PHYTON

ANNALES REI BOTANICAE

VOL. 51, FASC. 2 PAG. 177–327 20. 12. 2011

Phyton (Horn, Austria)	**Vol. 51**	Fasc. 2	177–199	20. 12. 2011

Conservation Status of Two Isolated Populations of *Gentiana verna* (*Gentianaceae*) in the Czech Republic: Insights from an Allozyme Analysis

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With 4 Figures

Received April 28, 2011

Key words: *Gentiana verna, Gentianaceae.* – Allozymes, conservation, polyploidy, population genetics, alien genotypes. – Flora of the Czech Republic.

Summary

KIRSCHNER J., KIRSCHNEROVÁ L. & BARTISH I. 2011. Conservation status of two isolated populations of *Gentiana verna* (*Gentianaceae*) in the Czech Republic: Insights from an allozyme analysis. – Phyton (Horn, Austria) 51 (2): 177–199, with 4 figures.

Gentiana verna L. consists in the Czech Republic of two populations which are 300 km apart and which are more than 100 km distant from the nearest populations abroad. The distribution pattern reflects a formerly much wider distribution. Samples from the two populations were studied using allozyme analysis. The population at Rovná, S Bohemia, is small and shows an almost complete homozygosity associated with a long period of inbreeding (indicated by unchanged allozyme patterns in

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repeated samples in 1998 and 2005), and a low genotype diversity. The larger population at Velká kotlina (Glacial Cirque), located in the Hrubý Jeseník Mts., N Moravia, is also homozygous in the majority of loci studied, and dispersal of seeds and pollen is not limited by spatial distance within this population. As a probable consequence of allotetraploidy, both populations exhibit fixed heterozygosity at *6Pgdh-1*. Three new plants suddenly appearing at Rovná during the monitoring of the site in 2005 were suspected to be a result of introduction of alien genetic material and were treated as a separate subpopulation. The three plants proved to be extremely distant from both natural populations and their alien status was confirmed. The problem of introduction of alien material into populations of threatened plants is briefly discussed. Comparison of gene frequencies, genotype composition and unique alleles of the two natural populations revealed that they are genetically very remote from one another and, from the conservation viewpoint, cannot be considered as a single management unit with mutually interchangeable individuals.

Zusammenfassung

Kirschner J., Kirschnerová L. & Bartish I. 2011. Conservation status of two isolated populations of *Gentiana verna* (*Gentianaceae*) in the Czech Republic: Insights from an allozyme analysis. [Schutz-Status zweier isolierter Populationen von *Gentiana verna* (*Gentianaceae*) in Tschechien: Einblicke aus einer Allozym-Studie]. – Phyton (Horn, Austria) 51 (2): 177–199, with 4 figures.

Von *Gentiana verna* L. kommen in Tschechien zwei Populationen vor, die voneinander mehr als 300 km und von den nächsten Populationen außerhalb des Landes 100 und mehr km entfernt sind. Die Disjunktionen lassen eine früher weitere Verbreitung vermuten. Proben von beiden Populationen werden mittels Allozym-Analyse untersucht. Die Population von Rovná, S Böhmen, ist klein und zeigt nahezu komplette Homozygotie verbunden mit offenbar lange dauernder Inzucht (angezeigt durch unveränderte Allozym-Muster in wiederholten Proben 1998 und 2005) und geringer Genotypen-Diversität. Die größere Population von Velká Kotlina (eiszeitliches Kar) in den Hrubý Jeseník Bergen, N Mähren, ist ebenso an der Mehrzahl der studierten Loci homozygot und die Ausbreitung von Samen und Pollen ist innerhalb dieser Population räumlich nicht limitiert. Als mögliche Konsequenz von Allopolyploidie zeigen beide Populationen fixierte Heterozygotie am 6Pgdh-1-Locus. Drei neue Pflanzen, die in Rovná während des Monitorings des Standortes im Jahre 2005 plötzlich vorhanden waren, werden als Resultat des Einbringes von fremden genetischen Materials betrachtet und als eigene Subpopulation behandelt. Die drei Pflanzen sind von beiden natürlichen Populationen extrem verschieden, was den Fremd-Status bestätigt. Das Problem des Einbringes fremden Materials in Populationen von gefährdeten Pflanzen wird kurz diskutiert. Der Vergleich der Gen-Häufigkeiten, Genotyp-Zusammensetzung und seltenen Allelen der beiden natürlichen Populationen zeigt, daß sie genetisch sehr voneinander verschieden sind. Vom Standpunkt des Naturschutzes können sie daher nicht als eine Management-Einheit mit austauschbaren Individuen betrachtet werden.

1. Introduction

Conservation genetics as a recently established field of quantitative population genetics (DeSalle & Amato 2004) offers a number of models of

genetic patterns of rare or threatened plants (for a review, see also FRANKHAM & al. 2007; an early survey also in SOULÉ 1986). Most of the allelic models explicitly or implicitly deal with diploid plant material, and the results are frequently interpreted as if the diploidy or diploid behaviour is self-evident. Polyploidy is a very common phenomenon in plants but sometimes it is difficult to detect (cryptic polyploidy or paleopolyploidy), and it is a phenomenon with a potentially serious impact on genetic diversity. A departure from Hardy-Weinberg equilibria indicates selection (e.g., overdominance), migration/hybridization effects or non-random mating or recombination; many causes of higher proportions of heterozygotes (heterozygote excess) can be attributed to polyploidy. However, polyploidy is frequently overlooked, as shown on a few examples below.

KIKUCHI & al. 2011, for instance, published a detailed analysis of the distribution of genetic variation among populations of the rare endemic *Salix hukaoana* KIMURA in Japan. Negative FIS found in the analysis was attributed to heterozygosity excess due to genetic bottlenecks. *Salix*, however, is a paleopolyploid/neopolyploid complex with 2n=38 and 76, and the influence of this fact should not be neglected (the chromosome number of *Salix hukaoana* remains unknown). Another example of a similar approach is that of MASCHINSKI & al. 2010 who examined the extent of introgression between an invasive *Lantana strigocamara* R. W. SANDERS and rare native *Lantana* taxa. A sophisticated ISSR analysis was performed to detect hybridization and introgression between taxa with probably different chromosome numbers (2n=22 vs. 2n=44). No genome size or chromosome number analysis was used in this study. Excessive heterozygosity was also found by HONJO & al. 2007 during the genetic study of an endangered species, *Primula sieboldii* E. MORREN. This phenomenon is attributed to higher fitness associated with heterozygosity while no attention is paid to the fact that *P. sieboldii* is a polyploid complex with 2n = 24, 36 and 48. A heterozygote excess attributed to bottlenecks is also emphasized in SEVERNS & al. 2011 for *Lupinus oreganus* A. HELLER, without regard to the common polyploidy known in the genus. In the case of relatively recent polyploids, results of basic population genetic analyses are prone to a serious bias, unless polyploidy is taken into consideration.

