2	-	-	-	1	-
G. terglouensis s.str.		1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2	2	-	1	2	2	1	1	2	?	?	-	-
G. terglouensis subsp. schleicheri		1	3	-	1	1	2	-	-	-	-	-	-	-	-	-	-	-	2	2	2	-	1	2	3	-	-	-	-	-	-	-
G. utriculosa		?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	2	1	-	2	-	-	-	-	-	-	-	-	-	-
G. verna s.str.	Alps, Jura	1	3	-	1	2	-	-	1	?	?	?	-	-	-	-	-	-	2	2	2	-	1	2	3	-	-	-	-	-	-	-
G. verna s.str.	Pyrenees	1	3	-	1	2	-	-	1	2	-	1	-	-	-	-	-	-	2	2	2	-	1	2	3	-	-	-	-	-	-	-
G. verna s.str.	Ural	1	3	-	1	2	-	-	1	2	-	2	-	-	-	-	-	-	2	2	2	-	1	2	3	-	-	-	-	-	-	-
G. verna subsp. balcanica		1	3	-	1	2	-	-	2	-	-	-	2	2	1	-	-	-	2	2	2	-	2	-	-	-	-	-	-	-	-	1
G. verna subsp. pontica		1	3	-	1	2	-	-	2	-	-	-	2	1	-	-	-	-	2	2	2	-	2	-	-	-	-	-	-	-	-	?
G. verna subsp. tergestina		1	3	-	1	2	-	-	?	?	-	?	?	?	?	-	-	-	2	2	2	-	2	-	-	-	-	-	-	-	-	1

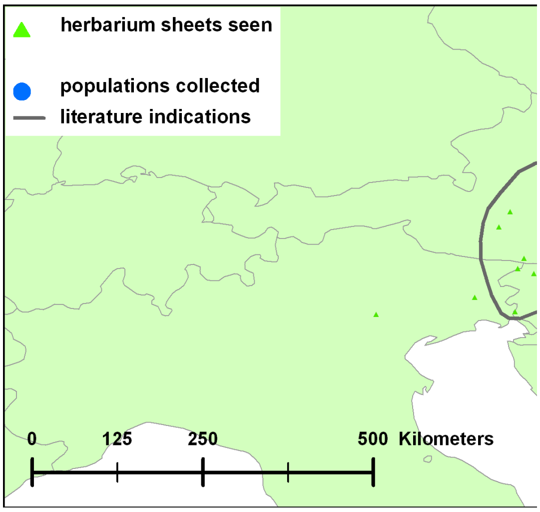
Table 1: Character matrix elaborated for consensus analysis upon parsimony trees. For precise information about coding see text, location of nodes are indicated in Fig. 1

bifurcations were therefore coded with characters 1 and 2, unresolved polytomies which 1, 2, 3 and so on. Taxonomic units not examined in one of the studies above were represented with "?" for those markers. Same character was used if one unit appeared on two branches descending from a particular node. Schemata of trees recoded are shown in Fig. 1, the final character matrix in Tab. 1.

Each character transformation was estimated as equivalent. To provide about the same impact on the final result by each tree coded, different weight factors were applied to each marker. An alternative matrix was done without weighting. "-" was estimated as character equivalent to all branch numbers instead of being interpreted as gap as usual, "?" signed for missing information.

Phylogenetic analysis was performed on PAUP* 4 (Swofford 1998) performing a heuristic search with parsimony criterion and random sequence addition with 10000 repetitions. Bootstrap analysis was performed on same parameters but 1000 sequence additions per bootstrap replicate for overall 1000 replicates.





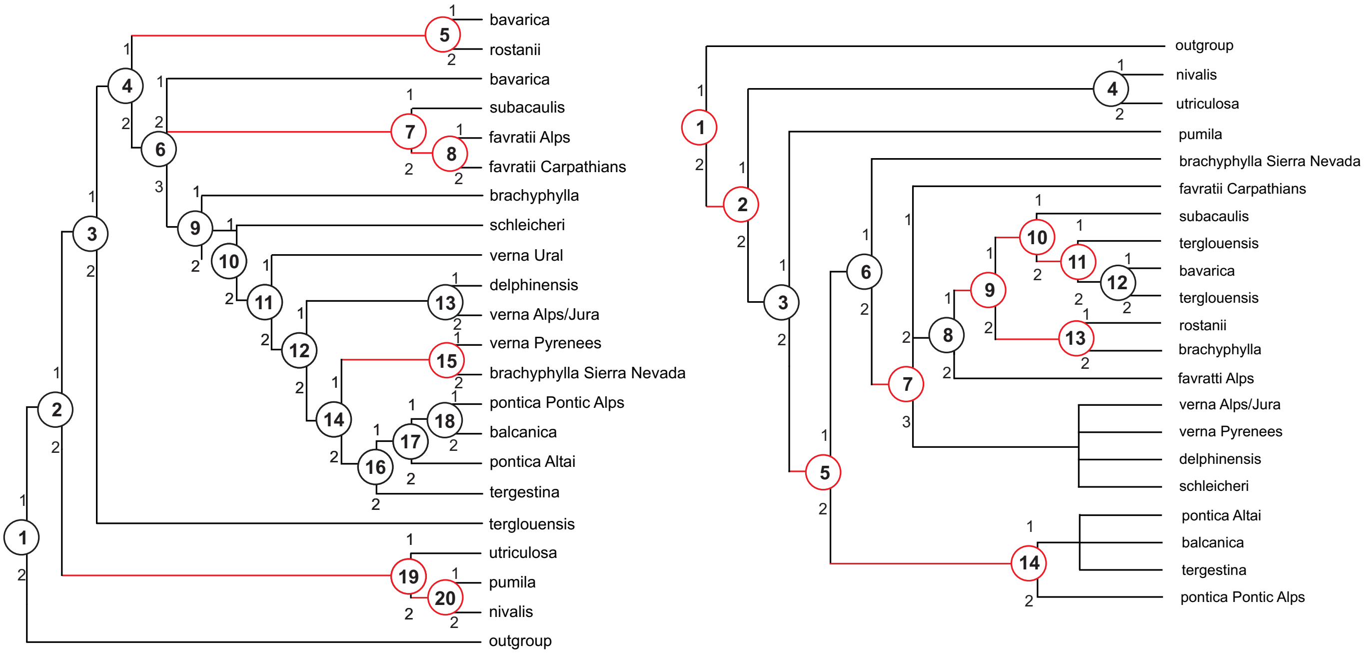


Fig 1: 4 parsimony trees recoded for calculation of consensus on tree topology: A: 5S-NTS (Haemmerli in publication c), B: maturase K (Haemmerli and Küpfer in publication a), C: ncpGS (Haemmerli in publication b), D: chloroplast PCR-RFLP (Haemmerli and Küpfer in publication e). Trees were simplified by removing all branches without bootstrap support higher than 50%. Branches and joined nodes with significant support of 95% or more are colored in red. Numbers in circles represent the marker index, numbers beside circles the value represented by that marker.

Branches from parsimony trees included in consensus analysis as described above didn't have a significant bootstrap support in each case. Therefore the resulting consensus tree also lacks significance indices. To overcome that problem, strict consensus trees from each study got reduced to branches with at least 95% bootstrap support. These 4 trees were base for a direct calculation of each a strict and a semi-strict consensus tree on * 4 (Swofford 1998) without preliminary recoding as done above. In Fig. 1 nodes and branches with bootstrap support of 95% and more as used for that posterior significance analysis are specially marked.

Combined sequence analysis

Sequences from trnL intron (Gielly and Taberlet 1996), 5S-NTS sequencing (Haemmerli *in publication c*), ncpGS sequencing (Haemmerli *in publication b*) and maturase K sequencing (Haemmerli *in publication a*) were combined into one alignment providing 3253 base pairs. GenBank accession numbers are provided in Tab. 1. Where one taxonomic unit covered several different sequences, a consensus using the terminology as proposed by Tipton (1994) was established. 5S-NTS sequences of *G. nivalis* L. were cropped from 174 base pairs to the end since the initial bases found no parallels any other accessions. The *G. acaulis* accession for the same sequence was used only between position 127 and 311 since flanking regions didn't permit an alignment to other accessions. The unknown bases were replaced by "?". A region of uncertainty in alignment between position 599 and 618 had to be excluded also from ncpGS sequence due to alignment ambiguity. Also missing was the initial 129 bps of *G. acaulis* L. ncpGS due to incomplete sequence. Likelihood parameters were established upon that alignment using Modeltest 3.7 software (Posada and Crandall 1998). Afterwards a Bayesian analysis on MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) performing a Markov chain Monte Carlo algorithm (Metropolis, Rosenbluth et al. 1953; Hastings 1970).

A parsimony analysis was performed on Paup* 4 (Swofford 1998) running 10000 repetitions with random sequence addition parameter and branch swapping in effect. A 50% majority consensus was calculated upon the resulting trees. For the same parameters as above but with only 1000 addition sequence repetitions, a bootstrap analysis was performed calculating 1000 replicates.

taxon	provenience	sequences			
		trnL	5S-NTS	maturase K	ncpGS
		(Gielly and Taberlet 1996)	(Haemmerli *in publication c*)	(Haemmerli *in publication a*)	(Haemmerli *in publication b*)
Alligned length		397 bp	397 bp	961 bp	1498 bp
G. acaulis		X77869	EF626794	EF552125	EF571643
G. bavarica s.str.		X77873	[EF626753-EF626755]	[EF552071-EF552074], EF552079	EF571635, EF571634, EF571637, EF571636, EF571638
G. bavarica subsp. *subacaulis*		-	EF626791, EF626756	EF552115	EF571601, EF571600
G. brachyphylla s.str.	Alps	X77875	[EF626757-EF626759]	[EF552116-EF552117]	EF571603, EF571602
G. brachyphylla s.str.	Sierra Nevada	-	EF626773	EF552100	EF571607
G. brachyphylla subsp. *favratii*	Alps	X77876	EF626761, EF626760	[EF552102, EF552112]	EF571639, [EF571640, EF571641]
G. brachyphylla subsp. *favratii*	Carpathians	X77876	[EF626792, EF626793]	[EF552080-EF552082]	EF571642
G. nivalis		X75703	EF62678, EF626783, EF626782, [EF626776-EF626780]	EF552121, EF552122, [EF552123, EF552124]	[EF571610-EF571617], [EF571619, EF571620]
G. pumila s.str.		X77891	EF626788	EF552120	EF571618
G. pumila subsp. *delphinensis*		-	EF626764	[EF552084-EF552086]	EF571630
G. rostanii		-	[EF626789, EF626790]	EF552113, EF552114	EF571599, EF571598
G. terglouensis s.str.		X77897	EF626786, EF626784, [EF626785, EF626787]	[EF552075-EF552078]	EF571621, EF571627
G. terglouensis subsp. *schleicheri*		-	EF626763, EF626762	EF552087	EF571623, EF571622
G. utriculosa		X77898		[EF552118, EF552119]	EF571609
G. verna s.str.	Alps, Jura	X75704	EF626769, EF626771, EF626766	[EF552088-EF552093, EF552095]	EF571625, EF571606, EF571605, EF571623
G. verna s.str.	Pyrenees	-	EF626770	[EF552096-EF552098]	EF571608
G. verna s.str.	E Carpathians	-		[EF552103, EF552105], EF552104	
G. verna s.str.	Ural	-	EF626767	EF552099	EF571604
G. verna subsp. *balcanica*		-	EF626772	EF552106, EF552111	EF571631
G. verna subsp. *pontica*	Altai	-	EF626768	EF552112	EF571633
G. verna subsp. *pontica*	Pontic Alps	-	-	EF552109, EF552110	EF571632
G. verna subsp. *tergestina*		-	EF626774, EF626775	EF552107, EF552108	EF571626, EF571628, EF571629

Table 2: Genbank accessions of sequences included in combined sequence analysis. Accessions in squared brackets "[]" represents identical sequences with different accession codes, without brackets means different sequences. If different sequences were existent, a consensus was built according (Tipton 1994)

Results

Consensus between tree topologies

Data from 5S-NTS-sequencing (Haemmerli *in publication c*) derived 17 characters (weight factor 420), from ncpGS-sequencing (Haemmerli *in publication b*) 20 characters were obtained (weight factor 357), maturase-K (Haemmerli *in publication a*) gave 6 characters (weight factor 1190) and chloroplast PCR-RFLP (Haemmerli *in publication e*) 14 characters (weight factor 510) resulting in a matrix providing 57 distinct characters for each unit (Tab. 1).

Parsimony analysis from weighted characters resulted into 75 best trees with a score of 62414 (CI = 0.931, RI = 0.927), whereof a 50% majority consensus was calculated (Fig. 2). The resulting tree provides a separation of the section into two groups: a first one (Subsect. *Calathianae*) covering the both annual *G. nivalis* L. and *G. utriculosa* L. as well as the perennial *G. pumila* Jacq. s.str.; the second group (Subsect. *Vernae*) hosts all the other perennial taxa. Also in regard to chromosome numbers (Müller 1982) that first node separates taxa with low numbers from 2n=14 to 2n=22 in the first group from higher ones with 2n=28 to 2n=38 in the second group. While for the first group no common pattern can be recognized, the second one provides hypothetically a number of 2n=30 at its base with later changes within some taxa. Basal bootstrap support for the fist group was 78%, for the second 99%. Within the first group the both annuals are separated from the perennial *G. pumila* Jacq. s.str. with a support of 67%.

The second group provides an a) one branch a large subgroup covering taxa *G. verna* L. subspecies, *G. pumila* Jacq. subsp. *delphinensis* Fourn. and *G. terglouensis* Hacq. subsp. *schleicheri* Tutin as much as *G. brachyphylla* Vill. subsp. *favratii* Tutin with a basal support of 54% in bootstrap analysis and a basal division that separates the two groups. Within *G. verna* L. and close related accessions eastern taxa such as *G. verna* L. subsp. *tergestina* Hayek, *G. verna* L. subsp. *balcanica* Pritch. and *G. verna* L. subsp. *pontica* Hayek as well as the southern Spanish population of *G. brachyphylla* Vill. s.str. from Sierra Nevada are placed together with a support of 91% in bootstrap analysis for the base and 95% for the subspecies of *G. verna* L.) and the western European taxa *G. verna* L. s.str., *G. pumila* Jacq. subsp. *delphinensis* Fourn and *G. terglouensis* Hacq. subsp. *schleicheri* Tutin as well as the eastern European Ural populations of *G. verna* L. s.str. on the other hand (with a low basal bootstrap support of 62%) form two distinguishable subgroups. Beside that first subgroup b) the Alpine *G. brachyphylla* Vill. s.str. as well as *G. bavarica* L. s.str. and subsp. *subacaulis* Müller, *G. rostanii* Reuter and finally *G. terglouensis* Hacq. s.str. That second branch provides a bootstrap support of 75% at its base and almost no supported internal structure. Taxa are mainly of 2n=30 except *G. brachyphylla* Vill. s.str. with 2n=28 and *G. terglouensis* Hacq. s.str. with 2n=38

The same analysis performed on characters with equal weights (instead of equal weights for entire trees) resulted into 24 most parsimonious trees with a optimal score of 118 (CI = 0.924, RI = 0.924). The overall topology of resulting consensus (Fig. 3) is equal to the one obtained through weighted characters except a certain gain in unresolved polytomies respectively a rearrangement around taxa *G. verna* L. As such all *verna*-taxa are arranged on a branch sister to *G. terglouensis* Hacq. subsp. *schleicheri* Tutin: while the eastern subspecies form a somehow closer linked group, the western accessions of *G. verna* L. including the material from Russian Ural Mts. as well as *G. pumila* Jacq. subsp. *delphinensis* Fourn. provide almost no further structuration.

Consensus analysis upon trees reduced to significantly supported branches resulted into an only weakly supported result. While a strict consensus tree delivered no internal structuration at all and results in a single unresolved, a semi-strict consensus that gave following result: in 50% of trees significantly support a branch hosting *G. nivalis* L. and *G. pumila* Jacq. s.str., in 1 tree *G. utriculosa* L. joins that group in a more basal position (in 1 out of these 4 tree *G. utriculosa* L. is not represented). In 1 tree all other perennials except the outgroup taxa are placed on unique branch in significant manner and in 50% of the involved trees the Alpine and the Carpathian populations of *G. brachyphylla* Vill. subsp. *favratii* Tutin are linked together in significant way.

Combined sequence analysis

Resulting alignment provided 2660 constant, 330 parsimony uninformative and 280 parsimony informative characters. Base frequencies of resulting alignment were A = 0.2815, C = 0.2101, G = 0.1912 and T = 0.3173. Likelihood parameters as established by ModelTest were gamma = 0.6854 and transition/transversion ratio = 0.4956 assuming the General Time Reversible Model (Rodriguez, Oliver et al. 1990), with unequal base frequencies and substitution rates, the use of gamma distributed rates and the presence of invariable sites. Substitution frequencies were: R_{AC}= 1.162, R_{AG}= 2.501, R_{AT}= 0.600, R_{CG}= 0.984, R_{CT}= 2.501 and R_{GT}=1.000. Bayesian analysis resulted into a consensus tree with lnL=-10584.16 (Fig. 4). That tree divides the section into two groups: a first one covering the annual *G. nivalis* L. and *G. utriculosa* L. as well as the perennial *G. pumila* Jacq. s.str. and a second one hosting all the other perennial taxa. Within the first group all branches are equipped with a posterior probability of 0.99. *G. nivalis* L. and *G. pumila* Jacq. s.str. are closer while the *G. utriculosa* L. gets separated first.

Within the second group providing itself a posterior probability of 0.99 an isolated position is occupied by *G. terglouensis* Hacq. s.str. supported by the same probability as above. Also with same support a next bifurcation separates between taxa *G. verna* L. and subspecies, *G. pumila* Vill. subsp. *delphinensis* Fourn. and *G. terglouensis* Hacq. subsp. *schleicheri* Tutin on one side and the remaining taxa on the other side. The first of these subgroups hosts *G. brachyphylla* Vill subsp. *favratii* Tutin separated in a next significant step while the remaining are only supported by a probability of 0.96. A distinct position has *G. terglouensis* Hacq. subsp. *schleicheri* Tutin while the other taxa form a further branch with maximal probabilities, providing mainly

G. verna L. from Alps and western Carpathian *G. verna* L. s.str. and *G. pumila* Jacq. subsp. *delphinensis* Fourn. on one side and in a second side branch eastern subspecies of *G. verna* L. as well as Pyrenean populations of that taxon and the Sierra Nevada population of *G. brachyphylla* Vill. s.str. These later two taxa form a closer group with maximal probability while the eastern *G. verna* L. subspecies group together in an order without significant support in some cases.

The second large subgroup within the perennial taxa beside the accessions around *G. verna* L. is built by Alpine *G. brachyphylla* Vill s.str., *G. rostanii* Reuter and *G. bavarica* L. s.str. as well as subsp. *subacaulis* Müller with maximal basal support. A basal most position is occupied by *G. brachyphylla* Vill., while the others are linked with significant to maximal posterior probability.

