



# The intraspecific genetic variability of siliceous and calcareous *Gentiana* species is shaped by contrasting demographic and re-colonization processes



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## ABSTRACT

The *Ciminalis* section of *Gentiana* comprises seven species, two of them growing on siliceous substrates (*G. alpina* and *G. acaulis*), the other ones being calcareous taxa (*G. clusii*, *G. angustifolia*, *G. ligustica*, *G. occidentalis* and *G. dinarica*). A total of 515 individuals from 183 populations over the entire *Ciminalis* distribution range was analyzed using four chloroplast loci (*trnH-psbA*, *matK*, *rpoB* and *rpoC1*) and the nuclear ribosomal marker ITS2. The siliceous species display only two chloroplast haplotypes each and are both characterized by patterns of range expansions all over the Alps. Conversely, the calcareous species are on average more diverse (two to 13 haplotypes per species) with strong patterns of local structuring. We suggest that the occurrence of many calcareous refugia at the periphery of the Alps must have led to local adaptation and morphological diversification, and helped preserving intraspecific diversities during the last glaciations for the associated taxa. ITS2 was more efficient in delineating species boundaries than the chloroplast markers for which several haplotypes are shared among species. This might be either due to chloroplast capture among species and/or to recent divergence. Species adapted to the same substrate are generally only distantly related when they co-occur in the same place. For both types of markers, *G. clusii* is found genetically distant from all other species.

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## 1. Introduction

Quaternary climatic oscillations are one of the most recent major elements that affected the composition and diversification of the European flora and fauna (Hewitt, 1999, 2000; Willis and Niklas, 2004; Schmitt, 2007). The sequence of long-lasting glacial periods, intersected by short times of warming during the last two million years at least, led to major modifications of the entire European biota (Cheddadi et al., 2005; Tzedakis et al., 2009). The main consequences resulting from climatic oscillations were extinctions (Svenning, 2003), range shifts as well as range contractions and expansions (Taberlet et al., 1998; Zhang et al., 2001; Kropf et al., 2003; Arenas et al., 2012), that sometimes resulted in secondary contacts and hybridization between close but previously disjoint species (Choler et al., 2004; Dixon et al., 2007).

Contractions as well as expansions or shifts might have occurred both during the glacial or the interglacial periods depending on species characteristics (Despres et al., 2002; Schönswetter et al., 2006; Dixon et al., 2007; Treier and Müller-Schärer, 2011).

Studies on temperate species mainly focused on the consequences of the last glaciation and the recent expansion that occurred with postglacial warming (Taberlet et al., 1998). For most of them, glacial oscillations involved latitudinal migration between North and South, with sources of diversity mainly found in the Iberian Peninsula and the Balkans as well as on the Italian Peninsula to a lesser extent (Sommer and Zachos, 2009). For cold adapted species however, the response to glacial oscillations involved both latitudinal and altitudinal shifts. Distinct parts of the range at different periods could have presented important constraints while others might have provided more opportunities for survival, leading to complex and dynamic processes. The number, type, location and accessibility of refugia must therefore have had an important influence on alpine species survival, also because they are more circumscribed for alpine than for temperate species (Holderegger and Thiel-Egenter, 2009). These refugia are now well known, among them the Eastern and

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Southwestern Alps (Tribtsch and Schönschwetter, 2003; Schönschwetter et al., 2005). Nunataks, defined as mountains or peaks that remained unglaciated within the ice sheet, were also proven to be important refugia for some alpine plant species (Stehlik et al., 2002; Holderegger et al., 2002; Bettin et al., 2007; Holderegger and Thiel-Egenter, 2009). This is the case of lowlands too, since long-distance migration and range shifts allowed for connections between different alpine habitats across the European mountain system (Schmitt, 2009). As for temperate species, the Iberian, Italian and Balkan peninsulas therefore helped to preserve the diversity of cold adapted species, leading also to diversification and hybridization (Vargas, 2003; Gomez and Hunt, 2007).

Many studies tried to find correlations between area of putative peripheral refugia or nunataks and species genetic diversity and abundance (Tribtsch, 2004; Schönschwetter et al., 2004, 2005; Mráz et al., 2007; Naciri and Gaudeul, 2007; Puşcaş et al., 2008 among others). Studies that investigated other factors, such as altitudinal range shifts (Soto et al., 2010; Casazza et al., 2008), or that compared patterns from different species (Comes and Kadereit, 2003; Tribtsch and Schönschwetter, 2003; Vargas, 2003; Médail and Diadema, 2009; Alvarez et al., 2009; Thiel-Egenter et al., 2011) usually draw complex patterns (Nieto Feliner, 2011). Strong substrate affinities, and ecological vicariance, are reported for many montane and alpine species, more than for temperate species (Zhang et al., 2001; Comes and Kadereit, 2003; Kropf et al., 2006). This aspect has been investigated in the light of diversity (Wohlgemuth and Gigon, 2003; Ewald, 2003) but few studies have correlated species genetic diversity and phylogeography with varying substrate affinities (Conti et al., 1999; Alvarez et al., 2009; Meirmans et al., 2011), despite the fact that peripheral refugia are highly structured according to substrate type (Tribtsch and Schönschwetter, 2003).