Gentiana verna L., is described as polyploid in several works (for summary, see HÄMMERLI 2007). Most of the chromosome numbers within the group of *G. verna* vary between 2n=28 and 2n=30, with occasional B-chromosomes reported (e.g., MÜLLER 1974, 1982); *Gentiana nivalis* L. as a reference diploid in the section *Calathianae* shows 2n=14. Another feature pointing to polyploidy is an enormous variation in genome size among species and populations showing 2n=28–30 (SCHISTEK & al. 2009). We therefore focused our analyses on the issues of genetic structure of populations and polyploidy in relation to conservation of this threatened taxon.

A scheme of rescue plans for selected threatened species was introduced by the Ministry of Environment in the Czech Republic. Among the first species chosen for an implementation of a rescue plan is *G. verna*, a species exhibiting a clear decline in number and size of populations. The species was studied also from other viewpoints relevant to conservation (Sýkorová & al. 2003, Kirschnerová, Moravcová & Rauch, in press). An introductory study of a seemingly simple case of the genetic variation in two extant populations of *G. verna* is given below.

1.1. *Gentiana verna* L. and the sect. *Calathianae* – Taxonomy, Variation and Distribution

The section *Calathianae* Froelich of the genus *Gentiana* L. comprises 15 taxa including *G. verna* L. The recent taxonomic circumscription of *G. verna* (Hämmerli 2007) removed the Balcanic complex of *G. tergestina* G. Beck from our species, and *G. verna* thus consists of two subspecies, subsp. *verna* and the SW Alpine and perhaps also Pyrenean subsp. *delphinensis* (Beauverd) H. Kunze. A relatively detailed molecular study of *G. verna* (Hämmerli 2007) revealed a geographic pattern where the species' range in Europe is dominated by a W European group of genotypes that reaches the Polish Western Carpathians in the east. An independent confirmation of the results achieved by Hämmerli 2007 was presented by Moosbrugger & al. 2008, 2009 and Schistek & al. 2009. In particular, both Hämmerli and Moosbrugger & al. concluded that the main phylogeographic unit identified within *G. verna* s. lat. comprises both Alpine and the W Carpathian plants; the dividing line between the two phylogeographic units is again drawn between the W Carpathians and the SE Carpathians.

Reticulate character of the evolution in the sect. *Calathianae* is difficult to prove by means of the methods employed by Hämmerli 2007 but some of his methods (cpDNA PCR-RFLP, cpDNA *mat*K sequences, AFLP) show that main genotype groups do not always correspond to taxonomic units, and *G. verna* s. str. from W Europe does not belong to the same group as the plants from the E Carpathians (Romania). In the AFLP study, Hämmerli 2007 found that the main multilocus genotypes are shared by populations of different taxa, e.g., *G. verna* s. str. and *G. pumila* s. lat., or *G. verna* s. str. and *G. tergestina* s. lat. This may be considered as an indication of the reticulation (including the phenomenon of alloploidy) in the evolutionary history of the group but further corroboration is needed by means of data from other sources, such as, for instance, nrITS cloning.

In the conception of Hämmerli 2007, generally also adopted in the present study, *G. verna* subsp. *verna* occupies a mainly Alpine-Pyrenean range (Austria, France, Germany, Italy, Slovenia, Spain and Switzerland), with isolated regions of occurrence in the Apennines (C. Italy), the Burren

(Ireland), Upper Teesdale (England), Arctic European Russia, the W Carpathians (Slovakia, Poland), SE Carpathians and adjacent ranges (Romania), and a scattered occurrence outside the main mountain ranges in Central Europe (Germany, Czech Republic). Czech material was not studied by HÄMMERLI 2007, neither it was used in any other study of molecular markers in the species.

It is important to consider polyploidy as a phenomenon substantially influencing the genetic make-up of *Gentiana verna* populations. The sect. *Calathianae* includes taxa with an array of somatic chromosome numbers ranging from 2n=14 to 2n=38 (HÄMMERLI 2007, MÜLLER 1974, 1982). The existence of the diploid with x=7 shows that the whole group of *G. verna* having 2n=28-32 represents a tetraploid. Thus, for the purposes of our study, we treat the Czech material as a paleoallotetraploid taxon. The support for this treatment was found in allozyme patterns of material from both the Alps (M. KŘÍŽ & al., unpubl.) and the Czech Republic – the Alpine material shows clear features of tetraploidy, while in the Czech material we found only inconspicuous remnants of tetraploid behaviour (fixed heterozygosity and gene duplication were looked for in the allozyme patterns). It is obvious that the paleoallotetraploidy and reproduction system are to be taken into consideration when gene frequencies are tested from the Hardy-Weinberg equilibrium viewpoint.

Reproduction and population characteristics of *Gentiana verna* were studied by BRADSHAW & DOODY 1978 and KOZUHAROVA & ANCHEV 2002, although the latter work might have been conducted on another, closely related taxon. Outcrossing with occasional selfing, an extensive clonality, rare sexual recruitment, high seed production and a several-year seed viability are the main features relevant for the present study.

1.2. *Gentiana verna* L. in the Czech Republic

Two major factors should be considered from the geographic point of view: in the Czech Republic, *Gentiana verna* occurs at the edge of its overall geographical range, and its current occurence in Southern Bohemia is a remnant of much wider distribution reduced by fragmentation of the range and disappearance of habitats and populations. The two extant populations are very isolated; they are more than 300 km from one another, the southern one being about 100 km far from the Bavarian populations in S Germany, and the northern one about 200 km from the nearest sites in the W Carpathians.

Historical distribution of *G. verna* in the Czech Republic is displayed in Fig. 1. It shows that before 1950 this species was relatively common in certain areas; the reasons for the considerable retreat of the species and its extinction from many localities were summarized by Z. MÜNZBERGOVÁ (unpublished, 2006) using a sample of 23 localities in S Bohemia: drainage,

ceased grazing, change into arable land and mechanical disturbance. Particularly after 1950, the rapid changes in the farming methods led to a massive disappearance of the species. Although the species was in the focus of nature conservation for a long time, the process of extinction could not have been stopped. Nowadays, there are only two localities of *G. verna* in the Czech Republic which are very distant from one another, and of a very different character (Fig. 1).