7 most parsimonious trees were obtained by Paup* 4 (Swofford 1998) with a score of 964 (CI = 0.788, RI = 0.748). The topology of a 50% consensus tree is identical to the one obtained by Bayesian Likelihood analysis. We identify both the known Subsections *Calathianae* and *Vernae* as above. The first one hosts the two annual taxa *G. nivalis* L. and *G. utriculosa* L. as well as the perennial *G. pumila* Jacq. s.str in the same order as proposed above, with maximal support for all braches. Subsection Vernae has an 89% bootstrap support at its base. The basal most taxon single on a branch is *G. terglouensis* Hacq. s.str. A further branch with 97% bootstrap support hosts the remaining taxa. *G. bavarica* L. s.str. and subsp. *subacaulis* Müller, *G. rostanii* Reuter and *G. brachyphylla* Vill. s.str. are separated into a group with

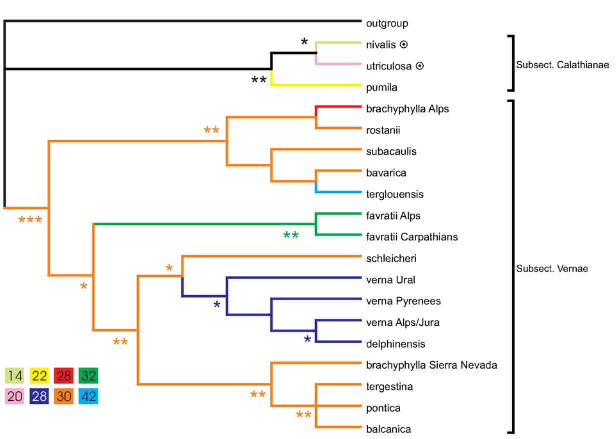


Figure 2: 50% consensus from 75 best trees out of parsimony analysis done on weighted characters as shown in Tab. 2. Annual taxa are indicated with ⊙, chromosome numbers as used for color code correspond to results obtained by Müller (1982). Bootstrap supports are indicated as asterisk: * = 50-70%, ** = >70-95%, *** = > 95%

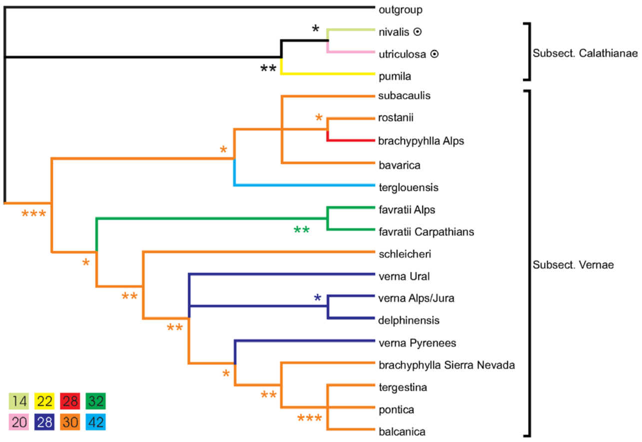


Figure 3: 50% consensus from 18 best trees out of parsimony analysis done on unweighted characters as shown in Tab. 2. Annual taxa are indicated with ⊙, chromosome numbers as used for color code correspond to results obtained by Müller (1982). Bootstrap supports are indicated as asterisk: * = 50-70%, ** = >70-95%, *** = > 95%

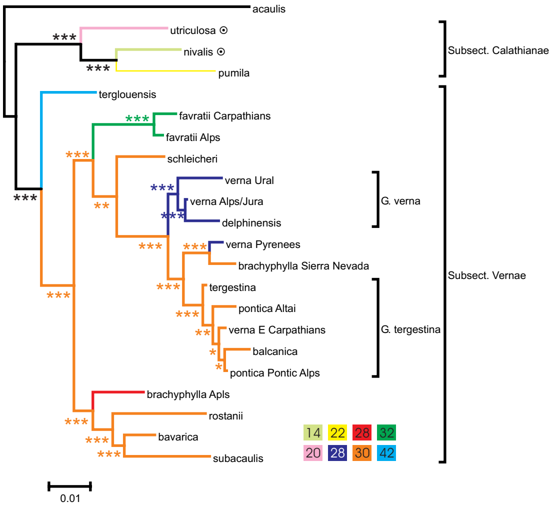


Fig. 4: Consensus tree obtained from a MCMC framework on MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Colors code for chromosome numbers similar to Fig. 2 Posterior probabilities are indicated as asterisk: * = 0.5-0.7, ** = >0.7-0.95, *** = > 0.95. Scale bar indicates substation rate per base.

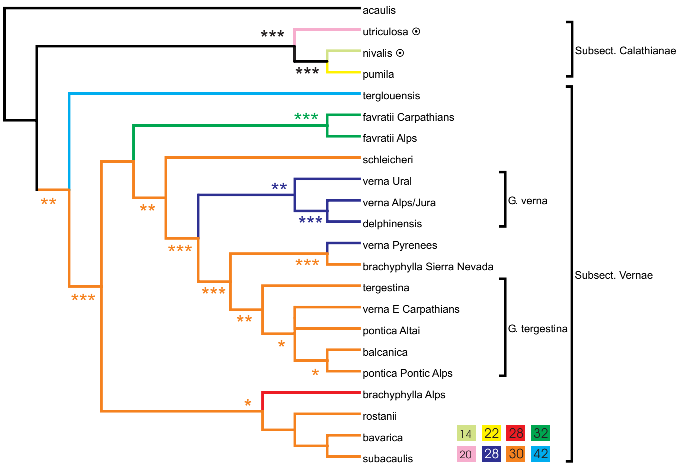


Figure 5: 50% consensus from 7 most parsimonious trees calculated upon combined sequences. Colors code for chromosome numbers similar to Fig. 2 Bootstrap supports are indicated as asterisk: * = 50-70%, ** = >70-95%, *** = > 95%

only 52% support at its base, but no further support within that group. The other branch at that node has no support at all, resulting in an unresolved polytomy between the taxa above, *G. brachyphylla* Vill. subsp. *favratii* Tutin and the remaining taxa. The branch hosting both, the Alpine and the Carpathian *G. brachyphylla* Vill. subsp. *favratii* Tutin is supported in bootstrap analysis by 97%. The third branch hosting all other taxa has only 73% support. Along that branch we find a next separation with *G. verna* L. s.str. from the Alps and the Jura, *G. pumila* Jacq. subsp. *delphinensis* Fourn as well as *G.* verna L. s.str. from the Ural Mountains. The first two are grouped together on a final branch with 96% support. The other taxa: all eastern subspecies of *G. verna* L. including populations from the southern Pyrenees and the Southern Carpathians as also *G. brachyphylla* Vill. from Sierra Nevada have a common branch with 90% support. The taxa from Southern Pyrenees and Sierra Nevada are significantly grouped together with 96% support in bootstrap replicates, while the other taxa form a cluster with only 79% support. Within that cluster only a weak internal order is recognizable. At its base we localize a unresolved polytomy which the eastern Carpathian *G. verna* L. s.str, *G. verna* L. subsp. *pontica* Hayek from the far eastern Altai and a group formed by populations of the latest taxon from Pontic Alps as well as *G. verna* L. subsp. *balcanica* Pritch. The polytomy is localized on a branch with only 53% support, the group covering taxa from Pontic Alps and Balkan Mountains by only 63% in bootstrap support.

Discussion

Consensus between tree topologies

Both phylogenetic consensus trees calculated upon tree topologies from included studies allow us to identify following unities: a) a group covering two annual taxa as well as one perennial, all these with a lower chromosome number (2n=14 to 2n=22) than the others as elaborated by Müller (1982). b) a large group covering most perennial taxa with chromosome numbers between 2n=28 to 2n=38; c) within that second group a subclade hosting *G. verna* L. and its subspecies as well as some other taxa.

A separation of *G.* Sect. *Calathianae* Froel. into two groups reflecting the life cycle has been proposed by several authors such as Rouy, Foucaud et al. (1893), Hegi (1927), Müller (1982). Zuev (1985), Tzevelev (1987) and Halda (1995) by separating the annual from perennial taxa. The annual group represents plants without a basal leaf rosette and branched stems with usually many flowers, while the perennials provide unbranched stems with few flowers and in many cases basal leaf rosettes, providing precise characters for differentiation. Nevertheless our interpretations would insert an additional perennial taxon *G. pumila* Jacq. S.str. within the first group beside *G. nivalis* L. and *G. utriculosa*. Based on the existence of the same discoid stigmata in both of these groups mentioned above a criteria exists to identify all taxa as member of a unique section. The declaration of *G.* Subgenus. *Calathianae* as done by Halda (1995) comprising of two sections, whereof one is only composed of annual taxa is rejected due to our results the main criteria for separation between the sections: the life cycle becomes second criterium behind chromosome numbers. We support therefore the conservation of a unique Section and the declaration of Subsections by adapting the terminology proposed as sectional names by Tzevelev (1987) and Halda (1995). Subsections as declared by Rouy, Foucaud et al. (1893) adapting section names following Gaudin (1828): *Hippion* and *Gentianella* are problematic since in use also as genus. Preferred should therefore be Subsect. *Calathianae* and Subsect. *Vernae*.

Subsect. *Vernae* has a maximal basal support in parsimony analysis. A main part of taxa included into that group provide chromosome number 2n=30, dispersed over different morphological and topological groups. Most probable is to propose that number as basal character for the entire subsection by revising interpretations done by Müller (1982), who defined two distinct groups with original n=15 for most of our taxa, but also a distinct group covering *G. terglouensis* Hacq. s.str. and subsp. *schleicheri* Tutin with n=10 at its base. According our interpretation all present chromosome numbers within Subsect. *Vernae* are derived from a common origin n=15.

Within Subsect. *Vernae* a large group is built by taxa covering *G. verna* L. providing a common root without maximal bootstrap support. Nevertheless the association of all subspecies of *G. verna* L. in one unity is a good criterion to identify that group as one species with different subspecies respectively haplotypes. These are *G. verna* L. s.str., *G. verna* L. subsp. *tergestina* Hayek, *G. verna* L. subsp. *balcanica* Pritch., *G. verna* L. subsp. *pontica* Hayek, *G. terglouensis* Hacq. subsp. *schleicheri* Tutin, *G. pumila* Jacq. subsp. *delphinensis* Fourn. as well as the Sierra Nevada population of *G. brachyphylla* Vill. s.str. Aligning chromosome counts as additional criteria we receive a separation in rather eastern taxa providing 2n=30 and rather western ones with 2n=28. Hegi (1927) and Kusnezow (1894) proposed *G. verna* L. to be a large and very diverse species. The later of these authors included also *G. brachyphylla* Vill. s.str. and subsp. *favratii* Tutin as varieties within that species what finds no support in our study. Haemmerli (*in press* b) has shown in AFLP study the lack of a separation between Alpine *G. verna* L. s.str. and *G. pumila* Jacq. subsp. *delphinensis* Fourn. since both taxa share in their overlapped distribution area two common genotypes. In present study a significant separation between the two taxa sharing the same chromosome number is not established upon tree morphology. Nevertheless it seems to be evident to accept them as different subspecies since morphological distinction can be made. In between the more eastern subspecies of *G. verna* L., *G. brachyphylla* Vill. from Sierra Nevada can be identified. Romo (1988) and later Rivas-Martinez, Asensi et al. (1991) compared that taxon to Cantabrian, Pyrenean and Alpine *G. verna* L., the later one by proposing *G. verna* L. subsp. *sierrae* for that *G. brachyphylla* Vill.-population. In consensus upon recoded parsimony trees the accession from Sierra Nevada is located basal to eastern subspecies of *G. verna* L. by proposing a strong east-(eastern Europe and Asia)-west-(Sierra Nevada) dispersal. The above mentioned AFLP study (Haemmerli *in press* b) has shown a similar situation for *G. verna* L. genotypes where the Pyrenees and Cantabric Mountains are closer related to eastern Mountains than to the Alpine Chain. Both analysis, the parsimony provide a highly significance for a separation between Sierra Nevada *G. brachyphylla* Vill. and the eastern *G. verna* L. subspecies reflected also in different morphology. *G. terglouensis* Hacq. subsp. *schleicheri* Tutin with a chromosomal number of 2n=30 is basal to Alpine *G. verna* L. but providing low support in both analysis. Nevertheless it has to be included within *G. verna* L. in order to keep that taxon monophyletic. Morphological and ecological differences provide enough possibilities for a clear separation between *G. terglouensis* Hacq. subsp. *schleicheri* Tutin and *G. verna* L. s.str., same as the difference in chromosome number, but still Bolòs and Vigo (1984) interpreted that first taxon as *G. verna* L. subsp. *schleicheri*. Also linked to the Alpine *G. verna* L. s.str. is the accession from the same taxon from Russian Ural Mountains. Different to the rather south western subspecies *G. verna* L. subsp. *tergestina* Hayek, *G. verna* L. subsp. *balcanica* Pritch., *G. verna* L. subsp. *pontica* Hayek that north eastern taxon is significantly related to the Alpine plants, same as shown in AFLP analysis (Haemmerli *in press* b) for the western Carpathians. (Meusel, Jäger et al. (1978) proposes the plants from arctic European Russia as recent expansion.

Beside the large *G. verna* L. Subsect. *Vernae* covers a serie of other taxa. The position of *G. brachyphylla* Vill. subsp. *favratii* Tutin since bootstrap support for the placement of that taxon is weak. Within that taxon, accessions from the Alps and the Carpathians are always grouped together. Based on consensus between tree topologies accessions from both geographical origins can be accepted as belonging to the same species. Always strongly supported is the association of remaining *G. bavarica* L. s.str., subsp. *subacaulis* Müller, *G. rostanii* Reuter, *G. terglouensis* Hacq. s.str. and the alpine *G. brachyphylla* Vill. s.str., but within that group an internal order is less significant. We recognize a tendency of *G. bavarica* L. s.str. to be linked to *G. terglouensis* Hacq. s.str. On the other hand *G. bavarica* L. s.str. and subsp. *subacaulis* Müller are morphologically relatively close and intermediates between the typical forms can be found depending on altitude of occurrence (Hess, Landolt et al. 1967). Therefore it should be our interest to preserve the species *G. bavarica* L. and since *G. terglouensis* Hacq. provide very different placements in phylogeny derived from combines sequences, the position of these two taxa in topology analysis could lead to misinterpretations. Basically the outcome of maturase-K sequencing (Haemmerli *in publication a*) providing identical sequences for almost all accessions of these taxa could be responsible for that link between them, while in 5S-NTS and in ncpGS-sequencing *G. terglouensis* Hacq. s.str. becomes a basal most position, clearly separated from *G. bavarica* L. The position of the two other taxa *G. rostanii* Reuter and Alpine *G. brachyphylla* Vill. finds also not sufficient support to make a reliable proposal. For these four taxa we summaries therefore that a consensus of four parsimony trees from different provenience doesn't deliver enough information to legitimate any changes on present taxonomy. Informations from further markers are needed, providing more resolution upon these close related taxa.

Combined sequence analysis

An analysis of 4 sequences (trnL intron, maturase K, ncpGS and 5s), two of them chloroplast and therefore maternally inherited, of these two (one covering a coding and one a non-coding sequence), the others two nuclear and biparental inherited, one a single copy sequence with both coding and non-coding content and the last a non-coding multi copy sequence, results in tree topologies basically supporting the conclusions made upon a comparison between different parsimony trees obtains from the sequences just described except trnL, but including also results from an AFLP study. The 4 sequences included in that combined sequence analysis were of different length, resulting therefore in a potentially different impact on the result.

A division of the section into two subsections is supported by the combined sequences. We obtain two highly supported basal branches. Also an internal one for Subsect. *Calathianae* is predicted: *G. utriculosa* L. is more basal than the others two. But we also have to face the problem of low taxa number within that part of the tree providing a relatively high number of different base pairs in between and therefore making the branch susceptible to long branch attraction (LBA) (Bergsten 2005). Both, the likelihood access done by the Bayesian analysis as the Maximum Parsimony access delivers the same composition of that branch hosting Subsect. *Calathianae*. Also we find support in all included studies in particular and a strong support in special by the ncpGS sequence, showing a long indel structure, separating the three taxa plus *G. terglouensis* Hacq. s.str. from all others. The question remains whether the real order within that branch conforms to that combined sequence analysis derived, or if rather more the basal most position of *G. pumila* Jacq. s.str., as reported by parsimony consensus upon tree topology is realistic. The question can be redefined: was the annual life cycle invented twice respectively did *G. pumila* Jacq. s.str. regain his perennial status if the real internal order of that subsection conforms to the outcome of combined sequence analysis or has the type of life cycle to be considered as strong indication for a closer relationship between the two annual taxa in a terminal position in intra-subsectional phylogeny. To resolve that problem based on sequence analysis more different accessions (we remember *G. nivalis* L. often identical in its entire distribution area, same as *G. utriculosa* L.) (Hillis 1996; Swofford, J. et al. 1996; Page and Holmes 1998) respectively more suitable sequences (Xiang, Moody et al. 2002) would be necessary to prevent from artificial influences on the result effected by the algorithms used (Bergsten 2005) since the exclusion of taxa potentially affected to LBA within that small subsection seems not practicable.

A fundamental difference to the consensus on tree topology is the basal most position of *G. terglouensis* Hacq. s.str. within Subsect. *Vernae*. In chloroplast sequences (trnL and maturase-K) as well as in chloroplast RFLP that taxon finds a position embedded within the Subsection, while in both nuclear sequences (5S-NTS and ncpGS) propose that basal placement dominating the combined sequence analysis. The dominance is caused by the predominance of nuclear sequences in number of bases over the chloroplast ones.

An only weak supported group is built by the four taxa *G. brachyphylla* Vill s.str. (Alps), *G. rostanii* Reuter, *G. bavarica* L. s.str. as well as subsp. *subacaulis* Müller. The posterior probability is remarkably deeper than elsewhere indicating an uncertain phylogeny. Therefore no reliable predictions can be maid based on combined sequence analysis. We can remark anyway: the Alpine taxa *G. brachyphylla* Vill. s.str. has a clearly distant position to its populations from Sierra Nevada as well as to its subspecies *favratii* Tutin resulting into a conflict with the principle of monophyly. No justifications or falsifications can be made for the arrangement of *G. rostanii* Reuter, *G. bavarica* L. s.str. and subsp. *subacaulis* Müller as above.

While in consensus analysis between tree topologies *G. terglouensis* Hacq. subsp. *schleicheri* Tutin has an intermediate position between the eastern and western accessions of *G. verna* L. and subspecies. In sequence analysis this position somehow changed to basal to all *G. verna* L. and subspecies. If we consider the bootstrap supports respectively the posterior probabilities, a weak predominance for the topology proposed by the actual analysis can be established. As well as in the problem around *G. terglouensis* Hacq. s.str. also here we face a difference between sequences originating from nuclear and chloroplast sequences. Chloroplast data integrate *G. terglouensis* Hacq. subsp. *schleicheri* Tutin fully within western European *G. verna* L. s.str. while in nuclear data, the taxon has its basal position as in final analysis. It has to be remarked that also that taxon is clearly distant to *G. terglouensis* Hacq. s.str. and somehow related to *G. verna* L.

