Cyclamen fatrense, myth or true Western Carpathian endemic? Genetic and morphological evidence

Je brambořík fatranský mýtus nebo skutečný západokarpatský endemit?

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Cyclamen fatrense is described as a narrow endemic of the Western Carpathians, but because of its uncertain morphological distinction from the closely allied *C. purpurascens* it is not generally accepted as a separate species. Genetic, morphological and karyological analyses were used to revise the taxonomic status of the Western Carpathian populations of *C. fatrense* and explore potential differentiations in the widely distributed *C. purpurascens*. Our results show that the Western Carpathian populations are genetically and morphologically separate from those of *C. purpurascens* and should be considered a separate endemic subspecies (*C. purpurascens* subsp. *immaculatum*). The most important diagnostic characters discriminating it from the nominate subspecies include the absence of ornamentation on the upper leaf surface, the shape of the leaves and sepals, the width of the petals and the position of the widest part of the leaf lamina. All populations studied are karyologically uniform and diploid ($2n = 2x = 34$) with negligible differences in their absolute DNA content ($2C = 6.63–6.92$ pg).

Keywords: AFLP, *Cyclamen*, endemism, genome size, multivariate morphometrics, systematics

Introduction

The evolution and spatial distribution of endemics is not random but result from the interplay of ancient and recent processes. The most important factors influencing speciation and spatial pattern of endemism are considered to be the long-term isolation and overall stability of biomes that harbour endemic organisms (Cox & Moore 2005). Since oceans and seas are considered to be the most important physical barriers to dispersal for the majority of terrestrial plants, the highest concentration of endemics are likely to occur on islands and oceanic archipelagos. Furthermore the “island like” continental areas in tropical rain forests and on high mountain ranges, characterized by special eco-climatologic conditions substantially different from those of adjacent regions, are also likely to be important centres of endemism (Kier et al. 2009). The regions in Europe rich in endemics, the so-called “evolutionary hotspots”, are predominantly located on southern-European peninsulas. These also include a number of high mountain ranges, which were characterized by a relatively mild and stable climate during Quaternary climate changes (see Hewitt 1996, 2004, Schmitt 2007). A large number of endemics evolved there because these regions served as glacial refugia for the majority of temperate European organisms during the Pleistocene (Hewitt 1996, 2004, Finnie et al. 2007, Médail & Diadema 2009). In addition to the southern peninsulas, there are also important centres of endemism located in more northerly parts of Europe in the Alps and Carpathians (Pawłowski 1970, Finnie et al. 2007). Endemism in the Alps was extensively studied over recent decades and the highest concentrations of endemic taxa there occur at the southern and southeastern margins, which served as glacial refugia, not only for high mountain arctic-alpine species (see Pawłowski 1970, Tribsch & Schönswetter 2003, Tribsch 2004, Schönswetter et al. 2005 for review) but also for temperate trees and woodland organisms (e.g. Willis & van Andel 2004, Magri et al. 2006, Bhagwat & Willis 2008, Kramp et al. 2009, Willner et al. 2009, Michl et al. 2010). There are also a high number of endemic species in the Carpathians, which is one of the northernmost centres of endemism in Europe (Pawłowski 1970, Finnie et al. 2007). The most comprehensive list of endemics and subendemics recorded in the Carpathian arc was published by Pawłowski (1970). In contrast, other authors only record endemics occurring in limited parts of the Carpathians, which are located in particular countries (Kliment 1999, Piękoś-Mirkowa & Mirek 2003, 2009, Oprea 2005, Tasenkevich 2011). It is unknown how many endemics there are in the Carpathians, as the majority were described only on the basis that they differ morphologically from closely related taxa and their status has yet to be verified using modern taxonomic methods (but see Goliašová 1985, Hodálová & Marhold 1998, Hodálová 1999, Mráz 2001, 2003, 2005, Zhang et al. 2001, Dvořáková 2003, Zhang & Kadereit 2004, Kochjarová et al. 2006, Ronikier & Szeląg 2008, Šingliarová & Mráz 2009, Šingliarová et al. 2011, Štěpánek et al. 2011).