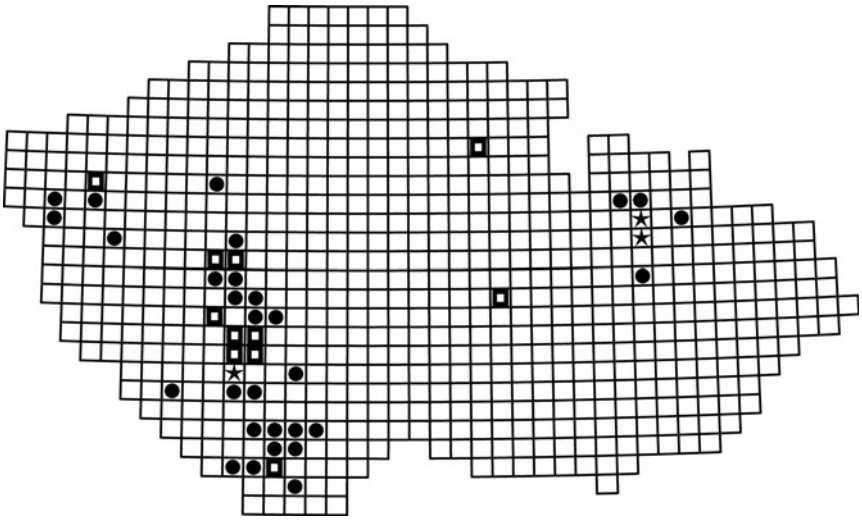

Fig. 1. Grid-map of historical and current distributions of *Gentiana verna* in the Czech Republic. Black circles – historical records before 1950; framed squares – records documented after 1950, but not extant currently; stars – current occurrences (S Bohemia: RO; N Moravia: VK). Modified from KIRSCHNEROVÁ & KLAUDISOVÁ 2008. Geographic position of the map: 12°–15°E, 48°30'–51° N.

The locality at Rovná (RO), district of Strakonice, South Bohemia, was known to consist of more than 1000 individuals in the late 1950s (MORAVEC 1959), at a wet low altitude (418 m) meadow on a mineral rich substrate. The change of a grazed and mown meadow into a unofficial soccer pitch did not disturb the population very much, which cannot be said of an extensive application of fertilizers in the 1980s. In spite of this, the population survived and the number of flowers fluctuates between four (in 1998) and 63 in 2002 (two to 31 individuals). A detailed monitoring of the population since 1998 shows changes in position and size of patches of *G. verna* (KIRSCHNEROVÁ, MORAVCOVÁ & RAUCH, unpubl.). A remarkable change was observed between 1998 and 2005: an apperance of three new clones, two of them in totally new squares of the permanent plot grid (plants revealed in 2004, first flowering in 2005). For the sake of cautiousness, these three new

ramets are treated as a separate 'subpopulation' (ALI) in our analyses. For details, see also KIRSCHNEROVÁ & ALBRECHT 2003.

The other population is found at the upper edge of the glacial cirque of Velká kotlina, the Hrubý Jeseník, N Moravia, between 1140 and 1410 m a. s. l. (VK). The population can be considered as a stabilized one, with a population size amounting to 2500–3000 individuals. The mountain grassland slopes soaked with calcium rich spring water determine the character of the population: several patches of common occurrence are connected by links with sparse or scattered presence of the species.

1.3. Objectives of the Study

The first question to be answered is how closely related genetically are the two existing populations (genetic distances are to be identified). The second question is what is the level of genetic diversity at Rovná (RO), as compared with the Velká kotlina population (VK), with its possible impact on the population's survival. And the third question to be dealt with is whether the possible genetic differences between populations support different conservation approaches.

2. Material

The sampling and other analyses using threatened plants at protected sites were done on the basis of a permit no. 1916/98-OOP/787/98, issued by the Ministry of Environment.

Sampling was not destructive, only a minor part of a ramet was removed.

The following samples were used; they include 76 plants:

a. – GARD – For the sake of comparison, a couple of samples of unknown origin from rock-garden cultivation was included (probable provenance: the Alps or mountains of Balkan Peninsula). The rock garden is in Prague vicinity.

b. – RO1 – All four polycormons (each sampled twice) observed at Rovná (49°17' N, 13°56' E) in 1998; the locality has been monitored for 15 years in detail, using a grid of 10×10 cm to micromap all visible individuals.

c. – RO2 – All 23 plants observed at Rovná (49°17' N, 13°56' E) in 2005. The increase between 1998 and 2005, after a detailed analysis of micromaps on permanent plots (not shown), is partly attributed to expansion and disintegration of individual patches of occurrence, partly to seedling recruitment.

d. – ALI – Because of the appearance of three new remotely isolated individuals outside the microregion of the species' occurrence at permanent plots, these three plants were treated as a separate subpopulation.

e. – VK – A sample of 40 individuals at Velká kotlina (glacial cirque) in the Hrubý Jeseník Mts. (50°03' N, 17°14' E). The population is large (over 1000 individuals) and consists of a series of patches of common occurrence connected by areas of scattered or rare occurrence; four of the former patches were sampled at random (with a minimum distance of ca. 0.5 m among samples), and the patches were chosen to cover the whole locality.

3. Methods

3.1. Allozyme Analysis

At the beginning of the development of population genetics based on molecular markers, LEWONTIN & HUBBY 1966 introduced isozymes as a breakthrough in this field. One of the essential issues of conservation biology and conservation genetics is the correlation between fitness and heterozygosity (FHC). In very different organisms, such as plants, vertebrates or molluscs, positive FHC was observed (for review, see DAVID 1998, who also points out the problems), and we can refer to the classical work of WATT 1977 where causal relationships leading from the enzymological data (PGI) to the behavioral properties of *Colias* butterflies and to the overdominance were shown. Further cases are discussed in BRITTEN 1996. Allozymes, as selectively non-neutral markers, are useful in this type of study.