Within *G. verna* L. two groups can be recognized: a) one group covering *G. verna* L. s.str. except the accessions from the south eastern Carpathians but also *G. pumila* Jacq. subsp. *delphinensis* Tutin; b) another group hosting the eastern subspecies of *G. verna* L. plus the accessions from south eastern Carpathians. Intermediate are somehow *G. brachyphylla* Vill. s.str. from Sierra Nevada as discussed above and similar to topology consensus analysis and *G. verna* L. from Pyrenees. The latest accession has a similar position in overall sequence analysis as in ncpGS sequence analysis (Haemmerli *in publication* b). In all other sequences included the accession was embedded within western European *G. verna* L. respectively not represented. The dominance in number of base pair from ncpGS sequence could cause the preference for that arrangement. Since in AFLP study (Haemmerli *in press* b) relations of Pyrenean populations to eastern European accessions of *G. verna* L. were proved the not purely Alpine provenience of that taxon has further support. Müller (1982) has also reported about forms of *G: verna* L. somehow morphologically different to those from the Alps. Nevertheless he counted a plant from Col de Somport in the southern Pyrenees and found the same chromosome number (2n=28) as in Alpine accessions. A special treatment of that one outstanding position basing mainly on one population from Southern Pyrenees seems therefore to be exaggerated.

<u>A closer look to *G. verna* L. in Europe</u>

In this paragraph we try to put results from the above analysis into relations to data delivered from AFLP study (Haemmerli *in press* b). It is remarked the existence on a eastern haplotype of *G. verna* L. and several western ones: one haplotype local to the Alps and Western Carpathians, another restricted to the south western Maritime Alps and a third in the Pyrenees and Cantabric Mountains. Haemmerli (*in press* b) has shown the closer relationship between most western types and the eastern ones, but also the existence of some possibly mixed populations within the south western

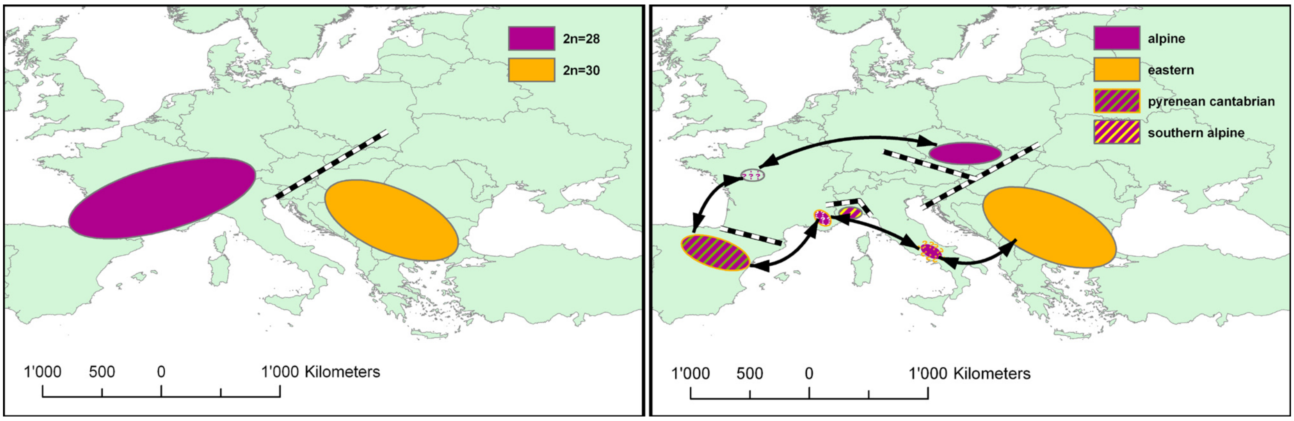


Figure 6: Hypothetical pathway of species and genotype formation within *G. verna* L. s.l. within Europe. Left: initial differentiation into two types covering different chromosome numbers. Numbers conform to (Müller 1982), dashed line: hypothetical border between the types; right: second phase of differentiation. 4 different types are indicated. Central color indicates main provenience chromosome numbers. Outline color indicate secondary influences. Question marks indicate unclear situation due to missing information. Dashed lines: barriers preventing from direct contact, arrows: potential pathways for exchange.

Alps (Col de Festre and Col de Restefonds, France). We also remember the displaced position of *G. verna* L. from Pyrenees in sequence analysis, close the plants from Sierra Nevada. As basal assumption we propose to accept chromosome numbers as elaborated by Favarger (1949), Müller (1974, 1982) and Gagnidze, Küpfer et al. (1992): 2n=28 for western Europe eastward to the eastern border of the Alps and including the western Carpathians (Tatra Mts.), as well as 2n=30 for everything further east.

Sequence and topology analysis illustrate a common origin of all these taxa mentioned above, closer linked than to the other perennial species. From the existence of a east-west distributed haplotype complex surrounding the Alps, also if that type has a weak internal correlation and host a wide number of internal variation we could possibly deduce the existence of former distribution of an ancient taxon covering the entire European mountain ranges and the afterward isolation into distant distribution patterns hypothetically to glaciation effects (Haemmerli *in press* b). But there are points contradicting that theory. As derived from Andersen (1981), Denton and Hughes (1981) the areas home to today's east-west distributed haplotype were possible refuges. Unclear is the situation within the southern Apennine, where no sample material was collected. To its close position to the Dinaric Alps a possible exchange between these two mountain ranges is possible. The existence of populations with rather east-western than alpine haplotype within the southern Alps also indicates the possibility on a further exchange via the most southern Alps and further westward to the Pyrenees. Müller (1982) has counted chromosome numbers in the Pyrenees and the Cantabric Mountains and found 2n=28 in *G. verna* L., equal to the ones from the Alps and in opposite to the eastern subspecies with 2n=30. Our phylogenies impose the conclusion that a number of 2n=30 could represent an ancestral character and that the reduction in *G. verna* L. s.str. and *G. pumila* Vill. subsp. *delphinensis* Fourn. took place afterwards.

A more probable hypothesis (Fig. 6) as above seems therefore to an initial separation the common ancestor with 2n=30 into two taxa, one reduced to 2n=28 and more dominant in Western Europe and the other to Eastern Europe. During periods of isolation as described above a further fragmentation of that 2n=28 type into the proposed refuge area took place forming type located to Spain in possible exchange via southwestern Alps and Apennine toward the Dinaric Alps, while two refuges for Alpine types were existing: one in the south western Alps and another north or northeast the Alps respectively in the western Carpathians. While the northern Alpine type was isolated from the Pyrenean/Cantabrian and the Dinaric one, these two effectuated a certain exchange, reflected by the intermediate position of plants from Pyrenees as shown in present study, but also in the proximity between the AFLP genotypes (Haemmerli *in press* b) of these two regions. Its thinkable that a certain exchange took also place between the Alps and the Pyrenees via the Central French Mountains (Auvergne), since *G. verna* L. is also indicated for that region (Fournier 1938, Braun-Blanquet 1923), explaining the missing measures to separate plants from Alps, Cantabric Mountains and Pyrenees based on chloroplast PCR-RFLP (Haemmerli *in publication e*).

A closer look to *G. terglouensis* Hacq.

We face an uncertainty in results concerning the taxon *G. terglouensis* Hacq. s.str., restricted to the eastern Alps and vicariate to *G. terglouensis* Hacq. subsp. *schleicheri* Tutin. Our results illustrate an intermediate position of that taxon between the two subsections based on nuclear sequences and a placement embedded close to *G. bavarica* L. s.str. based on chloroplast sequences and chloroplast PCR-RFLP. Chromosome numbers for *G. terglouensis* Hacq. s.str. were established by Müller (1982) as 2n=38 while Ho and Liu (2001) indicates 2n=40, and therefore much higher than the other taxa within the Section. Subsect. *Vernae* provides at its base possibly a chromosome number of 2n=30, within Subsect. *Calathianae* no clear structure is visible. Müller (1982) proposes for all these gentians a most ancestral state of x=5, a number that is no more existent within *Gentiana* nowadays

The intermediate position of *G. terglouensis* Hacq. s.str. between Subsect. *Calathianae* and *Vernae* in biparental inherited nuclear sequences but not in maternally inherited chloroplast markers, in combination with the elevated chromosome number (Favarger 1965; Müller 1974; Müller 1982; Löve 1986) is interpreted as result from a hybridization between each a descendent from the two subsections as origin for that taxon. Which possibly ancestral taxa were involved in that process and how the actually high chromosome number of present *G. terglouensis* Hacq. s.str. has to be explaines is subject to speculations.

Conclusion

Gentiana Sect. *Calathianae* Froel. is a monophyletic section with two subsections. Subsect. *Calathianae* and *Vernae*, no longer differing by the life cycle (annual or perennial), but chromosome numbers. Subsect. *Calathianae* comprises *G. nivalis L., G. pumila* Jacq. s.str. and *G. utriculosa* L., while *G. pumila* Jacq. subsp. *delphinensis* Fourn. is excluded from that last species and finds place in Subsect. *Vernae.* The second subsection *Vernae* covers taxa *G. bavarica* L. s.str., *G. bavarica* L. subsp. *subacaulis* Müller, *G. brachyphylla* Vill. s.str. comprising only of the Alpine accessions*, G. brachyphylla* Vill. subsp. *favratii* Tutin*, G. rostanii* Reuter and *G. terglouensis* Hacq. s.str.. *G. brachyphylla* Vill. subsp. *favratii* Tutin represents a unique taxon dispersed in the Carpathians and the Alps, and the name correctly postulated by Schur (1852, 1866) for Carpathian material: *G. orbicularis* becomes priority above the later definition as *G. favratii* by Rittener (1887) and Favrat (1887). The binomial *G. brachyphylla* Vill. should be only applied to Alpine plants of that species in strict sense, but not to those from southern Spanish mountains. *G. rostanii* Reuter*, G. bavarica* L. s.str. and *G. bavarica* L. subsp. *subacaulis* Müller represent taxa that can be distinguished upon molecular markers. Since no phylogeny with significant support can be established, they should be accepted the way as proposed by previous authors.

Further part of Subsect. *Vernae* is *G. verna* L. covering the mainly western European distributed *G. verna* L. s.str. G. pumila Jacq. subsp. *delphinensis* Fourn. is clearly distant from *G. pumila* Jacq., located even in a different subsection and becomes *G. verna* L. subsp. *delphinensis* H. Kunz. *G. terglouensis* Hacq. subsp. *schleicheri* Tutin shows no relation to *G. terglouensis* Hacq. s.str. and becomes an independent taxon as *G. schleicheri* Kunz, close to *G. verna* L., but providing a different chromosome number. In regard of missing monophylie within former *G. verna* L. in our molecular trees, the Dinaric subsp. *tergestina* Hayek, the south eastern European subsp. *balcanica* Pritch. and the Asian subsp. *pontica* Hayek. become excluded from *G. verna* L. Based on its common morphological criterion: a strongly winged calyx, they form a common species. Priority has the definition of Beck (1887): *G. tergestina* Beck, the others become *G. tergestina* Beck. subsp. *balcanica* Haemmerli and *G. tergestina* Beck. subsp. *pontica* Haemmerli. Separation criteria between *G. verna* L. and *G. tergestina* G. Beck is the presence of a stronger winged calyx in the later one {Ronniger 1915, Ho and Liu 2001).

The Sierra Nevada populations of *G. brachyphylla* Vill. are somehow close to *G. verna* L. and *G. tergestina* Beck, but clearly separated from Alpine *G. brachyphylla* Vill. They become excluded from that taxon and should be called *G. sierrae* Briquet.

Taxa not included in this study are *G. verna* L. subsp. *oschtenica* Halda, due to its geographical distribution in the eastern Black Sea region and the Caucasus as well as its morphology (Halda 1995; Halda 1996; Ho and Liu 2001) it is most probably also part of the eastern subspecies of former *G. verna* L. and would therefore become a subspecies of *G. tergestina* Beck. Also we miss the North African accessions of *G. brachyphylla* Vill. s.str. Following Romo (1988) who pointed out the proximity between these plants and those from Sierra Nevada, the North African taxon could hypothetically become either a subspecies to *G. sierrae* Briquet or then a close related independent taxon *G. penetii* Romo. Another point of question remains the identity of Apennine and southern Turkish populations of *G. brachyphylla* Vill. subsp. *favratii* Tutin. Tammaro (1986) established morphological differences between the Alpine, the Carpathian and the Apennine accessions of that taxon. Since we were able to unite the both first ones in *G. orbicularis* Schur, we estimate the third to be also part of the same species, but if that conclusion can be extended on the far isolated Turkish populations are unanswered. Finally we worry about the large and scattered distribution of *G. tergestina* Beck subsp. *pontica* Haemmerli, occurring in the Pontic Alps, Caucasus and connected ranges, then isolated and further east within the Elbours, the Pamir, the Tienschan and finally in a larger scatter in the Altai Mts. both in Russia but also China (Huang and Yang 1999). Far from enough sample material was obtained to enlighten the genetic variation within that taxon but the widespread occurrence over different mountain ranges worth's a closer look.

Taxonomy

New circumscription of *Gentiana* Sect. *Calathianae* Froel.:

Gentiana* Sect. *Calathianae Froelich, Gent. Dis. 57 (1796).
Lectotype: *G. nivalis* L., designated by Ho and Liu (2001)
= *Hippion* F.W. Schmidt, Fl. Boem. 2: 18 (1794).
= *Calathianae* Delarbre, Fl. Auvergne ed. 2, 1: 28 (1800).
= *Ericala* Renealm. ex. S.F. Gray, Net. Arr. Brit. Pl. 2: 335 (1821).
= *Ericoila* Renealm. ex Borkh. in Roem. Archiv i. I. 23 (1796)
= *Gentiana* Sect. *Ericoila* Dumortier, Fl. Belgica 51 (1827).
= *Gentiana* Sect. *Gentianella* Gaudin, Fl. Helv. 2: 270 (1828), non Dumortier.
= *Gentiana* Sect. *Hippion* (F.W. Schmidt) Gaudin, Fl. Helv. 2: 270 (1828).
= *Chiophila* Rafinesque, Fl. Tellur. 3: 25 (1837).
= *Gentiana* Sect. *Cyclostigma* Grisebach, Gen. Sp. Gent. 259 (1838).
= *Gentiana* Sect. *Vernae* Tzvelev. Fl. Russ. 3: 72 (1978).
= *Gentiana* Sect. *Cyclostigma* Ser. *Angulosae* Gagnidze et Ser. *Nivales* Gagnidze, Fl. Gruzii. 10: 311 (1985).
= *Gentiana* Subgenus *Calathianae* (Delarbre) Tzvelev, Bot. Zur. 78(6): 135 (1993).
= *Gentiana* Subgenus *Calathianae* (Froel.) Halda, Act. Mus. Richnov. 3: 32 (1995), non Tzvelev
= *Gentiana* Sect. *Calathia* DC, in Lam et DC, Fl. Fr. Ed. 3: 655

Annuals or perennials. Stems are sympodial, branched or unbranched arising from stolons or fibrous roots. Calyx tube angled or winged; corolla salverform, slender cylindrical tube, corolla lobes spreading; short plicae with 2-clef margin. Anthers free, stigma lobes expanded and connate or contiguous, forming a discoid structure closing the corolla tube, not free after anthesis. Seed coat thickened reticulate, winged or wingless.

Key to subsections

1a Annuals with fibrous roots, 2 to 35 cm tall, without sterile rosette; stems branched and many-flowered.*G.* Subsect. *Calathianae*
1b Perennials with stolons and freshly roots, stems unbranched with solitary and terminal flowers
  2a Plant 3-7 cm, leaves linear or linear lanceolate, apex acuminate and apiculate, calyx angled, not winged, corolla lobes more that twice as long as large .... *G.* Subsect. *Calathianae*
  2b Leaves elliptic to lanceolate, if lanceolate and apex acuminate and apiculate then calyx angled winged .... *G.* Subsect. *Vernae*

I. Subsect. *Calathianae*
Lectotype: *G. nivalis* L., designated by Ho and Liu (2001)
= *Gentiana* Sect. *Cyclostigma* Ser. *Nivales* Gagnidze, Fl. Gruzii. 10: 311 (1985)
= *Gentiana* Sect. *Cyclostigma* Griseb. sensu Tzevelev ex Fedorov, (1978). Flora Evropeiskoi Chasti SSSR. 3: 72 (1978)

Annuals or perennials. If annual then branched stems arising from a basal rosette with 1 to many terminal flowers. If perennial then unbranched stem with solitary terminal flowers, leaves lanceolate to linear lanceolate with an acuminate and apicular apex. Chromosome numbers 2n=14 to 2n=22.

Key to taxa

1a Perennial with unbranched stems ending in a terminal flower .... *G. pumila*
1b Annual with branched stems and many flowers.. 2
  2a Calyx tube (3-)4-7 mm wide, winged, wings 2-4 mm wide.. *G. utriculosa* L.
  2b Calyx tube 2-3 mm wide, angled but not winged.. *G. nivalis* L.

1. Gentiana nivalis L., Sp. Pl. 1: 229 (1753).
Type: not designated, sheet Linnaeus 328.16 [LINN] “Habitat in Lapponiae, Helvetiae summis alpibus.” proposed as lectotype by (Ho and Liu 2001).
Icon: Hess et al., Fl. Schweiz III, 31 (1972), Aeschimann et al. Fl. Alpina II, 22 (2003), Pignatti. Fl. Italia III, 340 (1982).
= *Gentiana prostrata* Schur, Enum Pl. Transs.: 459 (1866), non Haenke

2. Gentiana utriculosa L., Sp. Pl. 1: 229 (1753).

Type: not designated, sheet Linnaeus 328.18 [LINN] “Habitat in Alpibus Helvetiae, Italiae.” proposed as lectotype by Ho and Liu (2001).
Icon: Hess et al., Fl. Schweiz III, 31 (1972), Aeschimann et al. Fl. Alpina II, 24 (2003), Pignatti. Fl. Italia III, 340 (1982).
= *Gentiana bucovinensis* Herbich, Select. Pl. Rar. Galic. Bocov.: 10 (1836).

3. Gentiana pumila Jacq., Enum Stirp. Vindob. 41, 215 (1762).
Isotype: Austria, Wiener Schneeberg, N.J. Jacquin s.n. [BM]
Icon: Aeschimann et al. Fl. Alpina II, 20 (2003), Pignatti. Fl. Italia III, 339 (1982).
= *Gentiana imbricata* Willk. non Froel. in Nyman, Consp. 499 (1878).