In this study, we investigate the phylogeography of one of the 12 sections of the *Gentiana* genus (Gentianaceae), the *Ciminalis* section (Adans) Dumort. (=sect. *Megalanthe* Gaudin). The diversification center of this genus is located in Asia while three sections are found in Europe, with two being endemic there (*Gentiana* and *Ciminalis*; Ho and Liu, 1990). Phylogenetic studies showed that the three European *Gentiana* sections (*Gentiana*, *Ciminalis* and *Calathianae*) plus *G. asclepiadea* are close to each other and diverged early in the *Gentiana* history (Gielly and Taberlet, 1995; Yuan et al., 1996; Gielly et al., 1996). The *Ciminalis* section comprises seven species and one to several subspecies depending on the authors. For this study, we accepted the classification of Hungerer and Kadereit (1998) that describes seven species. All species display clear morphological characteristics that distinguish them from each other, such as leaf width, length and substance, presence/absence of a green color and/or papillae inside the corolla, stalk length, among others traits (Hungerer and Kadereit, 1998; Christe, 2010). These species are distributed in Central and South European high mountains. They are typical edaphic vicariant species: Two of them are siliceous and widespread (*G. alpina* and *G. acaulis*) whereas the five remaining are calcareous and more geographically limited (*G. ligustica*, *G. occidentalis*, *G. dinarica* and *G. angustifolia*), excepting *G. clusii*. For all species, seeds are barichorous and are not dispersed far from the mother plant. Pollinators are insects, mostly Hymenoptera, Lepidoptera and Diptera (Wenk, 2008), with pollen suspected to migrate further than seeds.

The *Ciminalis* section has been extensively studied since the early ages of botany because it brings together many aspects that are specific to the European flora, especially the one of high altitude and cold biota. The scientific interest on the *Ciminalis* section remained constant through time with studies on morphology, complemented with ecological and historical aspects that aimed at determining the number of species and subspecies as well as their relationships (Jakowatz, 1899; Wilczek, 1910; Favarger, 1949; Tutin, 1970; Nègre, 1975; Vivant, 1978; Magnin-Gonze,

1992a,b, 1998; von Hagen and Kadereit, 2000). Different reasons explain why gentianas of the *Ciminalis* section are seen as good model species: their analyses indeed allow addressing questions such as ecological and/or geographical vicariance (Ozenda, 1985; Comes and Kadereit, 2003), endemism (Aeschimann et al., 2011a,b) and hybridization (von Hagen and Kadereit, 2000; Wenk, 2008). The recent phylogeny and phylogeography studies on the *Ciminalis* section (Hungerer and Kadereit, 1998; Diadema et al., 2005; Kropf et al., 2006) answered some of these questions. The deepest node in *Ciminalis* section was dated, for different markers, between 1.8 Myr and 600,000 years (Hungerer and Kadereit, 1998). Such a recent divergence leaves a small time window for the completion of the speciation process, with possibly associated phenomena such as incomplete lineage sorting, ancestral polymorphisms or hybridization that are all expected to blur species boundaries, and render their genetic delineation unclear (Bacon et al., 2012), despite clear morphological and ecological arguments for the recognition of seven species within the section (Hungerer and Kadereit, 1998). Moreover, the diversification within the section must have been highly influenced by climatic oscillations of the Quaternary as for other montane and alpine taxa (Kropf et al., 2003) with periods of genetic drift leading to disappearance of parts of the section evolutionary history. The latest phylogenetic hypothesis is given by von Hagen and Kadereit (2000), who suggested, based on a compilation of former studies and on the most complete phylogenetic tree using the nuclear ribosomal internal transcribed spacer 2 (ITS2) (Hungerer and Kadereit, 1998), that the *Ciminalis* section is divided into two groups, the first one containing *G. alpina* and *G. clusii*, and the second one comprising the remaining species, with *G. dinarica* supposedly being paraphyletic. In this latter group, *G. angustifolia* is found close to *G. occidentalis* and to *G. ligustica* to a lesser extent.

To analyze all *Ciminalis* species, we used three cpDNA coding regions (*matK*, *rpoC1* and *rpoB*), one cpDNA spacer (*trnH-psbA*) and the nrDNA ITS2. Chloroplasts are usually inherited through seeds in most Angiosperms (Petit et al., 2005; Birky, 2008) and in *Gentiana* in particular (Corriveau and Coleman, 1988). Therefore, chloroplast markers are useful for tracking the colonization histories as well as hybridization events when they result in chloroplast captures from one species to the other. Conversely, nuclear data give insights into pollen flow. As pollen is usually more mobile and more widely dispersed than seeds, which is also suspected to be the case in the *Ciminalis* section, species are usually more homogenized for nuclear markers. According to Petit and Excoffier (2009), species should then be better characterized using such nuclear markers; a prediction that was recently confirmed for plants (Naciri et al., 2012). Comparing the two data sets therefore allows analyzing colonization histories in face of gene flow and species boundaries. Using markers from the two genomes, we addressed three main questions in this study: (1) what are the phylogeographic patterns of the *Ciminalis* species and what do they tell about the influence of the Quaternary glaciations in shaping the genetic diversity and structuring? (2) What was the evolutionary influence of the different substrate types within the *Ciminalis* group? (3) What are the relationships between the previously recognized seven *Gentiana* species and their respective boundaries? Are all these species taxonomically best treated at the species level or do they fall into one or several superspecies as defined by Mallet (2006).

## 2. Material and methods

### 2.1. Sampling and marker choice

Leaf material from one to four plants from a total of 183 populations of *Gentiana acaulis*, *G. alpina*, *G. angustifolia*, *G. clusii*, *G. dinarica*,

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