Allozyme analysis was chosen as a method to characterize genetic diversity of the material. The data were first evaluated from the viewpoint of the appropriateness of this method; the overall mean number of alleles per locus was found satisfactory for data analysis (2.66) and a mean number of unique alleles per population (3.66) also supports this decision. There are several reasons for this choice: a direct interpretability of known and potentially non-neutral markers is important for conservation purposes, small size of some samples (see NEI 1978), by far the lowest costs of analyses, and the opportunity to combine and compare the results of analyses with those of analyses performed earlier. However, the main reason was the necessity to study easily interpretable co-dominant markers able to detect traces of old gene duplication during polyploidy. The high probability of the paleoalloploid status of *Gentiana verna* follows from the chromosome numbers (see above) and gene duplication at several loci observed on Alpine material (KŘÍŽ & al., in preparation). In order to document phenomena such as fixed heterozygosity, we eventually decided to use the allozyme approach. As the questions to be answered are mostly of qualitative nature, possible underestimation of heterozygosity using allozymes is not a substantial problem; many of the PCR-based DNA markers have the same "inherent defects" as allozymes (associated with aneuploidy and null allele effect, see DAVID 1998).

The number of variable loci and gene diversity is satisfactory for the type of inferences made in the present paper (WIDÉN & al. 1994).

3.2. Electrophoretic Techniques

A method of discontinuous separation using vertical electrophoresis on polyacrylamide gels was used (Hoefer SE 600 set, Amersham). Electrophoresis was performed on crude protein extracts of roughly 60 mg of leaf material, originally frozen and kept at –75 °C; most techniques were modified from VALLEJOS 1983, and WENDEL & WEEDEN 1989. Tissue was ground in 400–600 µl of ice-cold tris-HCl extraction buffer: 0.1 M Tris-HCl pH 8.3; 1% glutathion, 5% sucrose, 0.1% 2-mercaptoethanol, 10 mM $MgCl_2$. Extracts were centrifuged for 10 min at 15,000 rpm and clear supernatants were stored at –75 °C. All enzyme systems were investigated on polyacrylamide gels (8.16% 1.82 M separation gel, 8% acrylamide, discontinuous tris-glycine buffer system). Initially, the following enzyme systems were tested: EST (esterase, EC 3.1.1.-), AAT (aspartate aminotransferase, EC 2.6.1.1), LAP (leucine aminopeptidase, EC 3.4.11.1), ADH (alcohol dehydrogenase, EC 1.1.1.1.), SKDH (shiki-

mate dehydrogenase, E.C. 1.1.1.25), MDH (E.C. 1.1.1.37), SOD (superoxide dismutase, E.C. 1.15.1.1), 6PGDH (phosphogluconate dehydrogenase, EC 1.1.1.44), PGM (phosphoglucomutase, E.C. 5.4.2.2), PGI (phosphoglucoisomerase, E.C. 5.3.1.9). The following variable enzyme systems (Table 1) and loci with clear interpretation were scored and the data used for further analyses (PGM yielded useful results but was used only in analyses excluding RO1 because of missing data, and PGI was visualised only on gels with RO1 and was excluded from statistical analyses).

Table 1. Characterization of allozyme loci

Locus	Subunit structure: Monomer (M) Dimer (D)	No. of alleles
Adh-1	D	2
6Pgdh-1	D	3
6Pgdh-2	D	2
Skdh	M	2
Lap-2	M	4
Aat-1	D	3
Pgm	M	3
Pgi	D	2

3.3. Data Interpretation

Loci are numbered according to the mobility of bands. For each locus, the alleles were labelled and ordered following the alphabetical sequence in order of their mobility towards the anode. Intergenic heteromers were observed in dimeric enzymes; no null allele situations were detected. As the material from both Czech localities largely lacks features of tetraploid behaviour (no gene duplication nor clearly uneven dosage of allelic products observed), the tetraploid patterns (aaaa/bbbb) are encoded as aa/bb. This approach does not affect the calculation of gene frequencies and the tests of fit to Hardy-Weinberg equilibria were not calculated. The source data are available in Table 2.

3.4. Data Analyses

For the two natural populations, elementary gene diversity parameters were estimated [percentage of polymorphic loci, mean number of alleles per locus, mean effective number of alleles per locus, observed heterozygosity within population (H_o) and estimate of expected heterozygosity within population (H_{ep})] together with the average for all variable loci genetic differentiation (F_{st}) and gene flow between these populations. The calculations were performed by POPGEN32 (see ‹http://www.ualberta.ca/~fyeh/popgene.html›).

The index of genotype diversity was calculated as in Hughes & Richards 1988: $GD = 1 - \Sigma\, x_i^2$ (where x_i is the frequency of multilocus genotype *i*, and GD range is $<0; 1>$). This parameter is useful for population sets with expected variation in re-

Table 2. Allozyme genotypes of 76 individuals of *Gentiana verna* from the Czech Republic. ALI- three isolated plants from the Rovná population (suspected to be an alien introduction); GARD- garden cultivation; RO1- population Rovná, sample from 1998; RO2- all 23 individual plants of Rovná population, observed in 2005; VK- sample of the population from Velká kotlina.

Individuals	Adh-1	6Pgd-1	6Pgd-2	Skd	Lap-2	Aat-1	Pgm-2
ALI-01	AB	BC	BB	AB	BD	AC	BB
ALI-02	AB	BC	BB	AB	BD	AB	BB
ALI-03	AA	BC	BB	BB	DD	BC	AA
GARD-01	AB	BC	BB	BB	DD	BC	nn
GARD-02	AB	BC	BB	BB	DD	BC	nn
RO1-01	BB	BC	BB	AA	BB	AA	nn
RO1-02	BB	BC	BB	AA	BB	AA	nn
RO1-03	BB	BC	BB	AA	BB	AA	nn
RO1-04	BB	BC	BB	AA	BB	AA	nn
RO1-05	BB	BC	BB	AA	BB	AA	nn
RO1-06	BB	BC	BB	AA	BB	AA	nn
RO1-07	BB	BC	BB	AA	BB	AA	nn
RO1-08	BB	BC	BB	AA	BB	AA	nn
RO2-01	BB	BC	BB	AA	BB	AA	BB
RO2-02	BB	BC	BB	AA	BB	AA	BB
RO2-03	BB	BC	BB	AA	BB	AA	BB
RO2-04	BB	BC	BB	AA	BB	AA	BB
RO2-05	BB	BC	BB	AA	BB	AA	BB
RO2-06	BB	BC	BB	AA	BB	AA	BB
RO2-07	BB	BC	BB	AA	BB	AA	BB
RO2-08	BB	BC	BB	AA	BB	AA	BB
RO2-09	BB	BC	BB	AA	BB	AA	BB
RO2-10	BB	BC	BB	AA	BB	AA	BB
RO2-11	BB	BC	BB	AA	BB	AA	BB
RO2-12	BB	BC	BB	AA	BB	AA	AA
RO2-13	BB	BC	BB	AA	BB	AA	AA
RO2-14	BB	BC	BB	AA	BB	AA	AA
RO2-15	BB	BC	BB	AA	BB	AA	AA
RO2-16	BB	BC	BB	AA	BB	AA	AA
RO2-17	BB	BC	BB	AA	BB	AA	BB
RO2-18	BB	BC	BB	AA	BB	AA	BB
RO2-19	BB	BC	BB	AA	BB	AA	BB
RO2-20	BB	BC	BB	AA	BB	AA	BB
RO2-21	BB	BC	BB	AA	BB	AA	BB
RO2-22	BB	BC	BB	AA	BB	AA	BB
RO2-23	BB	BC	BB	AA	BB	AA	BB
VK-01	AA	AC	BB	BB	CC	AA	CC
VK-02	AA	AC	BB	BB	CC	AA	CC