II. Subsect. *Vernae* (Tzvelev) Haemmerli stat. nov.
Type: *G. verna* L., designated by Zvelev (1978)
≡ *Gentiana* Sect. *Vernae* Tzvelev ex Fedorov,. (1978). Flora Evropeiskoi Chasti SSSR. 3: 72 (1978)
= *Gentiana* Sect. *Cyclostigma* Ser. *Angulosae* Gagnidze, Fl. Gruzii. 10: 311 (1985).
Perennials with sterile rosettes and unbranched flowering stem. Leaves of different shape, flowers solitary and terminal. Chromosome numbers 2n=28-38 (40)

Key to taxa
1a Leaf apex rounded..2
  2a Basal leaf rosette present, leaves broadly elliptic to ovate, up to 10 x 8 mm, stem 0 to 5 cm .... *G. orbicularis*
  2b Basal leaf rosette absent, leaves crowded at the bottom or distant, leaves elliptic to oblong and more than twice as long as large, plant up to 15 cm or then leaves suborbicular to orbicular, stem very short to almost absent 3
    3a Basal leaves crowded forming a rosette-like arrangement, leaves suborbicular to orbicular, stem almost absent ....*G. bavarica* subsp. *subacaulis*
    3b Basal leaves not crowded into a rosette, leaves oblong to oval, stem short to long*G. bavarica* s.str.
1b Leaf apex acute to obtuse..4
  4a Basal leaf rosette absent, leaves crowded at the bottom or distant 5
    5a Leaves elliptic, 3 times as long as large or longer, apex without incurved tip ....*G. rostanii*
    5b Leaves ovate-lanceolate, ovate-orbicular or rhomboid, less than 3 times longer than large, apex with incurved tip..6
      6a Basal leaves more or less erect, not forming a rosette-like assembly ....*G. terglouensis*
      6b Basal leaves compressed into a rosette-like assembly, outer leaves covering the inner ones ....*G. schleicheri*
  4b Rosette at the base, largest basal leaves 1.5-3 times as long as stem leaves 7
      7a Leaves ovate-orbicular to rhomboid, not longer than 1 cm, apex acute ....*G. brachyphylla*
      7b Basal leaves rhomboid, elliptic to lanceolate, (0.5)1-3cm, apex obtuse to acute (then leaves lanceolate)..8
        8a Calyx angled or very narrowly winged, wings 1-2 mm wide
          9a Basal leaves lanceolate..*G. verna* subsp. *delphinensis*
          9b Basal leaves elliptic to ovate 10
            10a Basal leaves rhomboid..*G. sierrae*
            10b Basal leaves elliptic to ovate ....*G. verna* s.str.
        8b Calyx broadly and distinct winged, wings 2-4 mm wide..11
          11a Basal leaves broad, ovate .... *G. tergestina* subsp. *pontica*
          11b Basal leaves lanceolate to elliptic 12
            12a Basal leaves lanceolate, about 4 times as long as large, corolla 40-46 mm .... *G. tergestina* subsp. *tergestina*
            12b Basal leaves elliptic, not more than twice as long as large, corolla 28-37 mm.. *G. tergestina* subsp. *balcanica*

4. Gentiana verna L. Sp. Pl. 1: 228 (1753).
Type: not designated, sheet Linnaeus 328. 12 [LINN] "Habitat in alpibus Helvetiae." proposed as lectotype by (Ho and Liu 2001)
= *Gentiana arctica* Grossh, Acta Acad. Sci. Azerb. S.S.R. 3 (1): 32 (1947). ≡ *Gentiana verna* L. subsp. *arctica* (Grossh.) V.G.Sergienko, Fl. poluostrova Kanin: 85 (1986). ≡ *Gentiana verna* L. var. *arctica* (Grossh.) Tolm. Sensu Czerepanov, Vasc. Pl. Russ: 270 (1995)
= *Gentiana verna* L. var. *willkommiana* P.Monts. & L.Villar, Doc. Phytosoc., 914: 214 (1975). ≡ *Gentiana verna* L. subsp. *willkommiana* (P.Monts. & Villar) Rivas Mart., Asensi, Díaz, Fern. Gonz. & Loidi, Itinera Geobot., 5: 70 (1991).

4a. Gentiana verna L. subsp. ***verna***
Icon: Hess et al., Fl. Schweiz III, 30 (1972). Müller in Fed. Rep. 93 (9-10): 714 (1982). Pignatti. Fl. Italia III, 337 (1997), Aeschimann et al., Fl. Alpina II, 18 (2003)

4b. Gentiana verna L. subsp**. *delphinensis*** (Beauv.) H. Kunz, Exs. Genav. 23 (1971)
Type: France, Hautes Alpes, Orcières, "flanc septentrional de l'Autane d'Orcières", 2100 to 2600 m, 12.08.1932, Beauverd [G]
Icon: Aeschimann et al., Fl. Alpina II: 20 (2003). Müller in Fed. Rep. 93 (9-10): 714 (1982)
≡ *Gentiana delphinensis* Beauverd, Bull Soc. Bot. Geneve ser 2, 25: 314 (1934)
= *Gentiana pumila* Vill., Hist pl. Dauph. 2: 527 (1787) non Jacq..
= *Gentiana verna* L. var. villarsiana Rouy, Fl. Fr. 10:264 (1908).

5. Gentiana tergestina G. Beck, Ann. Naturh. Hofmus. Wien 2: 130 (1887).
Holotype: Yugoslavia, Bosnia, Trebovie, 31.5.1885, G. Beck s.n. [PRC]
≡ *Gentiana verna* L. subsp. *tergestina* (G. Beck) Hayek, Prodr. Fl. Penins. Balcan. 2: 420 (1930).
= *Gentiana chalybaea* G. Beck, Ann. Naturh. Hofmus. Wien 2: 130 (1887). ≡ *Gentiana tergestina* G. Beck var. *chalybaea* (G. Beck) Jovanovic-Dunjic, Fl. SSR Srbije 5: 424 (1973).

5a. Gentiana tergestina G. Beck subsp. ***tergestina***
Icon: Müller in Fed. Rep. 93 (9-10): 714 (1982). Pignatti. Fl. Italia III, 337 (1997)

5b. Gentiana tergestina G. Beck subsp. ***balcanica*** (Pritch.) Haemmerli, comb. nov.
Holotype: Yugoslavia, Bosnia, in mountains, calcicole, April 1885, G. Beck 99 [E, holotype; BM, isotype]
Icon: Rochel, Pl. Banat. Rar. T. 22 (1828), Halda, Gen. Gent. 128 (1996)
≡ *Gentiana verna* L. subsp. *balcanica* Pritch., Notes Roy. Bot. Gard. Edinbourg 35: 312 (1977)

5c. Gentiana tergestina G. Beck subsp. ***pontica*** (Soltok) Haemmerli, stat. nov.
Lectotype: Turkey, Trabzon, Zigana Pass, 23.5.1894, Sintenis 5626 [WU, E, designated by N.M. Pritchard, 1977].
Icon: Bieb. Cent. Pl. Rar. 1: t47 (1810), Halda
≡ *Gentiana pontica* Soltok., Oesterr. Bot. Z. 51: 169 (1901). ≡ *Gentiana verna* var. *pontica* Kusn., Mat. Fl. Kavk. 4: 352 (1904). ≡ *Gentiana verna* L. subsp. *pontica (Soltok.)* Hayek, Prodr. Fl. Penins. Balcan. 2: 419 (1930). ≡ *Gentiana angulosa* Bieb. var. *pontica* (Soltok.) Gagnidze, Fl. Gruzii, 10: 138 (1985).

6. Gentiana sierrae Briquet, Candollea 4: 323 (1931).
Type: Spain, Sierra Nevada, Willkomm, Iter Pl. Hisp. Nr. 199 & 200, June 1844, 752294 [BM]
Icon: Müller in Fed. Rep. 93 (9-10): 714 (1982)
≡ *Gentiana verna* L. subsp. *sierrae* (Briquet) Rivas-Martinez, Asensi, Molero Mesa et Valle, Rivasgodaya 6: 35 (1991)
= *Gentiana nevadensis* Solt., Oesterr. Bot. Zeitschr. 51: 170 (1901), non Gilg (1896). ≡ *Gentiana verna* L. subsp. *nevadensis* (Solt.) Litard. & Maire, Mem. Soc. Dci. Nat. Maroco 4: 15 (1924).
= *Gentiana favratii* Rittener sensu Küpfer, Bull. Soc. Neuchât. Sci. Nat. 91: 97 (1968).
= Gentiana brachyphylla Vill. sensu Tutin, Flora Europaea 3: 62 (1964).

7. Gentiana schleicheri (Vaccari) H. Kunz, Ber. Schw. Bot. Ges. 49: 160 (1939).
Type: not defined
Icon: Pignatti, Fl. Italia II, 340 (1997), Hess et al., Fl. Schweiz III, 30 (1930), Aeschimann et al., Fl. Alpina II, 24 (2003), Kunz, Ber. Schw. Bot. Ges. 49: 161 (1939)
≡ *Gentiana verna* L. var. *imbricata* f. *schleicheri* Vaccai, Bull. Trav. Soc. Murith. 36: 242 (1911). ≡ *Gentiana terglouensis* Hacq. subsp. *schleicheri* (Vaccari) Tutin, Bot. J. Linn. Soc. 64: 378 (1971). ≡ *G. verna* L. subsp. *schleicheri* (Vaccari) Bolo et Vigo, Fl. P. Cat. 3: 127 (1995).
≡ *Gentiana verna* L. var. *terglouensis* Hacq. f. *schleicheriana* Vaccari, Nuov. Giorn. Bot. Ital. 24 (4): 230 (1917)

8. Gentiana rostanii Reuter ex. Verlot, Cat. Pl. Dauphine 242 (1872).
Type: France/Italy, Monte Viso, August 1864, C. Rostan 77 [G, holotype; GH, K, isotypes]
Icon: Müller in Fed. Rep. 93 (9-10): 713 (1982), Aeschimann at al., Fl. Alpina II, 22 (2003), Hegi et al., Fl. Schweiz III, 30 (1972), Pignatti, Fl. Italia II, 338 (1927). Bonnier, Fl. ill. Fr. VII, pl. 408 (1850)
≡ *Gentiana bavarica* L. subsp. *rostani* (Reut.) Coste. Fl. Fr. 2: 565 (1901). ≡ *Gentiana bavarica* L. var. *rostani* Car et St. Lag. sensu Hegi, Ill. Fl. M. Europ. 5(3): 2018 (1927)

9. Gentiana bavarica L., Sp. Pl. 1: 229 (1753).
Types: not designated, sheets Linnaeus p.81 [BM] and 23:16 [S] "Habitat in alpibus Helvetiae, Bavaria." proposed as lectotype by Ho and Liu (2001)

9a. Gentiana bavarica subsp**. *bavarica***
Icon: Müller in Fed. Rep. 93 (9-10): 713 (1982), Pignatti, Fl. Italia II, 339 (1927), Hess et al., Fl. Schweiz III, 30 (1972), Bonnier, Fl. ill. Fr. VII, pl. 408 (1850)

9b. Gentiana bavarica subsp**. *subacaulis*** (Schl.) G. Müller in Fed. Repert. 93 (9-10): 658 (1982)
Types: not designated
Icon: Müller, Fed. Repert. 93 (9-19), 713 (1982).
≡ *G. bavarica* L. var. *subacaulis* Schleicher, Cat. Pl. Hel. Ed. 4, 18 (1821).

10. Gentiana terglouensis Hacquet, Pl. Carniolicae 9, t. 2, f. 3 (1782).
Type: Slovenia, Mt. Triglav, no date or collector information [LJM]
Icon: Müller in Fed. Rep. 93 (9-10): 713 (1982), Pignatti, Fl. Italia II, 340 (1997), Aeschimann et al., Fl. Alpina II, 22 (2003)
≡ *Gentiana verna* L. var. *terglouensis* (Hacquet) Rouy, Fl. France 10: 263 (1908). ≡ *Gentiana bavarica* L. subsp. *terglouensis* (Hacquet) P. Fourn. 4 Fl. Fr.: 858 (1961).
= *Gentiana imbricata* Froel., Gent. Diss. 74 (1796).

11. Gentiana brachyphylla Villars, Prosp. Hist. Pl. Dauphine 23 (1779).
Type: France, Dauphine, no further collection data [GRM]
Icon: Müller in Fed. Rep. 93 (9-10): 714 (1982), Aeschimann et al., Fl. Alpina II, 20 (2003), Pignatti, Fl. Italia II, 338 (1997), Hess et al., Fl. Schweiz III, 30 (1972). Bonnier, Fl. ill. Fr. VII, pl. 408 (1850)
≡ *Gentiana verna* L. var. *brachyphylla* (Vill.) Reichenbach, Pl. Crit. 3:11 (1825). ≡ *Gentiana verna* L. var. *brachyphylla* (Vill.) Grisebach, Gen. Et spec. Gent.: 263 (1839). ≡ *Gentiana verna* L. subsp. *brachyphylla* (Vill.) P. Fourn. 4 Fl. Fr: 858 (1961).

12. Gentiana orbicularis Schur, Verh. Mitt. Siebenb. Ver. Naturw. 3: 88 (1852).
Type: Romania, Mt. Arpas, 21.7.1850, Schur [W and WU, designated by (Ronniger 1926)]
Icon: Müller in Fed. Rep. 93 (9-10): 714 (1982), Aeschimann et al., Fl. Alpina II, 20 (2003), Hess et al., Fl. Schweiz III, 30 (1972), Pignatti, Fl. Italia II, 338 (1997)
= *Gentiana favratii* Rittener, Bull Soc. Vaud. Sci. Nat. 22: 198-201 (1887).. ≡ *Gentiana brachyphylla* Vill. subsp. *favratii* (Rittener) Tutin, Bot. Jour. Linn. Soc. 64: 378 (1971). ≡ *Gentiana verna* L. subsp. *favrati* (Rittener) P. Fourn. 4 Fl. Fr: 858 (1938).
= *Gentiana magellensis* (Vaccai ex Ronninger) Tamaro, Arch. Bot. Biogeogr. Ital. 62: 54 (1986).

Unplaced taxa

Gentiana penetii (Litard. & Maire) Romo, Lagascalia 15 (Extra): 228 (1988).
Lectotype: Morocco: "In Atlantis Majoris valle Reraya […] ad Izgoun Ouagens in con valle Ouenkrim, solo porphyrico, 3000-3150 m, 20.7.1924, R. Maire [MPU, designated by Romo (1988)].

Biogeography of G. Sect. Calathianae Froel.

Prior to sample collection for molecular analysis (Haemmerli *in publication c* ; Haemmerli *in press* b (aflp). Haemmerli *in publication a* (matK). Haemmerli *in publication b* (ncpGS). Haemmerli *in publication e*), a qualitative study on biogeography of that section was established upon herbarium collections, databases and literature. Digital information was obtained for Switzerland form the CRSF centre (Bäumler 1999) and for Europe and Asia from the Biological Collection Access Service (www.biocase.org). From 1834 observations providing precise locality information 1320 coordinate points for particular taxa were extracted. These digital sources were consisted of data from the Herbaria of Nancy, France [NCY], Strasbourg, France [STR], Harmas de Fabre, Sérignan-du-Comtat, France [FABR], Paris, France [P], Cherbourf, France [CHE], Linz, Austria [LI], Kiel, Germany [KIEL], Giessen, Germany [GI], Osnabrück, Germany [OSBU], Ulm, Germany [ULM], Reading, UK [RNG], Vienna, Austria [WU], Innsbruck, Austria [IBF], Oskarshamn, Sweden [OHN], Stockholm, Sweden [S], as well as the European Environment Information and Observation Network (EUNIS, The European Environment Agency EEA, Kopenhagen, Denmark) and the Zentralstelle für Phytodiversität Deutschland (ZePhyD, Bundesamt für Naturschutz BfN, Berlin, Germany).

Totally 843 sheets from European Herbaria were manually revised. Of 662 we were able to identify a precise location resulting into 495 geographical coordinates for particular taxa. These herbaria were: Neuchâtel, Switzerland [NEU], Geneva, Switzerland [G], Paris, France [P], Kiev, UK [K], London (British Museum), UK [BM], and Zagreb, Croatia [ZA, ZAHO]. Further Herbaria cited by literature are Munich, Germany [M], Vienna, Austria [W], Lausanne, Switzerland [LAU] and Barcelona, Spain [BC]. An illustrated overview is given in Fig. 7.

G. nivalis L.

Herbarium sheets were found from the Pyrenees, the Alps including Ligurian Alps toward the Apennine as well as the central Apennine itself, Scandinavia all over Norway and Sweden, along the southern Coastline of Greenland northward to 73°N (Ho and Liu 2001), Iceland, the western Carpathians (Tatra Mts.) as well as the Southern Carpathians and two smaller Areas in the central Dinaric Alps (Vranica Mts. and Bjelasnica Mts., Bosnia and Herzegovina, [ZAHO]) toward the Balkan Mts. (Rusoljia Mts., Montenegro and Sar Planina, FYR Macedonia [ZAHO]). From Bulgaria only one record (Mt. Vitosha [K]) was found. Literature indications are also made for the Caucasus region from NE Turkey over Georgia, Armenia and Azerbaijan (Ho and Liu 2001). The Northern Russian Caucasus Region is mentioned by Komarov (1967). Literature indications are also done for northern Arctic Russia (Komarov 1967; Ho and Liu 2001) extending the Scandinavian Area into Arctic Russia and Kola. For North America the Coastline of Labrador, Newfoundland and Quebec is indicated by Müller (1982)and Ho and Liu (2001). All authors refer also to populations in northern Scotland southward to 56°30’ without further indications. From the European Jura only few populations are known. Welten and Sutter (1982) indicate plants on Mt. Tendre (Switzerland) and a rare existence on Mt. Reculet (France) as on Mt. Chasseral (Switzerland).

G. utriculosa L.

G. utriculosa L. is a taxon restricted to European Mountain ranges. Herbarium indications were found for the French Alps southward to 45°45’ (Val d’Isère [BM]), covering Swiss Alps as well as the Bodensee Region (Welten and Sutter 1982) and Southern Germany along the Rhine. Specimens were found all along the Ligurian Alps down the Apennine and along the Dinaric Alps into the Balkan Mountains. An isolated area is located in the southern Carpathians (Vlahita, Romania [K]). Plants were also found in Munt Caraiman (Romania).

G. pumila Jacq.

That taxon is restricted to the south eastern end on the Alps, reaching from “Wiener Schneeberg” (Austria) in the north-east westward to the “Hohe Nock” (Windischgarsten, Austria) and southward to the Slovenian/Italian border as well as in the Karawanken between Austria and Slovenia. A single isolated population was identified from north eastern Italian Monte Palla (“Below Passo Mulaz, Gruppe di Pala, N. Italy”, 13.8.1928, W.C. Barton [BM]). The area of occurrence covers therefore Niederösterreich, Styria, Carinthia and Slovenia as well as Venetia. An often indicated presence of that taxon in the south western Alps (Rouy, Foucaud et al. 1893; Coste 1901; Fournier 1938) is due to its confusion with *G. verna* L. subsp. *delphinensis* Kunz and to misunderstandings based on synonyms as illustrated by Müller (1982). The presence of that taxon in the western Alps, the Pyrenees, the Cantabric Alps, the Apennine or even to the Carpathians found no prove in Herbarium collections.

G. bavarica L. s.str.

G. bavarica L. s.str. shows in combination with *G. rostanii* Reuter a vicariate distribution pattern. While the later one colonises the south western Alps, *G. bavarica* L. s.str. occurs in the western Alps southward to 45° N (Montgenèvre, M. Bélanger, 1862, [G]) as reported by Müller (1982). The taxon is found in the Swiss and Italian Alps and eastward, in southern German Alps and in Austria up to 14° E (Eisenhut bei Turrach, P. Strobl, 29.7.1875 [G]). Ho and

Liu (2001) also indicates a presence in former Yugoslavia, but in Herbaria no such indications were found. Soltokovic (1901) even indicates the presence on Raxalpe (Austria), slightly more eastward. Two populations of *G. bavarica* L. s.str. were also found in the Apennine (Mte Amoso, Majella, Italy, 2088 m, Pherdel, 7.1882 [BM Europe]; Mt. Vettore, Italy, "In alpis Mt. Settore a Vettore", Antoni Orsini [G]). Schur (1866) proposes the taxon for the Carpathians: "auf den Kronstädter Alpen", but any herbarium specimen for confirmation are missing so that we believe into confusion.

G. bavarica L. subsp. *subacaulis* Müller

Herbarium sheets clearly identified as *G. bavarica* L. subsp. *subacaulis* Müller were recorded from a restricted area covering mainly the central Swiss Alpine Chain with only four specimen outside that range (Cheval Blanc, Savoie, France, Briquet [G]; Passo Stelvio, Italy, Ambrosi, Francesco [G] and Cime di Gobetta, Italy, 2800m, s.n. [NEU], Gschnitz, Tirol, "In pratis alpinis montis "Muttenspitze" in valle Gschnitz", Schaffner [G]).

G. rostanii Reuter

As mentioned above, *G. rostanii* Reuter and *G. bavarica* L. s.str. share the Alpine chain as region of occurrence in a non-overlapping way. *G. rostanii* Reuter is strictly limited to the far south western end of the Alps (Cottian Alps, Ligurian Alps and Alp Maritime), reaching northward up to 45° N and colonizing both the French as also the Italian side of the Alps. We were not able to confirm an existence further northward as indicated one herbarium specimen (*G. bavarica* L., changed to *G. rostanii* Reuter by G. Müller, France, Basses-Alpes, "bords petit lac", Cormet de Roseland, ca 2400 m, 18.7.1963, [G]) by our own collections, same as for a presence in the Pyrenees or in Switzerland as proposed by Soltokovic (1901)

G. orbicularis Schur

In Europe *G. orbicularis* Schur is reported by Herbarium specimen from the Alpine chain east ward to central Austria, and from the Southern Carpathians (Muntij Bucegi, Romania, "Muntenia, distr. Prahova. In Alpe Caraiman montibus Bucegi. Alt cca 2100 m s. m., solo calc.conglom", 21.7.1925, A. Borza [BM]; Romania, Mt. Arpas, 21.7.1850, Schur [W]). Tammaro (1986) gives some further indications from the Southern Apennine as Campo Imperatore, Monte Corno, Mt. Arapietra and Campo Pericoli (Gran Sasso) as well as Mt. Majella, Mt. Rapina, Valle Orfenia, Valle Femina Morta and Mt. Amaro (Majella). A small occurrence is indicated by Pritchard (1977a) for Turkey: Kizil Tepe above Bulgar Maden, 2500m (Nigde, Taurus). The distribution area indicated by Kusnezow (1894) including also Sierra Nevada (Spain) and the Caucasus seems to be to large, possibly due to a confusion with *G. sierrae* Briquet and *G. tergestina* Beck subsp. *pontica* Haemmerli, while the existence in Turkey is often not mentioned by Soltokovic (1901). Halda (1996) splits the taxon into distant parts: one covering Alps, Spain, Balkan, Caucasus, Iran and Turkey; the other only the Carpathians, while Ho and Liu (2001) report the same distribution as we did above, understanding the taxon as *G. brachyphylla* Vill. subsp. *favratii* Tutin.

We were not able to prove the existence of that taxon within the Pyrenees since plants identified as *G. orbicularis* Schur (France, Haute-Pyrenees, Hourquette de Héas, 2288 m, R. Deutschle, M. Haemmerli, 10.07.2003, 398312 [NEU]) based on morphological characters turned out to be identical to *G. verna* L. s.str. from neighbouring populations in maturase K and 5S-NTS sequences (Haemmerli *in publication c* and c), while chloroplast PCR-RFLP gave a controversial result, rather pointing toward a connection of Pyrenean plants to Alpine and Carpathian *G. orbicularis* Schur (Haemmerli *in publication e*).

G. terglouensis Hacq.

G. terglouensis Hacq. is restricted to the south eastern Alps from Mt. Sciliar (Italy) in the west to the Hochobir in the east, occurring in Italy, Austria and Slovenia. An isolated population was also found on Hochschwab (Austria), northeast the main distribution area, as well as close to Passo Umbrail (Switzerland) at the Italian/Swiss borderline. Ho and Liu (2001) also indicate France and Soltokovic (1901) for the Italian maritime Alps, perhaps due to confusions with *G. schleicheri* Kunz. Meusel, Jäger et al. (1978) mention *G. pumila* Jacq. on Hochschwab, but not *G. terglouensis* Hacq. In Müller (1982) we can read about an austro-oriental occurrence in the Karawanken (Hochobir), Carinthia, Julian Alps, the Carniole Alps and the Tyrol toward Trentino/Alto-Adice with complete absence in the Rhetian Alps (Passo Umbrail)

G. schleicheri Kunz

That taxon represents the western Alpine opposite of the above *G. terglouensis* Hacq. with a presence from the Valais (Switzerland) westward, following the alpine chain though the Penninean, Savoyan and Cottian Alps into the Dauphine and Maurienne. Somehow isolated from its main distribution area, stations in the maritime Alps were identified: Mt. Mongioie (Italy) and Carsene-Margenaneis (France, Alpes-Maritimes, Tene, Massif Carsene-Margenaneis, "pelouses alpines rocailleuses, sur calcaire, versant S et SE du Carsene", Alt.2100 2100-2350 m, A. Charpin & R. Salamon, 6.8.1982 [G]). An occurrence in the Pyrenees as described by Müller (1982) found no proof neither in our herbarium research as in the field. One sheet was identified from Col de Puymorens (France, Southern Pyrenees) showing characters of *G. schleicheri* Kunz ("*Gentiana* tergestina, no 3136, Col de Puymorens, France 66" 11.7.58, J. Raynal [P]). Müller (1982) indicates them for Puigmal and Cambre d'Aze and identifies a herbarium sheet composed of *G. verna* L. and *G. schleicheri* Kunz from the Pyrenees (M197/647, *G. verna* L., Pyrenees orientals. Col de la Descargue east of Mont Canigou, above Corsavy, "Mattenhänge bei 1600-1450 m", 5.6.1967. H. Roessler, No 5588 [M]).

G. brachyphylla Vill.

In herbaria *G. brachyphylla* Vill. is reported from the entire Alpine chain starting in the French Maritime Alps through to central Austria (Admont, Steiermark). We weren't able to find any clear sheet from outside the Alps except one sheet identified as *G. brachyphylla* Vill. from the southern Pyrenees (*Gentiana verna*, No 3286, Eyne, France, 13.7.58, J. Raynal. [P]). Meusel, Jäger et al. (1978) postulate the taxon also for the Pyrenees and the central Cantabric Mountains, Ho and Liu (2001) even for the eastern European ranges in proposing an existence "throughout the range of species except Turkey" (and probably also except the far eastern mountains as Altai). Kusnezow (1894) even cites Grisebach by reporting a specimen from Scotland but remarks that a confirmation misses. Indications for the Carpathians can be found by Schur (1866): "auf den Keprereasze und Aroaser Alpen". Only one population from the Pyrenees was found by Müller (1982) on the Puigmal, together with *G. verna* L. and *G. schleicheri* Kunz. Other sheets from the Pyrenees are reported but not seen; "Pic du Midi de Bigorre, No. 27107", Merxmüller and Zollitsch [M] and "Port de Vénasque", Vetter, 18.7.1833 [LAU]. Bolòs and Vigo (1984) compares *G. brachyphylla* Vill. to *G. schleicheri* Kunz and doesn't mention a particular occurrence in Spain, not such as Rivas-Martinez, Izco et al. (1971), proposing the taxon for Picos de Europa. Müller (1982) saw specimen from that region and treated them as form of *G. verna* L. with short leaves as also found in the Pyrenees.

G. tergestina Beck s.str.

G. tergestina Beck s.str. has a distribution all over the Dinaric Alps. The northern border is linked with the end of the Alpine chain as defined by Aeschimann and Lauber (2004) respectively along a line from Gorizza (north of Trieste), passing south of Ljubiana and continuing toward Zagreb (Soltokovic 1901; Rogenhofer 1905). The taxon is present on the Istrian peninsula and follows the Dalmatic Coastline southward. Opposite to the sharp and well defined border at the northern end of the distribution with no or almost no overlapping which *G. verna* L. at the southern end of its occurrence in Albania, Greece and Bulgaria the taxon overlaps with an area also reported for *G. tergestina* Beck subsp. *balcanica* Haemmerli. The most southern specimen identified came from Greece (Mt. Vermion, Makedonia, Greece, "No. 8745, *Gentiana verna* L. var. aestiva (Schur.) Hayek, Macedonia occidentalis, In monte Vermion prope Maussa in pascuis alp., substr. Calc., alt 1900 m", K.H. Reichinger, identified as *G. verna* L. subsp. *tergestina* (G. Beck) Hayek by Ho T.N. 2000 [BM]; "Flora of Greece, No. L. 145, *Gentiana verna* L., Pindis Mts, Mr, Smolika summit, 8600 ft, open, among serpentine slopes", 26.07.1961 [K]). Halda (1996) proposes also an existence in France, same as two herbarium sheets that were found for France and Switzerland (France, Vallée de la Roya, Casterine, "*Gentiana* aestiva, Piemont: Casterino", 13-15.6.1933, identified as *G. verna* L. subsp. *tergestina* (G. Beck) Hayek by Ho T.n 2001 [BM]; Switzerland, Vaud, Mt. Jorat, "Praisies autour le Lac de But", Alt 650 m, R. Mason, 13. 4 1882 [BM]). Since the existence of plants of *G. verna* L. larger than usual in all parts, we doubt in the presence of *G. tergestina* Beck within the Alps which are also not accepted by Tutin (1964). Kusnezow (1894) is a possible source of irritation since he mentions the taxon for entire Asia and Europe.

G. tergestina Beck subsp. *balcanica* (Pritch.) Haemmerli

Ho and Liu (2001) indicates that taxon for the Balkan Peninsula and Turkey, same as Halda (1996). Hercarium collections predict a distribution area covering the Albanian, Greek, Bulgarian and FYR Macedonian Balkan Mountains as in Meusel, Jäger et al. (1978), overlapping with the taxon above. Unclear is the treatment for the southern Carpathians. Sheets as "Transsilvania, distr. Ciuc. In declivibus graminosis montis "Transsilvania distr. Ciuc. In pascuis montanis et subalpinis "Curmătura" montis Hăgimaşul Mare. Alt cca 1500-1600 m s. m., solo calc.", 27.05.1911, [BM, K] and "Transsilvania, Distr. Ciuc. In declivibus graminosus montis «Öcsém teteje» et «Terké». Alt cca. 1400 m s.m. solo calc.-hum. 22 May 1922" [BM, K] were identified as *G. tergestina* Beck subsp. *pontica* Haemmerli by T. Ho. But we could also face to integrate them within the actual taxon since Pritchard (1977b) mentions *G. tergestina* Beck subsp. *balcanica* Haemmerli beside the Balkan Mts. Also for Romania (Maja Pukus) and western Turkey.

G. tergestina Beck subsp. *pontica (Soltok.)* Haemmerli

G. tergestina Beck subsp. *pontica* Haemmerli represents an Asian taxon, dispersed from its most western population on Ulu Dagh (Bursa) all along the Pontic Alps eastward to the Caucasus further to the Caspian Sea as well as southward to the Lake of Van ("Flora of Turkey, No 31, *Gentiana verna* L., Doğubajazit -> Mt. Ararat (S. ascent) damper alpine flushes & nival zone species, alt 4000 m" 28.07.1966, D.J. Allen, H1349/74, 268 [K]; "Flora of Turkey, C10, Hakkari, Mor Dag. Mtn upland, stony patches, moist at melting snow dwarf flora, few, scattered, alt 3250m", A.R. Mitchell, 29.06.1967 [K]. From Ho and Liu (2001) indications for the Iraq are known. One herbarium sheet was also identified for the Iraq (" Region and District MRO, No 24825, *Gentiana verna*, Helgord Rande, 3000mm", A. J. Rais, 03.09.1957 [K]). In the Caucasus *G. tergestina* Beck subsp. *pontica* Haemmerli is present in the main ridge ("*Gentiana verna*, UDSSR: Georgian military highway, between Ordzhonikidze and Tbilisi, Gruzinskaya S.S.R, 1-2 km north of the Krestovyy Pereval. Rough meadow, scrub an steep hillside. 2300 m. Flowers bright sky-blue, stripes at side of tube. Coll: C.R. Fraser Jenkins, 4.7.71, No. 3045" [G]) close to Mt. Kazbek, but also from the southern small Caucasus ridge ("Transcaucasia, prov Tiflis distr Gori. In jugo Tzchra-Tzkaro prope pag. Bakuriani, in alpinis", W. Kozlowsky, 30.05.1923, H1056/87, 32 [K]). Meusel, Jäger et al. (1978) also report the taxon for the Iran, specially the Elbours Mountains north of Teheran, same as for far eastern Asia we found few herbarium indications for that taxon: "*Gentiana verna* L. var. *obtusifolia* Boiss., Mazandaran; E. side of Chalus Pass; 10'000 fts., in short alpine turf; dampish place", Furse & Synge 389, 5.6.1969, H.1349/74, 279 [K]; "*Gentiana verna* L. var. *obtusifolia* Boiss. Mts. Hazar Cham, near Khar Rustar, 9000 fts", A.L. Trodt, 8.1934, H.1349/74, 281 [K] and „ *Gentiana verna* L. v. *obtusifolia* Boiss. Persia borealis: Elbrus occid. in regione alpina montis Tachti Soleiman, ad nives prope Häsartschal, 40-4100 m s.m.", J. et A. Bornmüller, 29,6,1902, H.1349/74, 282 [K]. For eastern Russia Meusel, Jäger et al. (1978) indicate a scattered area is indicated in the Pamir and further the Tienschan as well as the Altai Mts. With an eastern border of distribution east of Lake Baikal. Further some isolated occurrences in neighbouring regions are also indicated. Descriptions from Komarov (1967) are relative vague. From Tienschan region one sheet was found („In descento Kirgisico vel in montibus Tarbagatai ant Ala-Tau" A. Schrenk, 1840, P00350806 [P]). Pritchard (1977b) treats *G. tergestina* subsp. *pontica* Haemmerli as central Turkish and far eastern taxon, while specimen from rather western Turkish Ulu Dag (Bursa) and Ilgaz Dag (Kastamonu) are called *G. verna* L. subsp. *balcanica* Pritch (*G. tergestina* subsp. *balcanica* Haemmerli). In Pritchard (1977b) we can read that the material from Ilgaz Dag has somehow an intermediate position between the two taxa, indicating the possibility of no sharp separation line between the distribution areas but rather more a zone of mixture.

The yellow flowering *G. verna* L. subsp. *oschtenica* Halda from the eastern black sea border and Iran ("*Gentiana verna* L. var. *pallida* Parsa, Iran borealis, Shahzade Kuh, Fields, 3100m s. m.", A. Parsa, 30.06.1941, H1349/74, 289, type [K]; "*Gentiana oschtenica* (Kusn.) Woren, Krasnoyarsky kray, Krasnaya Polyana, M. Aibga, alpine meadows", 13.06.1983, H. Mondav, No. 1952 [K]; "*Gentiana verna* var. *oschtenica* Kusnezov, Ciscasis: Mt. Khag, 2825 m", N.M. Albov, 17.8.1894 P00350785, [P]; Georgia, Napra Gora, "Abchazia, distr. Gutauty, jugum Bzybicum, in monte Napra, in lapidosis 2400 m", A. Kolakovsky, 20.07.1935, H.1344/74-297 [K]) was interpreted by Pritchard (1977b) as yellow form of *G. tergestina* Beck subsp. *pontica* Haemmerli.

<u>*G. verna* L. s.str.</u>

Herbarium indications for *G. verna* L. s.str. are extremely numerous, therefore only a small selection was recorded. From northern Spain specimen are known from Picos de Europa in Cantabric Mountains ("Picos de Europa: Fuente De. 1°7'2'' long, 43°9'20'' lat. Grid ref 50889510, Alt 1820-1880m, rocky limestone turf, area grazed by sheep & cattle, Flowers blue, 26 may 1969", K. Petrý, 26.07.1969 [BM]; Spain, Santander Province, "Above Fuente De, Picos de Europa, 15km due west of Pontes; in short mountain turf, abundant, Alt. 1750 m", R.K. Brummitt, 02.06.1972, 12839 [K]). The taxon is also known from all over the Pyrenees, and the entire Alpine chain including the Ligurian Alps as bridge to the Apennine, as well as the Jura mountains and the in an isolated pattern in the western Carpathians (Poland, Tatra, Barania Góra, "In montibus Tatra, ad Baranée, Com. Liptoviensis. Po miejsach trawaistych 1700 - 2000 m.n.p.m.", J. Ullepitsch, 1894 [BM, K]; Slovakia, Tatra, "Slovakia borealis, montes Malá Fatra supra pagum Kral'ovany: in pratis ed cacumen montis Stoh, solo calcareo, c. 1500 m s.m.", M. Deyl et P. Sillinger, 12.5.1930, [BM]). Sheets were also found from the southern Apennine ("Pastures of the Rafina: Mte Majella, Abruzzi, S. Italy, 7000' Aug. 1880", s.n. [BM]; "Mte Amoso, Abruzzi, Majella, 8000'", Pherdel, 7.1882 [BM]). Meusel, Jäger et al. (1978) indicates a separation between the Alpine pattern and the specimen from the central and southern Apennine by a zone of no inexistence in the northern Apennine.

Müller (1982) declares the taxon as missing in the Vosges and the Black Forest, but on the high plains east of the Rhine. Kusnezow (1894) and Fournier (1938) mention an existence in the Auvergne (France). An existence on the British Island is proved from several herbarium sheets ("Enclosure of Valance Lodge, Laugdon Beck", A.E. Gregor, 25.06.1918 [K]; "Harwood Beck nr Widdy Bank Fell", A.O. Hume, 19.05.1903 [K]; "*Gentiana* verna L., Vice-County Durham, Locality: Teesdale", A.G. More, 6.1837 [K]) as well as detailed indications in Elkington (1972), also providing localities for Ireland. Komarov (1967) also indicates the taxon for Scandinavia, but without that other references were found. Further he mentions *G. arctica* Grossh. as synonym to *G. verna* L. s.str. for the arctic European part of Russia, specially from Kolgoyev Island (Russia), as also proposed by Meusel, Jäger et al. (1978). Tzevelev (1987) also adds indications for Russia the basin of the Pinega River and the Timan Range, as well as an endemic existence close to village Maryan Gorka, Upper Dniepper Region (Belarus) with no proof by other authors.