Table 2, continued

Individuals	Adh-1	6Pgd-1	6Pgd-2	Skd	Lap-2	Aat-1	Pgm-2
VK-03	AA	AC	BB	BB	BC	AA	CC
VK-04	AA	AC	AB	BB	BC	AA	CC
VK-05	AA	AC	AB	BB	BC	AA	CC
VK-06	AA	AC	AB	BB	AB	AA	CC
VK-07	AA	AC	AB	BB	AB	AA	CC
VK-08	AA	AC	BB	BB	BB	AA	AA
VK-09	AA	AC	BB	BB	BC	AA	CC
VK-10	AA	AC	AB	BB	AB	AA	CC
VK-11	AA	AC	AB	BB	BB	AA	CC
VK-12	AA	AC	AB	BB	BC	AA	CC
VK-13	AA	AC	AB	BB	BC	AA	CC
VK-14	AA	AC	BB	BB	BC	AA	CC
VK-15	AA	AC	AB	BB	BC	AA	CC
VK-16	AA	AC	AB	BB	BB	AA	CC
VK-17	AA	AC	AB	BB	BC	AA	CC
VK-18	AA	AC	AB	BB	AC	AA	CC
VK-19	AA	AC	AB	BB	BB	AA	CC
VK-20	AA	AC	AB	BB	BB	AA	CC
VK-21	AA	AC	AB	BB	BC	AA	CC
VK-22	AA	AC	AB	BB	BB	AA	CC
VK-23	AA	AC	AB	BB	BB	AA	CC
VK-24	AA	AC	AB	BB	AC	AA	CC
VK-25	AA	AC	AB	BB	CC	AA	CC
VK-26	AA	AC	AB	BB	AC	AA	CC
VK-27	AA	AC	BB	BB	AC	AA	CC
VK-28	AA	AC	BB	BB	AA	AA	CC
VK-29	AA	AC	AB	BB	AC	AA	CC
VK-30	AA	AC	AB	BB	BB	AA	CC
VK-31	AA	AC	AB	BB	BB	AA	CC
VK-32	AA	AC	BB	BB	CC	AA	CC
VK-33	AA	AC	AB	BB	BC	AA	CC
VK-34	AA	AC	AB	BB	AC	AA	CC
VK-35	AA	AC	AB	BB	BB	AA	CC
VK-36	AA	AC	AB	BB	CC	AA	CC
VK-37	AA	AC	AB	BB	AC	AA	CC
VK-38	AA	AC	AB	BB	BB	AA	CC
VK-39	AA	AC	AB	BB	BC	AA	CC
VK-40	AA	AC	BB	BB	BB	AA	CC

production systems (i. e., a substantial departure from the Hardy-Weinberg expectations) and for situations where recombination is partially suppressed as a consequence of alloploidy; it reasonably reflects both richness and evenness and closely approaches the modified Simpson's Index (WIDÉN & al. 1994).

Using the package NTSYSpc (Exeter Software, see also at <http://www.exetersoftware.com/cat/ntsyspc/ntsyspc.html>), genetic distances (SIMGEND) among five populations were calculated (after calculating gene frequencies from allelic data

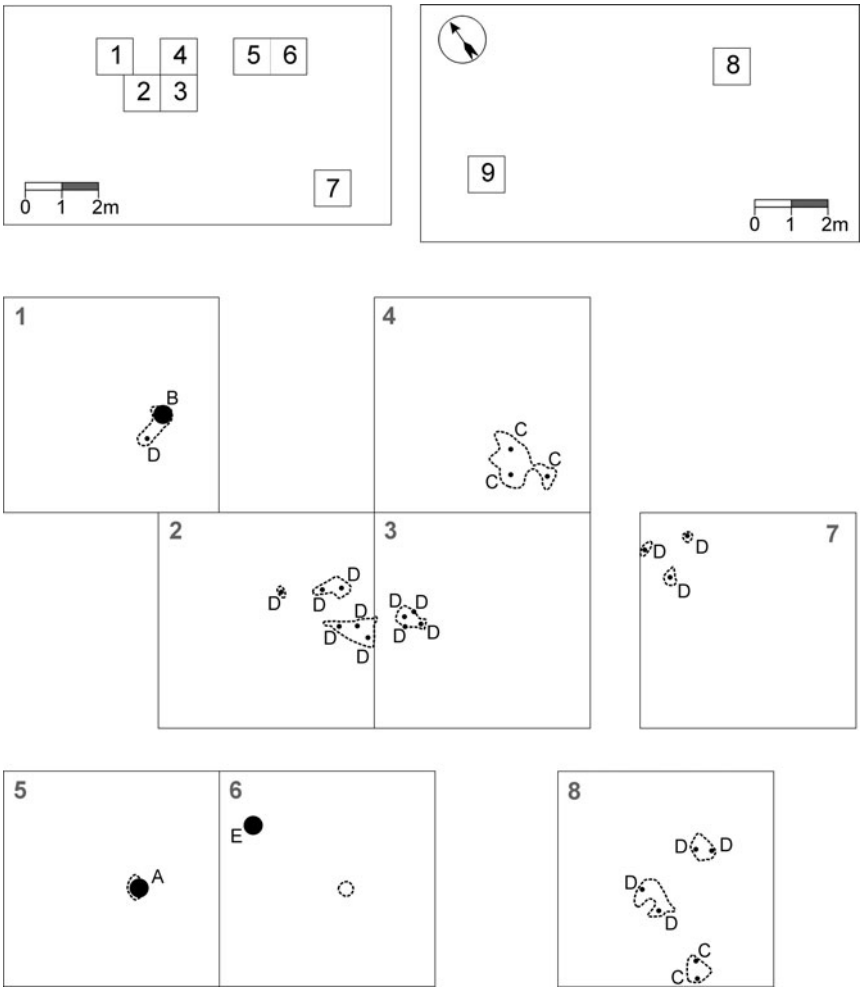

Fig. 2. Distribution of genotypes at Rovná (RO2) in 2005. Position of monitored plots (1 through 9) is displayed in the upper part of the figure. Grey capital numerals (in italics) label plots corresponding to those depicted within monitored areas. Individual genotypes are labelled in accordance with Table 6. Three individuals (ALI) considered to belong to alien genotypes A, B and E, are displayed as bigger black dots. Distance between the two monitored areas (shown in the upper part of the figure) is 43 m.

in FREQ). Two genetic distance coefficients were used, Nei's Genetic Distance (Nei 1972) and an unbiased version of it (Nei 1978). Various clustering alternatives were applied for the genetic distance matrices (NJOIN, SAHN with several alternatives). As the results did not differ substantially from one another, results of SIMGENDNEI72 and UPGMA are displayed. NTSYS was also used to perform Principal Co-

Ordinates (PCO) analysis of relationship between all genotypes found in the two largest populations (RO2 and VK). We used NEI'S (1973) genetic distances between all genotypes found in these populations for the analyses. The first two principal coordinates were used for graphical representation of relationships between these genotypes. Finally, the association between spatial and genetic distances among 40 individual plants in the largest and most genetically variable population (VK) was assessed using Mantel test option in NTSYS.

3.5. Flow Cytometry

HÄMMERLI 2007 includes in his sample all the populations of *G. verna* from the West Carpathians westwards in the 2n=28 western group of genotypes. In addition, we analyzed samples from RO2 and VK by means of flow cytometry. The task was to prove genome size identity of the two samples. All measurements were taken at the Laboratory of Flow Cytometry, Institute of Botany, Czech Academy of Science, Pruhonice, with Partec CyFlow equipped by green solid-state laser (532 nm) for PI excitation. A two-step procedure with buffers after OTTO 1990 (described in DOLEŽEL & al. 2007, see also LOUREIRO & al. 2010) was used.

Table 3. Basic parameters of allozyme variation

Locality	A_v*	*P*	*FH*
GARD	1.5000	3/7	–
RO1	1.1667	2/7	2
RO2	1.2857	2/7	1
ALI	1.8571	6/7	–
VK	1.7143	3/7	1

A_v – Mean number of alleles per locus; *P* – proportion of variable loci; *FH* – number of loci with fixed heterozygosity (not given for GARD and ALI because of a small sample size).
*) Number of alleles per variable locus averaged over all populations: 2.66.

4. Results

4.1. Elementary Descriptive Parameters

Results of the estimation of basic parameters of genetic diversity of *Gentiana verna* populations are summarized in Table 3.

In a paleotetraploid, the expected heterozygosity calculated on the basis of a seemingly diploid data set does not have the same importance as in a real diploid. Table 4 below thus mainly describes the situation observed. Within populations, monomorphic homozygous configurations prevail (five of seven loci in RO2, four of seven loci in VK). The more conspicuous is therefore the fixed heterozygous pattern observed at *6Pgdh-1* in both populations.

Table 4. Heterozygosity observed (H_o), heterozygosity expected (H_{ep}), effective number of alleles (N_e) according to KIMURA & CROW 1964, and Shannon's Information index (I), LEWONTIN 1972, for RO2 and VK

A) Heterozygosity Statistics for RO2

Locus	H_o	H_{ep}*	N_e	**I**
Adh-1	0.0000	0.0000	1.0000	0.0000
6Pgdh-1	1.0000	0.5111	2.0000	0.6931
6Pgdh-2	0.0000	0.0000	1.0000	0.0000
Skd	0.0000	0.0000	1.0000	0.0000
Lap-2	0.0000	0.0000	1.0000	0.0000
Aat-1	0.0000	0.0000	1.0000	0.0000
Pgm-2	0.0000	0.3478	1.5158	0.5236
Mean	0.1429	0.1227	1.2165	0.1738
St. Dev	0.3780	0.2148	0.3953	0.3009

* Computed using Levene 1949

B) Heterozygosity Statistics for VK

Locus	H_o	H_{ep}*	N_e	**I**
Adh-1	0.0000	0.0000	1.0000	0.0000
6Pgdh-1	1.0000	0.5063	2.0000	0. 6931
6Pgdh-2	0.7500	0.4747	1.8824	0. 6616
Skd	0.0000	0.0000	1.0000	0.0000
Lap-2	0.5500	0.6161	2.5869	0.0086
Aat-1	0.0000	0.0000	1.0000	0.0000
Pgm-2	0.0000	0.0494	1.0512	0.1169
Mean	0.3286	0.2352	1.5029	0.3543
St. Dev	0.4300	0.2818	0.6492	0.4223

* Computed using Levene 1949

Table 5. Number of unique alleles for each pair of populations

→	RO1, RO2	VK	ALI
RO1, RO2	×	3	0
VK	7	×	2
ALI	5	5	×

Mean number of unique alleles per population: 3.66.

To summarize the results of basic characterization of the allozyme pattern data set, we point out the following features:

(i) a prevailing homozygosity in the majority of loci;

(ii) a relatively high level of divergence in unique alleles between populations (Table 5);

(iii) fixed heterozygosity at some loci;

(iv) large differences in genotype diversity (see Table 6).

4.2. Fixed Heterozygosity

A phenomenon of utmost importance for the interpretation of our data is the occurrence of fixed heterozygosity in the allozyme patterns. It was observed in *Pgi* (RO1 only) and in *6Pgdh-1* (RO1, RO2, VK, ALI, GARD). Particularly in RO1 and RO2, the fixed heterozygosity is in striking contrast with uniformly homozygous patterns found at most loci. For the purposes of our study, we should point out the support for the alloploidy hypothesis, and the conservation importance of fixed heterozygosity in small, highly inbred populations (see Discussion).