G. verna L. subsp. *delphinensis* Kunz

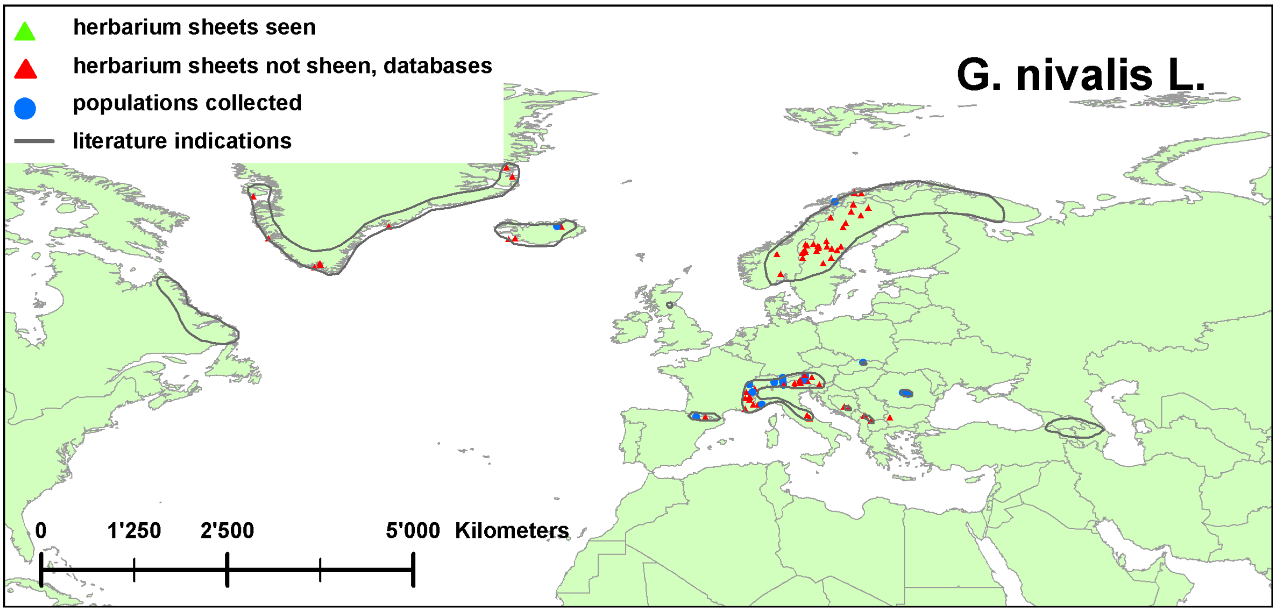
We were able to identify herbarium material from the south western border of the French Alps (France, Hautes-Alpes, “Aiguille d’Orsières, ca 2600”, G. Beauverd, 12.8.1933, 7717, type [G]; France, Hautes-Alpes, “Gallia: in jugo “pas de l’Echelle” ad septentrionem pagi “Saint-Geniez” provinciae Alpinum inferiorum, 1250 m supra maris aequor. In pascuis supra rubes calcareas abundans”, A. Charpin, W.R. Greuter, 12.05.1970 [BM], France, Hautes Alpes, “Chaillol-le-Petit en Valgaudemar (Htes-alpes): pâturages des montagnes”, Darnaud, 9.5/26.6.1883 [P]; France, Basses-Alpes, “2 km a pago “Majastres” meridiem versus secus viam (D.17), 1200 m. In clivis herbidis siccis ad boreooccidentum spectantibus, solo calcareo, inter Carices caryophylleas et Genistas pillosas abundans”, A. Charpin, W.R. Greuter, 25268 [G]; France, Vanoise, Pralognan-la-Vanoise, G. Beauverd [G]). Plants from the Pyrenees as indicated in Fournier (1938), Müller (1982) and Halda (1996) were not found in herbarium collections.

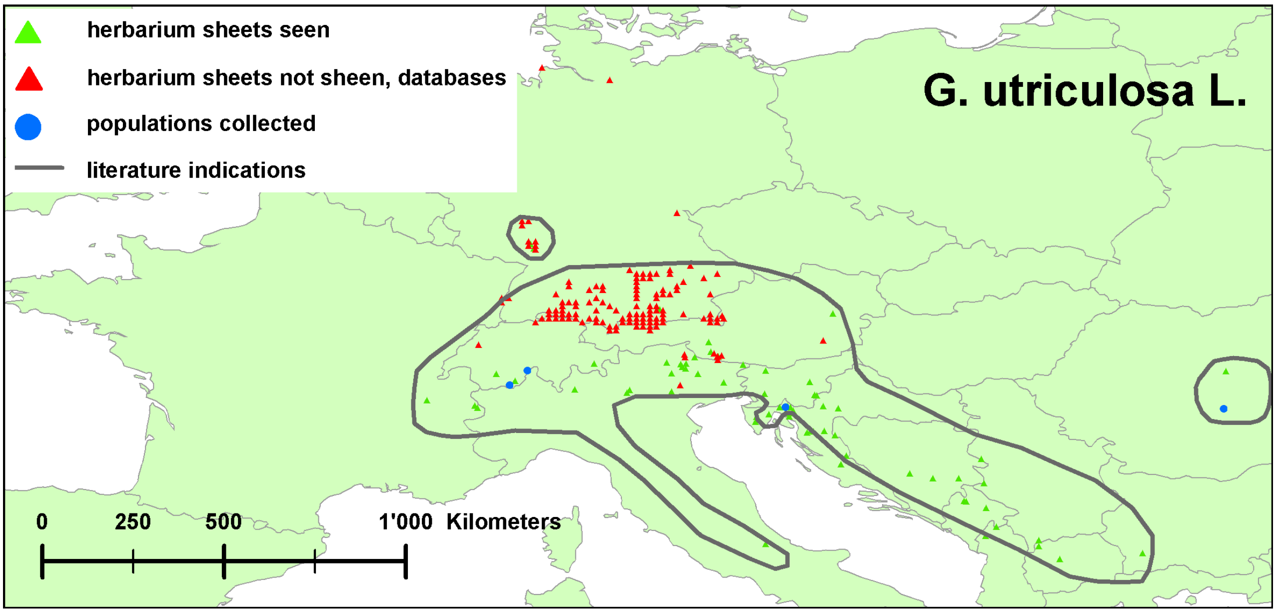
G. sierrae Briquet

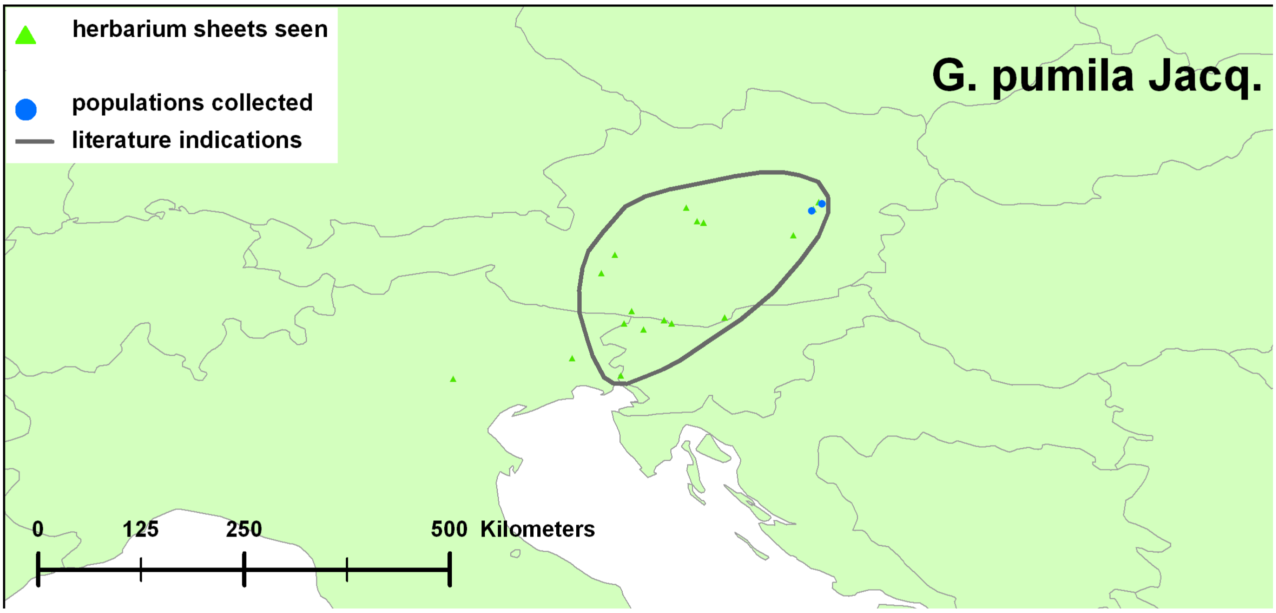
The taxon is isolated and restricted to southern Spanish Sierra Nevada range (Spain, Sierra Nevada, “8 km. N. of Laroles. UTM. VG. 90, Alt. 1800 m, Damp D-Facinf grassy slope, Fls deep blue, No 1634”, 12.06.1967, P.W. Ball [BM]; Spain, Sierra Nevada, “région neigeuse, au Barranco de S. Juan”, E. Bourgeau, 9.8.1851 [K]). Beside several specimen from Sierra Nevada. Romo (1988) also refers to a specimen from Sierra de Abrocema (Almeria, Spain): “Barranco de Campana, Gros, 2.7.1929 [BC].

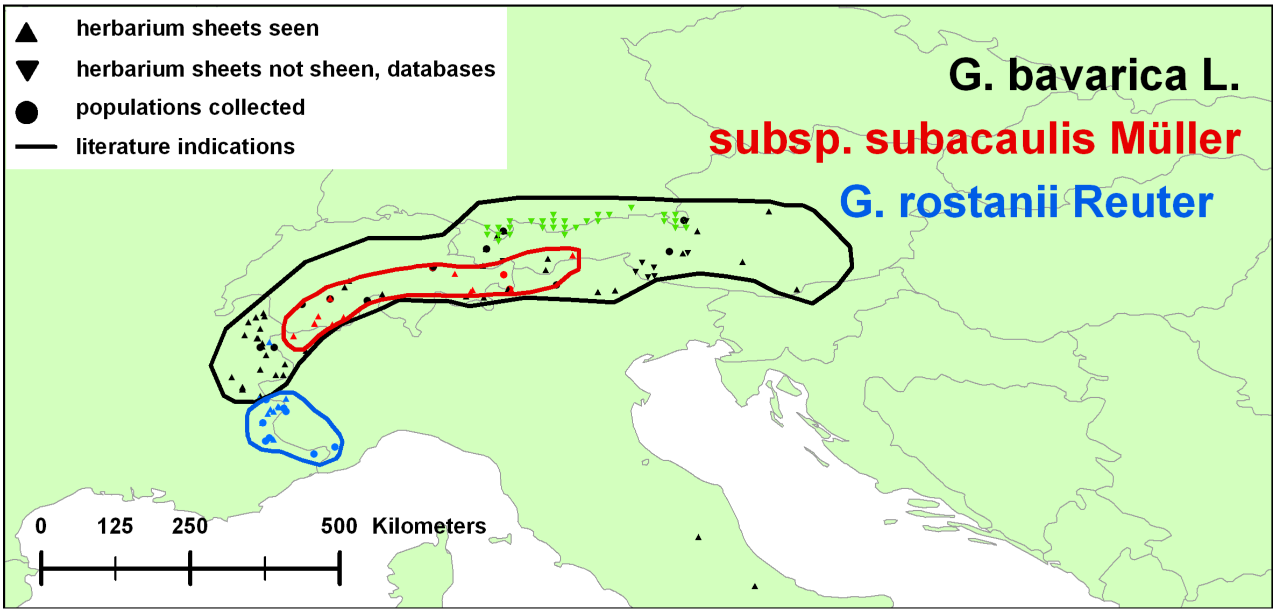
Müller (1982) threats *G. penetii* Romo from the North African Atlas Mts. as synonym to *G. sierrae* Briquet and includes therefore the High Atlas within the distribution area of the later taxon. Ho and Liu (2001) does the same by including *G. sierrae* Romo and *G. penetii* Maire within *G. brachyphylla* Vill. and extends its distribution area to Southern Spain and Northern Africa. Romo (1988)instead points out differences and indicates for *G. penetii* an occurrence in Ouemkrim, Tichka (Tizi n Tichka), M’Goun and Rhat Mountains beside the locus classicus close to Jebel Toubkal. Several sheets were found for that gentian in northern Africa: “Jebel Toubkal. Haut Atlas. Alt. ± 2200 m., No. 2278, Very moist mountain meadow. On places which are slightly elevated in the meadow.”, de Wilde and Dorgelo, 12.05.1961, 2278 [BM]; “*Gentiana verna*, Djebel Toubkal, 10500 ft. Wet turf” E.K. Balls, 21.6.1936, B2862 [BM], “*Gentiana verna* L. ssp. *penetii* R. Rit. et Maire, In Atlantis Majoris valle Reraya: in pascuis turfosci con valli Ouenkrim, solo porphyrico, 2700-3150 m.”, Maire, 20.7.1924, lectotype [P]).

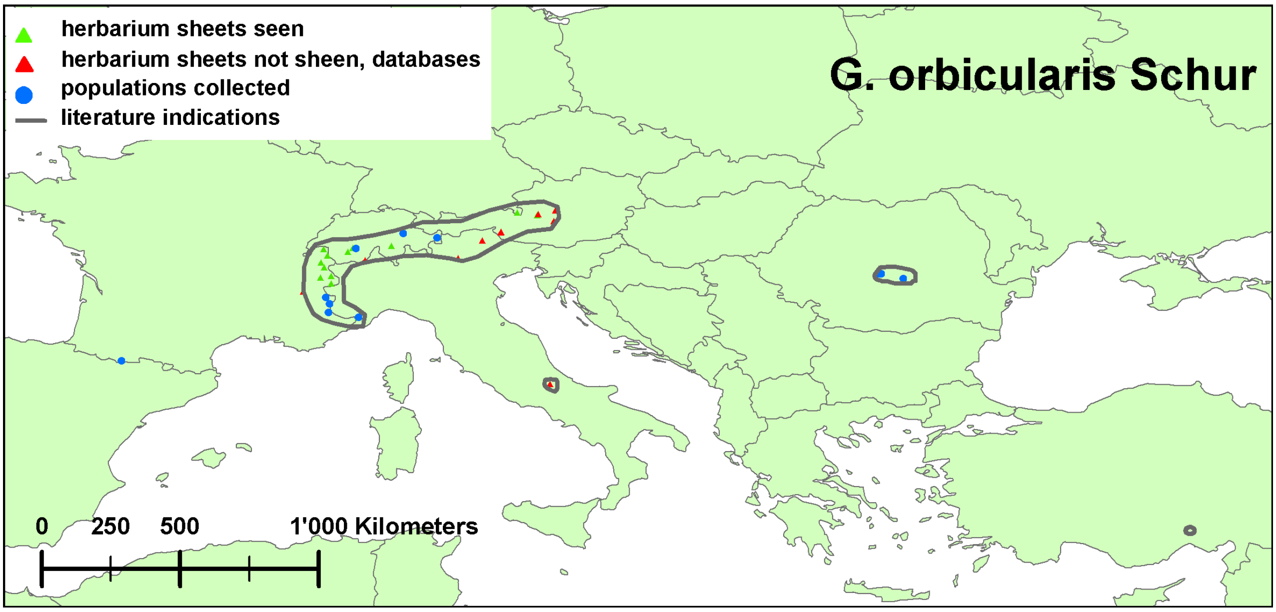
Figure 7 (3 pages): Distribution patterns of species from *Gentiana* Sect. *Calathianae* Froel. Derived from herbaria collections, database and literature references (for detailed list of included collections: see text).

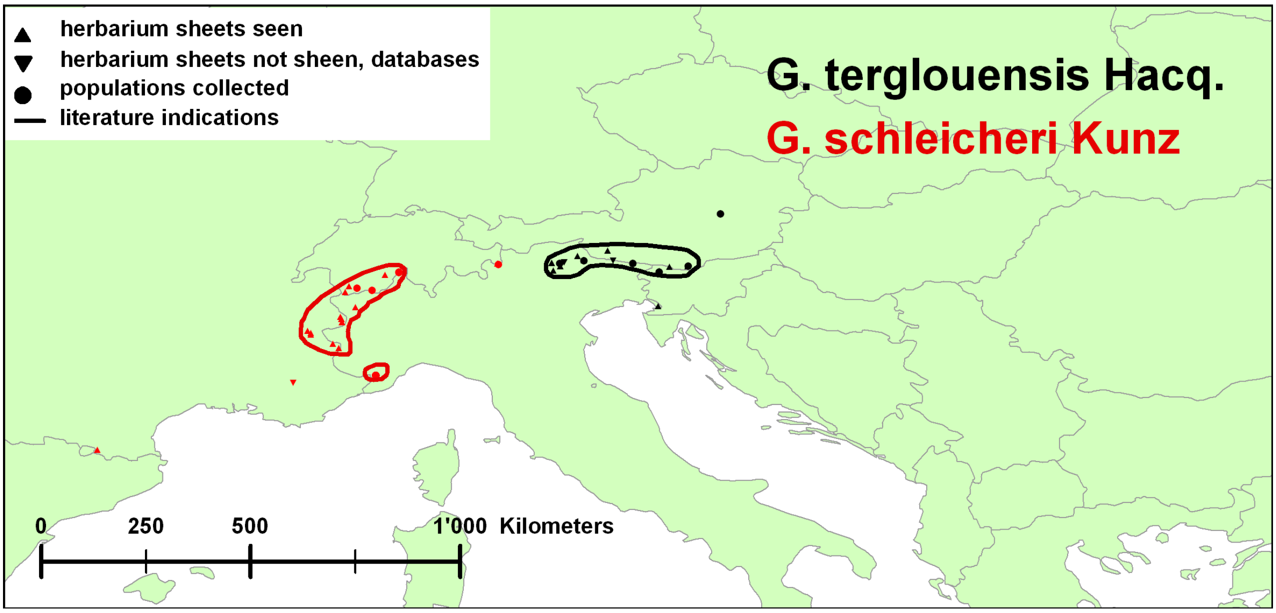

G. nivalis L.
▲ herbarium sheets seen
▲ herbarium sheets not sheen, databases
● populations collected
— literature indications
0
1'250
2'500
5'000 Kilometers

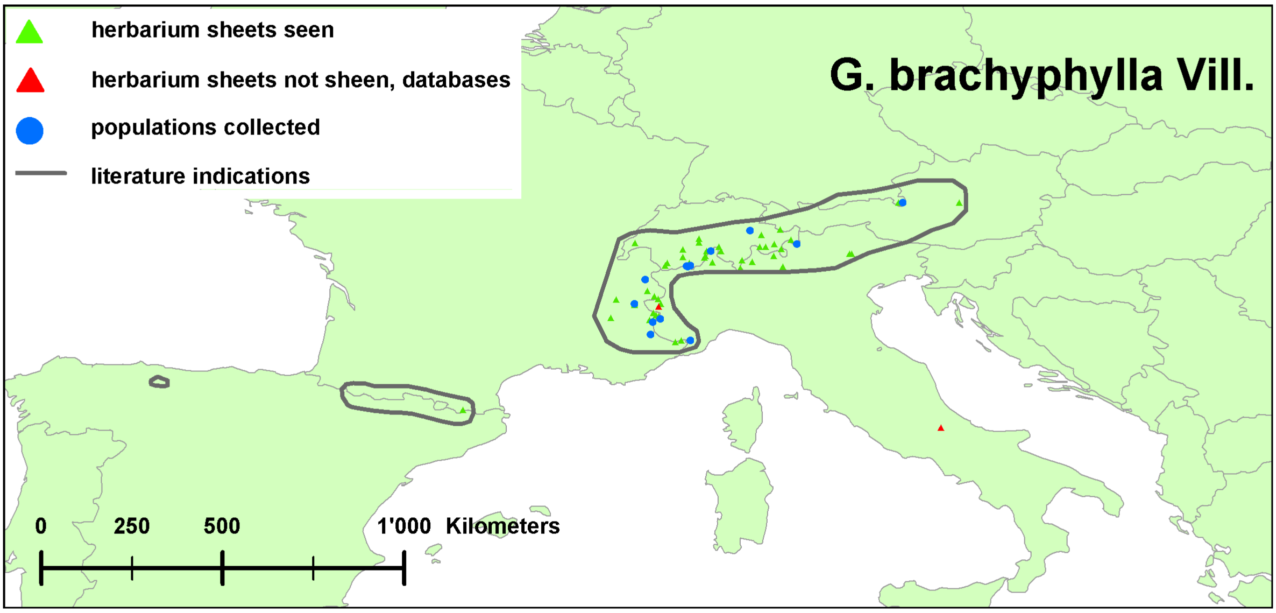

G. utriculosa L.
▲ herbarium sheets seen
▲ herbarium sheets not sheen, databases
● populations collected
— literature indications
0
250
500
1'000 Kilometers