4.3. Considerable Genetic Distance between Rovná and Velká kotlina

For conservation purposes in the case of the Czech *Gentiana verna*, the essential question is whether or not the two populations (RO2 and VK) can be considered as a single population genetic management unit. Unique alleles as the first indicator show that the two populations are quite distant (altogether 10 unique alleles [!!], see Table 5). The genetic distance UPGMA analysis (Fig. 4) on the basis of gene frequencies reveals how distant the two relevant populations are; they are the most distant units in the analysis. This means that it is not advisable to use material from VK to increase the genetic diversity at RO2; markers of non-neutral character, with probable influence on fitness, are incommensurable in the two populations (whose habitats are also ecologically remote). The F_{st} coefficient of genetic differentiation between these populations is also relatively high (F_{st}=0.562), whereas the gene flow is low (N_m=0.195). PCO analysis supports these inferences at the level of individual genotypes. The two genotypes found in population RO2 are both strongly different from all 11 genotypes of population VK (Fig. 3). This figure also illustrates relatively high genetic variability within population VK.

Fig. 3 and 4 also show the separate character of the three new plants treated as a subpopulation of its own (ALI); it is extremely different from both Czech populations.

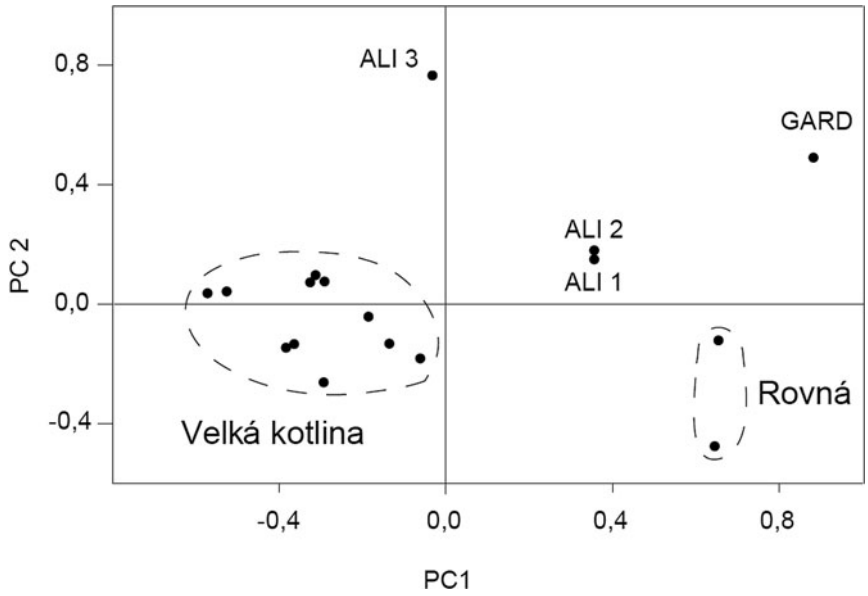

Fig. 3. Results of PCO analysis of genotype data.

4.4. The Depauperate Population at Rovná

Comparison of genotype diversities, numbers of genotypes (Table 6) and basic parameters (Table 4) clearly shows the low diversity at Rovná (RO1 & RO2). The eight plants analyzed as RO1 were identical according to the isozyme patterns available. The difference (one additional genotype in RO2) between RO1 and RO2 might be attributed to one or a few overlooked sterile individuals during the first sampling (or, with lower probability, to recruitment of seed bank material). If we disregard the fixed heterozygous pattern at one of the loci, the population is completely homozygous. The low heterozygosity and the difference from VK exclude alloploidy as the main reason of low genotype diversity. Consequences of this highly depauperate population status are obvious – a substantial decrease of fitness is to be expected (see Discussion).

4.5. Alien Genotypes at Rovná

The three newly found, isolated plants at RO2 were analyzed separately (as ALI). Each of the three plants represents a genotype unique within the set of plants studied. Another qualitative indicator of the separate character of the three plants, number of unique alleles (Table 5), corroborates the presumed alien character of them. ALI possesses five unique alleles against each of the two main populations. Also the UPGMA analysis of genetic distances among the five groups studied and (on the basis of

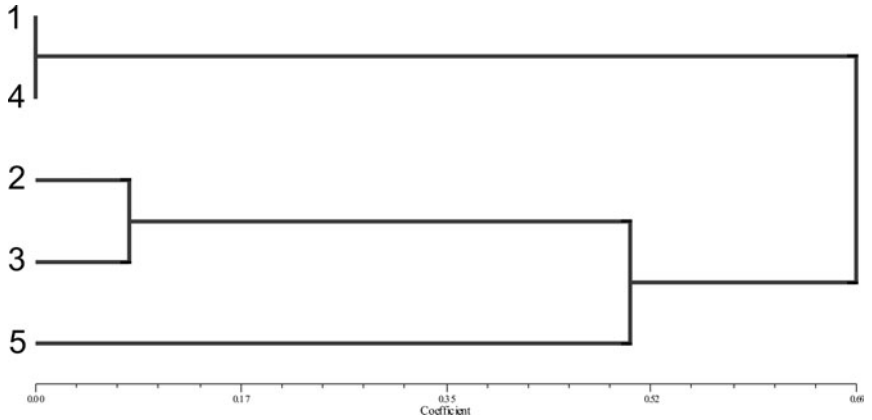

Fig. 4. UPGMA analysis of genetic distances on the basis of gene frequencies of the five plant groups (1 – RO1; 2 – GARD; 3 – ALI; 4 – RO2; 5 - VK). RO and VK represent natural populations.

gene frequencies, and PCO using individual allelic data, see Fig. 3 and 4) show that ALI is considerably different from both native populations in the Czech Republic. No great value was attributed to the relative resemblance between ALI and the garden sample (GARD) because of the very small size of these samples.

The above facts exclude all explanations other than the alien status of the three plants at Rovná. Either through sowing or direct transplantation, these plants were introduced into the protected, highly threatened population.

4.6. Absence of Spatial Structure within Population VK

We analyzed the association between spatial and genetic distances among individual plants within population VK by Mantel test and found no association (r=–0.014; P=0.375). The same high-frequency genotype (for instance, H and I) could be found in all spatial clusters of plants in this population, even though some of them were more than 500 meters apart. At the same time, strongly differentiated genotypes could be found no more than several meters apart. These results indicate that dispersal of seeds and pollen is not limited by spatial distance within this population.

4.7. Flow Cytometry

The analysis of the genome size in plants from the two natural populations showed a difference of almost 4% (RO bigger than VK) but not at the level corresponding to different chromosome numbers. The western *Gentiana verna* is known to exhibit a certain genome size variation (J. Suda, in litt.) and our findings are within the limits of this variation.

5. Discussion

5.1. Alloploidy and its Consequences for the Conservation of *Gentiana verna*

Polyploidy, and alloploidy in particular, has always been known as a common phenomenon in plants. Recent development of techniques of DNA analysis provides a historical perspective to understand the complicated evolutionary history of a number of species and genera. Ancient genome duplication events were detected in many taxa (e.g., BLANC & WOLFE 2004), and it is even hypothesized that most plants have polyploid ancestors. The evolution of polyploids involves substantial genome rearrangement and reduction processes such as chromosome loss and gene loss or silencing. Consequently, the majority of paleopolyploids now behave as diploids, and it is not easy to find traces of old genome duplication in their genomes. A classical example is that of *Arabidopsis thaliana*, with documented polyploidy in the past and up to 90% of genes returned to a single copy state (BLANC & al. 2003). Many polyploid plants are encountered at half-way to the reduced diploid stage, and paleoalloploidy can be detected by population genetic analyses. *Viola* subsect. *Viola* may serve as an example (MARCUSSEN 2000); phenomena such as gene duplication, fixed heterozygosity and striking departures from Hardy-Weinberg equilibria with excessive heterozygosity point to genome doubling through hybridization.

Table 6. Genotype diversity calculated as GD = $1 - \Sigma\, x_i^2$ (where xi is frequency of multilocus genotype *i*, and GD range is $<0; 1>$); see HUGHES & RICHARDS 1988.

VK (N=40)			RO2 (N=23)		
Genotype Code	genotype size	genotype frequency squared (x_i^2)	genotype code	genotype size	genotype frequency squared (x_i^2)
F	3	0.005625	C	5	0.047259
G	6	0.022500	D	18	0.612476
H	10	0.062500			
I	10	0.062500			
J	1	0.000625			
K	1	0.000625			
L	1	0.000625			
M	1	0.000625			
N	1	0.000625			
O	3	0.005625			
P	3	0.005625			
GD = 0.83250			**GD = 0.340265**		

Features relevant for our conservation study are cited from a review by Soltis & Soltis 2000: (i) Polyploids ... generally maintain higher levels of heterozygosity than do their diploid progenitors. (ii) Polyploids exhibit less inbreeding depression than do their diploid parents and can therefore tolerate higher levels of selfing.

Alloploid behaviour of *Gentiana verna* in the Alpine material is obvious (Kříž & al., in prep.; gene duplications and fixed heterozygosity observed), while the Czech material exhibits only traces of old polyploidy in its allozyme patterns (fixed heterozygosity). We can hypothesize that fixed heterozygosity at a few loci might have contributed to the survival of *G. verna* at Rovná under the conditions of selfing and high overall homozygosity (see also David 1998).

5.2. Genetic Distance between the Two Natural Populations of *G. verna* in the Czech Republic

Although Hämmerli 2007 published a convincing evidence for the phylogeographic pattern dividing *G. verna* s. str. into the western and eastern groups of populations, with a dividing line drawn east of the West Carpathians, our results show a finer structure of genetic diversification in the area between the Alps and the Carpathians. The flow cytometry analysis shows that both Czech populations belong to one of the groups, most probably to the western one with 2n=28. The great differences in gene frequencies and unique allele distribution between the two populations (RO and VK) are shown on Figs. 3 and 4 and in Table 5 and 6. The nature of the variability suggests different migration origin of the two populations. There are several case studies showing phylogeographic patterns of counter migrations from the Alps and the Carpathians (Těšitel & al. 2009, Mráz & al. 2007, Ronikier & al. 2008); several taxa of Alpine origin appear in S Bohemia while most of high mountain taxa in N Moravia (the Hrubý Jeseník Mts.) are expected to be of Carpathian origin, although outside the subalpine vegetation exceptions are known. The situation found in *G. verna* may correspond to this picture.

5.3. Uncontrolled Sowing or Transplantation of Protected or Rare Plants

Attempts to "improve" natural populations by means of introduction of plant material (seeds, fruits, plants) of unknown origin are rarely recorded in the literature. We have failed to find any serious discussion on this topic, other than the discussion forum taking place in the Czech Botanical Society, and, expectedly, opinions substantially differ both as to the general ideas and consequences of these controversial activities (for summary, see Kaplan & al. 2007). Most of these discussions, however, suffer from a lack of specific and well-documented examples. A monitoring of genetic erosion taking place after (unintentional) introduction of *Viola*

tricolor s. lat. in the populations of the subendemic *Viola sudetica* was described in KRAHULCOVÁ & al. 1996. This may be a situation with similar consequences but not similar causes. General concepts which might help to evaluate cases of intentional introductions are (i) hybridization, followed by genetic erosion, (ii) introduction of pathogens with alien material, (iii) disturbed competition network, and (iv) problems caused to research for the conservation purposes.

The case of the introduction of totally alien plants into the RO population of *G. verna* shows how radical change in the genetic make-up of the small population may be caused by a few plants with dramatically different genotypes. For the time being, there are probably no direct consequences for the population because of the extremely low rate of sexual recruitment, which prevents the possible hybrid progeny from establishing at the locality. An indirect evidence comes from the seed production and germinability data (KIRSCHNEROVÁ, MORAVCOVÁ & RAUCH, in prep., submitted) showing a sudden increase in seed quality in the mid 2000s.

6. Conclusions for Conservation Planning

The probably different migration origin of the two Czech populations (RO and VK) is to be documented by comparison with plants from source areas (the Alps and the Carpathians) but it is obvious that for conservation purposes they must be treated as separate management units.

Separate conservation strategies for the populations in South Bohemia and NE Moravia are necessary (population/locality approach with active rescue measures versus conservation of the existing habitat).

"Improvement" of genetic make-up of *Gentiana verna* at Rovná raises serious questions.

Heterozygosity (or fixed heterozygosity) preserved in the paleoallopoid, together with the prevailing clonal growth, may represent factors preventing this small, genetically depauperate population from total extinction.

7. Acknowledgements

We are grateful to I. PLAČKOVÁ, K. KOTTOVÁ, L. MORAVCOVÁ, J. MALÁ, J. RAUCHOVÁ and M. KŘÍŽ for allowing access to unpublished data and for valuable technical assistance. Thanks are due to Z. MÜNZBERGOVÁ for her comments on an early version of the manuscript. The staff of the Nature Conservancy offices at Protected Landscape Areas of Jeseníky and Blansky les provided important support for the field research. The work was prepared on the basis of a grant by the Ministry of Environment of the Czech Republic, no. SP/2d4/112/08, and supported by the Institute of Botany Research Plan no. AV0Z60050516.

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