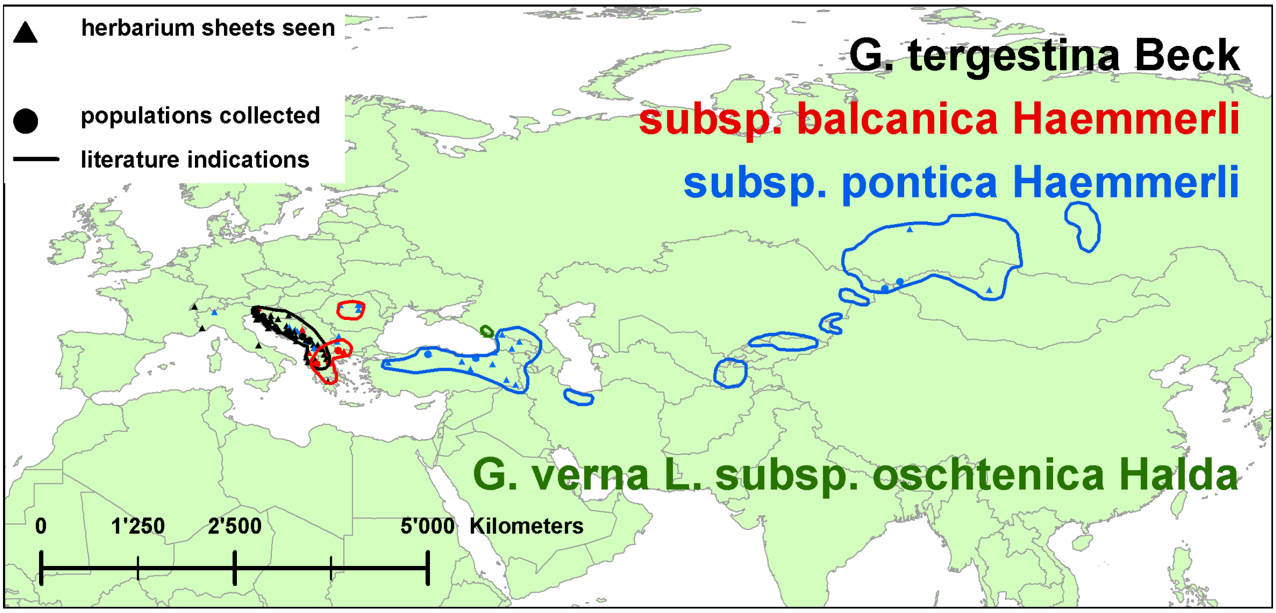

G. pumila Jacq.
▲ herbarium sheets seen
● populations collected
— literature indications
0
125
250
500 Kilometers

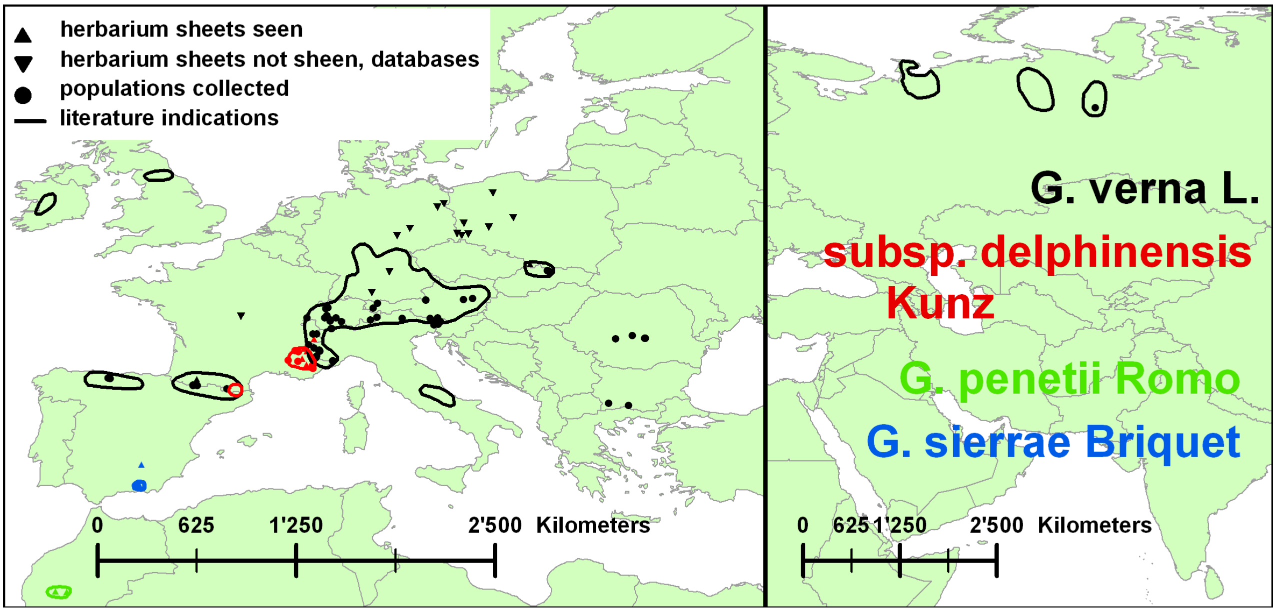

▲ herbarium sheets seen
▼ herbarium sheets not sheen, databases
● populations collected
— literature indications
G. bavarica L.
subsp. subacaulis Müller
G. rostanii Reuter
0
125
250
500 Kilometers


▲ herbarium sheets seen
▲ herbarium sheets not sheen, databases
● populations collected
— literature indications
G. orbicularis Schur
0
250
500
1'000 Kilometers


▲ herbarium sheets seen
▼ herbarium sheets not sheen, databases
● populations collected
— literature indications
G. terglouensis Hacq.
G. schleicheri Kunz
0
250
500
1'000 Kilometers


herbarium sheets seen
herbarium sheets not sheen, databases
populations collected
literature indications
G. brachyphylla Vill.
0
250
500
1'000 Kilometers


herbarium sheets seen
populations collected
literature indications
G. tergestina Beck
subsp. balcanica Haemmerli
subsp. pontica Haemmerli
G. verna L. subsp. oschtenica Halda
0
1'250
2'500
5'000 Kilometers


herbarium sheets seen
herbarium sheets not sheen, databases
populations collected
literature indications
0
625
1'250
2'500 Kilometers
G. verna L.
subsp. delphinensis Kunz
G. penetii Romo
G. sierrae Briquet
0
625
1'250
2'500 Kilometers

Synthesis

Final conclusions

Gentiana Sect. *Calathianae* Froel. is composed of two subsections, *Calathianae* and *Vernae* (Tzevelev) Haemmerli. Subsection *Calathianae* comprises three taxa: *G. pumila* Jacq., *G. nivalis* L. and *G. utriculosa* L., the first which is perennial, while the other two are annual. In common are the lower chromosome numbers compared to the remaining taxa within the section (Müller 1982). As shown in maturase K sequencing, the differences between these three taxa are significant compared to the remaining taxa. Subsection *Vernae* (Tzevelev) Haemmerli has the remaining perennial taxa: *G. verna* L. s.str., *G. verna* subsp. *delphinensis* Kunz, *G. bavarica* L. s.str., *G. bavarica* subsp. *subacaulis* Müller, *G. rostanii* Reuter, *G. brachyphylla* Vill., *G. orbicularis* Schur, *G. terglouensis* Hacq., *G. schleicheri* Kunz, *G. sierrae* Briquet and *G. tergestina* Beck s.str., subsp. *balcanica* Haemmerli and subsp. *pontica* Haemmerli.

Taxa in particular

G. verna L. is represented by subsp. *verna* from the Cantabric Mountains, the Pyrenees, the Alps, Jura, Apennine and the northwestern Carpathians (Tatra Mountains) as well as subsp. *delphinensis* Kunz in the Southwestern Alps. The AFLP analysis shows the existence of a geographic distribution pattern for genotypes within that species. While the northern and central Alpine chain and the Jura Mountains are populated by one only genotype, distinct genotypes are present within the Southwestern Alps and in another case in the Cantabric Mountains and the Pyrenees. The eastern European and Asian spring gentians represent different taxa to the western and central European ones as above. Gentiana *tergestina* Beck s.str. subsp. *balcanica* Haemmerli and *pontica* Haemmerli were represented as related taxa as much in chloroplast PCR-RFLP and maturase K as also in nuclear ncpGS and 5S-NTS sequencing. In the AFLP analysis all populations representing these taxa are placed in a very heterogeneous cluster clearly separated from *G. verna* L.

In Spanish Sierra Nevada the spring gentians are represented by *G. sierrae* Briquet that has relations to the eastern *G. tergestina* Beck s.l. in three sequences examined.

In relation to *G. verna* s.l. are the western Alpine *G. schleicheri* Kunz and the Alpine and Carpathian *G. orbicularis* Schur. Both taxa are overlapping in their distribution with *G. verna* but differ from those by chromosome numbers (Müller 1982) and in their ecological parameters (Ho and Liu 2001, Aeschimann 2004, Hegi 1927). *G. brachyphylla* Vill. subsp. *favratii* Tutin from the Alps were identified as synonymous with *G. orbicularis* Schur from the Carpathians. An existence of that taxon in the Pyrenees found no proof in our analysis.

G. bavarica L. s.str. and *G. rostanii* Reuter are two related but clearly distinguishable taxa. While the first populates the Eastern, Central and northwestern Alps southward to a line between Briançon (France) and Turin (Italy). Southward of that separation line *G. bavarica* L. is replaced in its ecological environment by *G. rostanii* Reuter. In the ncpGS analysis a relatedness between the geographical close western populations of G. *bavarica* L. and *G. rostanii* Reuter was shown while the farer eastern *G. bavarica* L. took a more distant position.

G. brachyphylla Vill. was identified as an independent species with no clear relations to the others. Excluded from that taxon were subsp. *favratii* Tutin that became part of *G. orbicularis* Schur, but also *G. sierrae* Briquet. Due to a lack of material the question whether the northern African *G. penetii* Romo also to be excluded also, can not clearly be answered but seems probable due to its proximity to *G. sierrae* Briquet (Romo 1988).

Two taxa with possible hybrid origin were identified: a) *G. terglouensis* Hacquet s.str. as close related taxon to *G. bavarica* L. in chloroplast markers but as basal most taxon within Subsection *Vernae* (Tzevelev) Haemmerli in nuclear ncpGS and 5S-NTS sequencing; b) *G. bavarica* L. subsp. *subacaulis* Müller with *G. bavarica* L. s.str. as closest relative in chloroplast PCR-RFLP and 5S-NTS sequencing, *G. brachyphylla* Vill. in maturase-K and *G. orbicularis* Schur in ncpGS sequencing.

Consequences of present study

Taxonomy in *Gentiana* Sect. *Calathianae* Froel. is revised in the present work. Different taxa are elevated into a higher taxonomic rank or were displaced to other groups compared to previous authors such as Ho and Liu (2001), Halda (1996), Müller (1982) and Tutin (1964). In consequence distribution patterns of taxa included have to be revised as done in our biogeography section.

As such we are able to show the value as independent taxon in case of *G. sierrae* Briquet from Spanish Sierra Nevada, representing local endemics together with the north African *G. penetii* Romo. Since both taxa are restricted to small areas of tourist and agricultural interest a adapted concept for protection different from those of other taxa is necessary.

Further action seems to be necessary to prevent European lowland populations of *G. verna* L. and *G. utriculosa* L. from extinction although these taxa are less endangered in higher altitudes. In Eastern Europe, *G. tergestina* Beck. s.str. is another species colonising lower summits and occurring even on lowland close to the Adriatic sea. As the example of possibly disappeared populations close to the Italian town Trieste illustrate, that taxon should also become subject to adapted protection measures, especially since the independence from *G. verna* L. is proved by our data.

As illustrated by AFLP data the southwestern populations of *G. verna* L. s.str. and subsp. *delphinensis* Beck partially represents a genotype restricted to that region, same as the Cantabric Mountains and the Pyrenees. Protection of these types has an elevated importance especially in the French Maritime Alps with their lower summits.

In case of *G. orbicularis* Schur we were able to show the relatedness of Alpine to Carpathian populations, although also by demonstrating the absence of complete equalness. Its separation from *G. brachyphylla* Vill. underlines the necessity of protection of that rather vulnerable taxon (Ludwig and Schnitter 1996, Conti, Manzi et al. 1997). It is obvious that both distribution areas host local types with their own local importance for protection. Even higher is this importance in case of the more restricted the Italian, Turkish and southern Carpathian populations not examined in present study.

G. schleicheri Kunz becomes separated from *G. terglouensis* Hacq. and represents a relatively rare and independent taxon for the western Alps with "near threatened" status (IUCN 1994, IUCN 2991) for Switzerland (Moser, Gygax et al., 2002) "rare" for France (Olivier, Galland et al. 1995) and "lower risk" for Italy (Conti, Manzi et al. 1997).

Perspectives

In both *G. verna* L. and *G. tergestina* Beck s.l. sample material from several interesting geographic regions is missing in the present study. As such a gap is present in our analysis for *G. verna* L. from the British and Irish islands (Elkington 1972), but also from the Apennines and in some case also from the southern Alpine chain. AFLP analysis retrieves for central and rather southern Alpine populations of this taxon a possible divergence from the northern Alpine genotype. In case of the Apennines even a contact to *G. tergestina* Beck s.str. from the Dinaric Alps is possible.

Of special interest should be the proof of proposed taxonomy for eastern European and Asian taxa of *G. tergestina* Beck s.l.. While the Dinaric Alps, Balkan Mountains and the Carpathians were sampled in a substantial way, of the largely distributed plants from the Asian continent an appropriate representation is missing. AFLP analysis groups these samples in one, very diverse cluster with low internal correlation indicating the possible existence of more but so far hidden characters for differentiation. It is highly probable that our study neglects important characters since they are not represented in our raff sampling of that region. An appropriate sampling of that large area would be necessary to enlighten the taxonomy of these taxa.

Another point of interest is the relationship of southern Spanish *G. sierrae* Briquet to the northern African *G. penetii* Romo respectively to the eastern European and Asian taxa. It was not possible to get actual material of *G. penetii* Romo from its *locus classicus* of from its other rare places of occurrence within the Atlas Mountains illustrating once more the importance of protection for that only taxon of *Gentiana* Sect. *Calathianae* Froel. endemic to Northern Africa.

The identity of *G. orbicularis* Schur has been proven within the Alps and the Carpathians. The taxon is also reported from the Apennine and from Southern Turkey. Material possessing a morphology and ecology similar to *G. orbicularis* Schur from the Pyrenees has been clearly identified as *G. verna* L. by all sequences as much as by PCR-RFLP analysis, demonstrating the capacity of morphological adaptation of *G. verna* L. The question upon the identity of *G. orbicularis* Schur from the Apennine and the highly isolated populations in southeastern Turkey remains therefore doubtful.

Müller (1982) published a large number of chromosome numbers for *Gentiana* Sect. *Calathianae* Froel. Present taxonomy is highly compatible to these results, but still the precise mechanism of chromosomal evolution is still unknown. Since a basal chromosome number of perennial Subsect. *Vernae* (Tzevelev) Haemmerli 2n=30 could be identified since represented in different distinct and far related taxa. 2n=28 as present in *G. verna* L. and *G. brachyphylla* Vill. should have been evolved therefore two times. Since the relatedness of Alpine and Carpathian *G. orbicularis* Schur was shown, these populations with both 2n=32 could lead back to a common origin. The large number of taxa possessing 2n=30 illustrates the reduced usefulness of chromosome numbers to explain the taxonomy of that section. It would be desirable to elaborate a deeper knowledge about morphology and structure of these chromosomes to understand their evolution and to find coincidences with present results. Still unexplained also is the large difference of chromosome numbers between the two subsections *Vernae*(Tzvelev) Haemmerli and *Calathianae*.

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Oral presentation

2007: Biology07, the Annual Meeting of the Swiss Zoological, Botanical and Mycological Societies, ETH Zürich, Switzerland, 15 to 16 February 2007

New insights into *Gentiana* Sect. *Calathianae* Froel. (*Gentianaceae*) based on chloroplast PCR-RFLP in combination with chloroplast matK and nuclear ncpGS-sequences

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Gentiana Sect. *Calathianae* Froel. is a small section defined by strong morphological similarities and growing mainly in European and Asian mountain ranges with each one taxon in Northern Africa and North America. Based on a chloroplast PCR-RFLP from 8 different non-coding regions, each digested by 4 enzymes, and chloroplast matK as well as nuclear ncpGS-sequences, a detailed new order of that is developed, composed by an annual and a perennial group of taxa, the perennial one itself divided into basically three components: one hosting most non-*G.-verna*-taxa, one comprising of western European *G. verna* and related taxa and the last built by eastern-*G.-verna* taxa. The classification is supported both by morphological and geographical criteria.

Poster presentation

2005: XVII International Botanical Congress, Vienna, Austria, 17-23 July 2005

Phylogeny of *Gentiana* Sect. *Calathianae* Froel. based on chloroplast PCR-RFLP

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Gentiana Sect. Calathianae is distributed in Europe, Asia, North America and North Africa, primarily occupying mountainous to alpine habitats. A phylogeny based on chloroplast PCR-RFLP is presented, including 18 taxa with 250 accessions, using 8 different non-coding regions, each digested with 4 enzymes covering about 15% of the plastid genome. Different monophyletic lineages can be recognised, such as the two annuals *G. nivalis*. and *G. utriculosa* and the eastern Alpine *G. pumila* s.str. that is separate from *G. pumila subsp. delphinensis*. The taxa of *G. verna* s.l. are divided into an Alpine/Pyrenean clade and another covering the southeastern European mountains. *G. brachyphylla* s.str. from Alpine populations is placed in a separate lineage where it groups neither with *G. brachyphylla subsp. favratii* nor with plants from southern Spain. *G. bavarica* and *G. rostani* represent two distinct monophyletic species placed in different subclades.

							5s		matK		ncpgs		rflp
taxon	mountain range	country	locality	coordinates	collector / date	herbarium voucher	haplo-type	GenBank accession	haplo-type	GenBank accession	haplo-type	GenBank accession	halpo-type
G. bavarica L.													
	Alps												
subsp. *bavarica*													
		Austria											
			Nassfeld, Grossglockner	N 47°7'2.5'' E 12°49'28.5'', Alt: 2212 m	MH, 16.08.2002	NEU 398304	26	EF626755	1	EF552071			ba01
			Spullersee	N 47°02'21.1'' E 10°04'12.300, Alt: 1841 m	MH, 07.08.2004						5	EF571637	
		France											
			Cormet de Roseland	N 45°40'20.6'' E 6°40'5'', Alt: 2356 m	MH, 02.08.2002	NEU 398305			1	EF552072	1	EF571635	ba01
		Germany											
			Hochbrett	N 47°35'2.9'' E 13°2'53.3'', Alt: 2317 m	MH, 09.08.2004	NEU 398315			1	EF552073	6	EF571636	ba01
			Gaissalp, Oberstdorf	N 47°25'14.1'' E 10°19'44.1'', Alt: 1873 m	MH, 08.08.2004	NEU 398314	26	EF626754	1	EF552074	7	EF571638	ba01
		Switzerland											
			Binntal	N 46°22'38.7'' E 8°16'37.1'', Alt: 2230 m	MH, 02.07.2002	NEU 079441	26	EF626753	2	EF552079			ba01
subsp. *subaccaulis* Müller													
		Switzerland											
			Flims	N 46°52'46.5'' E 9°15'57.2'', Alt: 2508 m	MH, 20.07.2002	NEU 079436	27	EF626791			11	EF571601	bs01
			Lötschenpass	N 46°23'0'' E 7°43'0'', Alt: 2690 m	MH, 27.06.2003	NEU 079437	28	EF626756	16	EF552115	12	EF571600	bs01
			Val Lischana	N 46°46'5.5'' E 10°20'3'', Alt: 2447 m	MH, 07.09.2003								bs01
G. brachyphylla Villars													
subsp. *brachyphylla*													
	Alps												
		France											
			Col de la Moutière	N 44°19'15.7'' E 6°47'47.3'', Alt: 2414 m	MH, 26.06.2002	NEU 398290			16	EF552117	13	EF571603	br03
			Col de Tronchet	N 44°37'0'' E 6°50'0'', Alt: 2000 m	AL, MH, 29.05.2003	NEU 398291							br02
		Switzerland											
			Flims	N 46°52'46.56'' E 9°15'57.24'', Alt: 2460 m	MH, 20.07.2002	NEU 079790	25	EF626758					
			Umbrail	N 46°32'55.56'' E 10°25'17.46'', Alt: 2768 m	MH, 19.08.2002	NEU 079789	25	EF626759					
			Zermatt	N 46°0'0.7'' E 7°41'21.7'', Alt: 2382 m	MH, 30.05.2002	NEU 079438	25	EF626757	16	EF552116	14	EF571602	br02
	Sierra Nevada												
		Spanien											
			Sierra Nevada	N 37°5'11.2'' W 4°37'29'', Alt: 2487 m	RD, MH, 23.07.2003	NEU 398292	21	EF626773	5	EF552100	24	EF571607	br01
subsp. *favratii* Tutin													
	Alps												
		France											
			Col de l'Izoard	N 44°49'6.29'' E 6°43'57.83'', Alt: 2248 m	MH, 05.08.2002	NEU 398307	10	EF626761	6	EF552112	8	EF571639	
			Col de Restefonds	N 44°20'18.54'' E 6°49'43.55'', Alt: 2517 m	MH, 04.08.2002	NEU 398303	11	EF626760			9	EF571641	
		Italy											
			Mt. Mongioie	N 44°10'7.5'' E 7°47'25.6'', Alt: 2145 m	PK, MH, 03.08.2002	NEU 398313			6	EF552102	9	EF571640	bf02
		Switzerland											
			Segans Sura	N 46°53'44.3'' E 9°14'50.6'', Alt: 2498 m	MH, 22.09.2003								bf03
			Val Lischana	N 46°45'44.5'' E 10°20'31.3'', Alt: 2647 m	MH, 07.09.2003								bf03
	Carpathians												
		Romania											
			Munt Caraiman	N 45°25'0'' E 25°27'0''	LN, 27.07.1999		9	EF626792	3	EF552080			bf01
			Muntii Bucegi	N 45°26'7.9'' E 25°27'15'', Alt: 2407 m	M&AR, 27.07.2004		9	EF626793	3	EF552081	10	EF571642	bf01
			Muntii Fagarasului	N 45°35'0'' E 24°45'0''	LN, 04.08.1999								bf01
			Muntii Fagarasului	N 45°35'24'' E 24°39'30.9'', Alt: 2330 m	M&AR, 03.08.2004				3	EF552082			bf01
	Pyrenees												
		France											
			Hourquette de Héas	N 42°45'21.3'' E 0°6'32.7'', Alt: 2288 m	RD, MH, 10.07.2003	NEU 398312	17	EF626765	4	EF552083			bf02

taxon	mountain range	country	locality	coordinates	collector / date	herbarium voucher	5s haplo-type	 GenBank accession	matK haplo-type	 GenBank accession	ncpgs haplo-type	 GenBank accession	rflp halpo-type
G. nivalis L.													
	Alps												
		Germany											
			Geissfuss, Oberstdorf	N 47°24'0" E 10°20'10.2", Alt: 1854 m	MH, 08.08.2004	NEU 398310	4	EF626776	19	EF552121	35	EF571610	ni01
			Pfaffenkegel, Berchtesgaden	N 47°34'36.9" E 13°2'31.1", Alt: 1873 m	MH, 09.08.2004	NEU 399330							ni01
		France											
			Cormet de Roseland	N 45°41'2.8" E 6°40'46.2", Alt: 2057 m	MH, 02.08.2002	NEU 398308	4	EF626777			35	EF571611	ni01
		Italy											
			Mt. Mongioie	N 44°10'22.4" E 7°47'30.4", Alt: 2316 m	PK, MH, 03.08.2002	NEU 398309			21	EF552123	36	EF571619	ni01
		Switzerland											
			Flims	N 46°52'2.9" E 9°15'47.4", Alt: 2059 m	MH, 20.07.2002	NEU 079787					35	EF571612	ni01
			Umblail	N 46°32'57.7" E 10°25'17.5", Alt: 2768 m	MH, 19.08.2002	NEU 079785	4	EF626778			36	EF571620	ni01
	Carpathians												
		Poland											
			Tatra Mts.	N 49°14'0" E 19°59'0", Alt: 1952 m	ZM, 25.08.2005		2	EF626783			35	EF571613	ni01
		Romania											
			Munt Caraiman	N 45°25'0" E 25°27'0", Alt: 2200 m	LN, 27.07.1999		1	EF626781					ni04
			Muntii Fagarasului	N 45°37'5.9" E 24°46'15.9", Alt: 2347 m	M&AR, 03.08.2005		3	EF626782			35	EF571614	ni03
	Island												
			Myvatn	N 65°38'25.9" W 17°5'8.4", Alt: 283 m	RA, 01.08.2003		4	EF626779	21	EF552124	35	EF571615	ni01
	Jura												
		Switzerland											
			Mont Tendre	N 46°36'0.5" E 6°19'8.9", Alt: 1672 m	PD, 15.07.2005						35	EF571616	ni01
	Norway												
			Ungsfjorden	N 68°37'54.4" E 16°35'30.2"	AG & DH, 29.06.2005	NEU 398429	4	EF626780	20	EF552122	35	EF571617	ni02
	Pyrenees												
		Spain											
			Col de la Bernatoire	N 42°43'7.5" W 1°53'53.2", Alt: 2305 m	RD, MH, 08.07.2003	NEU 398311							ni01
G. pumila Jacq.													
	Alps												
subsp. *pumila*													
		Austria											
			Raxalp	N 47°41'12.6" E 15°42'3.8", Alt: 1847 m	GK, MH, 13.06.2004								pu01
			Wiener Schneeberg	N 47°45'57.9" E 15°48'53.8", Alt: 1853 m	MH, 17.08.2002	NEU 398289	5	EF626788	18	EF552120	34	EF571618	pu01
subsp. *delphinensis* Fourn.													
		France											
			Col de Festre	N 44°40'10.3" E 5°50'43.7", Alt: 1538 m	AL, MH, 29.05.2003	NEU 398317			4	EF552084	18	EF571630	pd02
			Mt. Ventoux	N 44°10'53.1" E 5°15'19", Alt: 1411 m	AL, MH, 31.05.2003	NEU 399301			4	EF552084			pd03
			Mtgne de Lure	N 44°7'8.7" E 5°48'59.5", Alt: 1680 m	AL, MH, 31.05.2003	NEU 398316							pd03
			Vauplan, Picogu	N 43°52'54.2" E 6°38'30.3", Alt: 1685 m	MH, 26.06.2002	NEU 398325	14	EF626764	4	EF552084			pd01
G. rostanii Reuter													
	Alps												
		France											
			Col de Cayolle	N 44°16'7.5" E 6°44'48", Alt: 2213 m	MH, 04.08.2002	NEU 398279	29	EF626789	14	EF552113	3	EF571599	ro01
			Col de Var	N 44°32'28" E 6°42'8", Alt: 2091 m	MH, 25.06.2002	NEU 398278	29	EF626790	15	EF552114	4	EF571598	ro01
G. terglouensis Hacq.													
	Alps												
subsp. *terglouensis*													
		Austria											
			Hochobir	N 46°30'22.8" E 14°29'14.2", Alt: 2123 m	MH, 18.08.2002	NEU 398280	8	EF626785	1	EF552075			te02
			Hochschwab	N 47°37'30.3" E 15°10'42.6", Alt: 1854 m	MH, 11.07.2004	NEU 398281	6	EF626786					te02
		Italy											
			Passo Sella	N 46°33'48.6" E 11°43'55.3", Alt: 2330 m	MH, 15.06.2002	NEU 399297	7	EF626784	1	EF552076	32	EF571627	te01

							5s		matK		ncpgs		rflp
taxon	mountain range	country	locality	coordinates	collector / date	herbarium voucher	haplo-type	GenBank accession	haplo-type	GenBank accession	haplo-type	GenBank accession	halpo-type
G. terglouensis Hacq.													
	Alps												
subsp. *terglouensis*													
		Slovenia											
			Triglav Mts.	N 46°22'47.8'' E 13°51'58.3'', Alt: 2154 m	MH, 07.07.2004	NEU 398282	8	EF626787	1	EF552077	31	EF571621	te02
		Switzerland											
			Umbrail	N 46°32'55.5'' E 10°25'17.4'', Alt: 2768 m	MH, 19.08.2002				1	EF552078			ba01
subsp. *schleicheri* Tutin													
		Italy											
			Mt. Mongioie	N 44°10'2.5'' E 7°47'20.7'', Alt: 2099 m	PK, MH, 03.08.2002	NEU 398306	13	EF626762	4	EF552087	16	EF571622	ts02
		Switzerland											
			Binntal	N 46°22'32.7'' E 8°17'33.1'', Alt: 2340 m	MH, 02.07.2002	NEU 079442							ts01
			Val de Dix	N 46°2'29.0'', E 7°23'30.0'', Alt: 2595 m	LN, 08.08.1997		12	EF626763			15	EF571623	
G. utriculosa L.													
	Alps												
		Switzerland											
			Binntal	N 46°22'25.6'' E 8°13'4.3'', Alt: 1790 m	MH, 02.07.2002	NEU 079435			17	EF552118	33	EF571609	ut01
	Carpathians												
		Romania											
			Munt Caraiman	N 45°25'0'' E 25°27'0'', Alt: 2100 m	LN, 28.07.1999								ut02
	Dinaric Alps												
		Croatia											
			Risnjak	N 45°28'17.3'' E 14°37'1.8'', Alt: 972 m	MH, 06.07.2004	NEU 398266			17	EF552119			ut01
G. verna L.													
subsp. *verna*													
	Alps												
		Austria											
			Gartnerkofel	N 46°34'19.3'' E 13°17'49'', Alt: 1864 m	MH, 10.07.2004	NEU 398323	15	EF626769	4	EF552088	22	EF571623	ve04
			Hochschwab	N 47°36'58.7'' E 15°10'44.8'', Alt: 1621 m	MH, 11.07.2004	NEU 398302							ve04
			Raxalp	N 47°41'15.4'' E 15°42'53.4'', Alt: 1346 m	G. Karrer, MH, 13.06.2004	NEU 398296			4	EF552089			ve02
		France											
			Col de la Traversette	N 44°42'28'' E 7°2'53.8'', Alt: 2457 m	MH, 25.06.2002	NEU 398299			4	EF552090			ve04
			Col de Larche	N 44°25'21.5'' E 6°53'29.8'', Alt: 2013 m	MH, 25.06.2002	NEU 398405							ve05
			Mt. Gondran	N 44°53'31.6'' E 6°43'19.3'', Alt: 2360 m	MH, 24.06.2002	NEU 398298							ve04
			Pt St.-Bernard	N 45°40'39.7'' E 6°52'42'', Alt: 2177 m	MH, 01.08.2002	NEU 398300							ve04
		Germany											
			Hochbrett bei Berchtesgaden	N 47°35'2.9'' E 13°2'53.3'', Alt: 2317 m	MH, 09.08.2004	NEU 398301			4	EF552091	19	EF571625	ve04
		Italy											
			Mt. Majur	N 46°12'43.8'' E 13°31'45.5'', Alt: 1638 m	AL, MH, 03.06.2003	NEU 398272							ve04
			Passo Sella	N 46°33'48.6'' E 11°43'55.3'', Alt: 2330 m	MH, 15.06.2002	NEU 398406							ve04
		Slovenia											
			Mt. Krn	N 46°14'19.9'' E 13°45'3'', Alt: 1547 m	AL, MH, 02.06.2003	NEU 398275			4	EF552092			ve04
			Triglav Mts.	N 46°22'8.2'' E 13°52'7.3'', Alt: 1574 m	MH, 07.07.2004	NEU 398324			4	EF552093			ve04
		Switzerland											
			Gurnigel, Nünenenberg	N 46°43'1.9'' E 7°27'6.1'', Alt: 1690 m	MH, 21.05.2002	NEU 079444							ve04
			Pontresina, Alp Languard	N 46°29'10.9'' E 9°55'9.6'', Alt: 2248 m	MH, 31.05.2002								ve04
			Zermatt, Alp Hermettji	N 45°59'56'' E 7°43'6.2'', Alt: 2412 m	MH, 30.05.2002	NEU 079445							ve04
	Cantabrian Mountains												
		Spain											
			Peñas de Hachero	N 43°10'3.5'' W 5°12'1'', Alt: 1797 m	MLC, MH, 04.07.2005	NEU 398322							ve03
			Rosada de Valedon	N 43°10'19.4'' W 5°4'1.3'', Alt: 1642 m	MLC, MH, 05.07.2005	NEU 398320							ve04

							5s		matK		ncpgs		rflp
taxon	mountain range	country	locality	coordinates	collector / date	herbarium voucher	haplo-type	GenBank accession	haplo-type	GenBank accession	haplo-type	GenBank accession	halpo-type
G. verna L.													
subsp. *verna*													
	Carpathians												
		Poland											
			Przelecz miedzy	N 49°14’57” E 20°01’0”, Alt: 1560 m	MR				4	EF552094			
		Romania											
			Muntii Bucegi	N 45°26’7.9” E 25°27’15”, Alt: 2407 m	M&AR, 27.07.2004				7	EF552103			ve08
			Munt Caraiman	N 45°26’0” E 25°28’0”, Alt: 2250 m	LN, 28.07.1999				8	EF552104			ve10
			Muntii Fagarasului	N 45°35’0” E 24°45’0”, Alt: 2200 m	LN, 03.08.1999				7	EF552105			ve09
			Muntii Latoritei	N 45°24’45” E 23°47’45”, Alt: 1870 m	M&AR, 16.08.2004								ve07
	Jura												
		Switzerland											
			Chasseral	N 47°7’28.8” E 7°23’42.5”, Alt: 1609 m	MH, 26.05.2002		16	EF626771	4	EF552095	21	EF571605	ve01
			Mont Tendre	N 46°36’0.5” E 6°19’8.9”, Alt: 1672 m	MH, 22.06.2002	NEU 079440	20	EF626766			20	EF571606	ve04
	Pyrenees												
		France											
			Col de Puymorens	N 42°33’39.4” E 1°48’36.9”, Alt: 1923 m	MLC, MH, 06.07.2005	NEU 398321	18	EF626770	4	EF552096	23	EF571608	ve01
			Hourquette d’ Ossue	N 42°45’52.6” W 1°53’8”, Alt: 2436 m	RD, MH, 07.07.2003	NEU 398293			4	EF552097			ve01
			Hourquette de Héas, Gavarnie	N 42°45’18” E 0°6’35.9”, Alt: 2400 m	RD, MH, 10.07.2003	NEU 398297			4	EF552098			ve01
	Ural												
		Russia											
			Seydre	N 63°4’57.1” E 67°2’7.1”	AT, 01.07.2004		20	EF626767	4	EF552099	17	EF571604	ve06
subsp. *balcanica* Pritchard													
	Pindus Mountains												
		Greece											
			Olympos Mt.	N 40°4’37.2” E 22°22’14.6”, Alt: 2256 m	MH, 26.06.2004	NEU 398268			12	EF552111			vb02
			Timfi Mts.	N 39°57’11.2” E 20°51’23.2”, Alt: 2025 m	MH, 23.06.2004	NEU 398267	23	EF626772	8	EF552106	26	EF571631	vb01
subsp. *pontica* Hayek													
	Altai Mountains												
		Russia											
			Sarzhematy Peak	N 49°31’56” E 88°38’23.9”, Alt: 3000 m	AT, 05.08.2003		22	EF626768	13	EF552112	27	EF571633	vp01
	Pontic Mountains												
		Turkey											
			Ilaz Dag	N 41°4’4.8” E 33°46’56.4”, Alt: 2084 m	MH, 28.07.2005	NEU 398318			11	EF552110	25	EF571632	vp02
			Zigana Pass	N 40°38’45.3” E 39°23’41.9”, Alt: 2156 m	WI & MH, 30.07.2005	NEU 398319			10	EF552109			vp03
subsp. *tergestina* Hayek													
	Dinaric Alps												
		Bosnia & Herzegovina											
			Vran Planina	N 43°39’11” E 17°31’59.2”, Alt: 1233 m	MB, MH, 17.06.2004	NEU 398273							vt01
		Croatia											
			Mt. Ucka	N 45°17’2.4” E 14°12’6.8”, Alt: 1382 m	AL, MH, 01.06.2003	NEU 398274							vt02
			Risnjak	N 45°28’17.3” E 14°37’1.9”, Alt: 975 m	MH, 06.07.2004						30	EF571626	vt01
		Montenegro											
			Cakor Pass	N 42°39’43.1” E 19°59’30.9”, Alt: 1611 m	MH, 01.07.2004	NEU 398276	24	EF626775	9	EF552108	29	EF571628	vt01
			Njegovuda	N 43°8’40.2” E 19°13’29.2”, Alt: 1315 m	MH, 02.07.2004	NEU 398277							vt01
		Slovenia											
			Mt. Caven	N 45°55’48.2” E 13°51’31.7”, Alt: 1253 m	AL, MH, 02.06.2003	NEU 398271	19	EF626774	8	EF552107	28	EF571629	vt01

taxon	mountain range	country	locality	coordinates	collector / date	herbarium voucher	5s haplo-type	 GenBank accession	matK haplo-type	 GenBank accession	ncpgs haplo-type	 GenBank accession	rflp halpo-type
outgroup													
G. ciliata L.	Alps	Switzerland	Alp Mora	N 46°36’27.6” E 10°16’27.4”, Alt: 2308 m	MH, 02.10.2004	NEU 079443							out2
G acaulis L.	Alps	Switzerland	Jaun	N 46°35’0.0” E 7°20’0.0”, Alt: 1540 m	MH, 21.05.2002	NEU 079802	out	EF626794	out 1	EF552125	out	EF571643	
G. prostrata Haenke	Alps	Austria	Edelweisspitze	N 47°7’29.30“ E 12°50’1.63“, Alt: 2577 m	RD, MH, 30.07.2000				out 2	EF552126			out1

Collectors were: AG: A. Guggisberg; AL: A. Lendel; AT: A. Tribsch; DH: D. Heizmann; GK: G. Karrer; LN: L. Nussbaumer; M&AR: M. & A. Ronikier; MB: M. Bedalov; MH: M. Haemmerli; MLC: M.L. Cheung; DP: P. Druart; PK: P. Küpfer; RA: R. Aeschlimann; RD: R. Deutschle; WI: W. Imhof; ZM: Z. Mirek