A typical example of such a doubtful Carpathian endemic is *Cyclamen fatrense* Halda et Soják (*Primulaceae*), which is considered to be a narrow endemic in the Western Carpathians with distribution restricted to a few localities in the Veľká Fatra Mts, Nízke Tatry and Poľana Mts (Marhold & Hindák 1998, Bernátová & Feráková 1999, Turis 2008, 2009, Mereďa & Hodálová 2011). It was first described as a variety of *C. purpurascens*, namely as *C. europaeum* subsp. *orbiculatum* (Mill.) O. Schwarz var. *immaculatum* Hrabětová (Hrabětová 1950), later elevated to the subspecies level as *C. purpurascens* subsp. *immaculatum* (Hrabětová) Halda et Soják (Halda & Soják 1971a) and finally defined as the species *C. fatrense* Halda et Soják (Halda & Soják 1971b). In addition to its isolated distribution in the Western Carpathians, the absence of silver-grey ornamentation on the upper surface of its leaves and to a lesser extent a more obtuse leaf apex are the most important diagnostic character distinguishing it from *C. purpurascens* Mill. (Hrabětová 1950, Halda & Soják 1971a, b, Mereďa & Hodálová 2011). Since individuals without ornamentation sporadically occur in populations of *C. purpurascens* (Hrabětová 1950, Turis 2008, 2009, authors' personal observations) and other morphological characters appeared somewhat doubtful, *C. fatrense* was not accepted as a separate species by some European taxonomists (e.g. Grey-Wilson 2003).

In a previous comprehensive study, we explored the genetic pattern and putative glacial refugia of *C. purpurascens* and *C. fatrense* (henceforth *C. purpurascens* s.l. in the text) in Europe using amplified fragment length polymorphism (AFLP) and sequences of the *trn*D-*trn*T intergenic spacer of chloroplast DNA (Slovák et al. 2012b). AFLP data revealed the existence of five allopatric phylogeographic lineages (phylogroups) distributed as follows: I – the western parts of the Eastern Alps, ranging from the Alpi Orobie and Rätikon in the west to Berchtesgadener Alpen and Karawanken in the east; II – northwestern Dinarides, easternmost Alps, southwestern Pannonia and southern Moravia; III – the westernmost Alps and the Jura Mountains; IV – the Western Carpathians; and V – the eastern

Dinarides. Western Carpathian populations in phylogroup IV have the same chloroplast DNA haplotype as populations in the Alps, Dinarides, the Pannonian plain and Moravia (phylogroups I, II, V). However, the inference drawn from AFLP data is that they make up a homogeneous independent genetic lineage distinct from others (Slovák et al. 2012b). The question whether the Western Carpathian populations can be characterized morphologically and/or karyologically, and thus deserve recognition as a separate taxon, however remained unresolved. In this study, we apply a combination of genetic (AFLP), morphological and karyological data to definitely resolve the taxonomic status of the Western Carpathian populations and reveal their relationship with those of *C. purpurascens* in the other parts of its distribution. The following specific questions are addressed: (i) What is the overall morphological and karyological variation in *C. purpurascens* s.l. throughout its entire distribution? (ii) Are the patterns in the morphology and karyology of *C. purpurascens* s.l. congruent with the genetic groups revealed by AFLP? (iii) Is the overall distinction of the Western Carpathian populations sufficient for them to be considered an endemic taxon?

Material and methods

Study species

According to recently published studies, the genus *Cyclamen* comprises approximately 21 taxa that are predominantly restricted to the Mediterranean basin, but extend eastwards to the Caspian region and eastern Africa (Compton et al. 2004, Yesson et al. 2009). Cyclamens are long lived tuberous geophytes, which mainly inhabit the understory level of various woodland biomes. The majority of species are endemics with distributions restricted to islands or single mountain ranges, but there are a few taxa, such as *C. coum* Mill., *C. hederifolium* Aiton and *C. purpurascens*, which are widely distributed throughout large geographic regions (Grey-Wilson 2003, Yesson et al. 2009). *Cyclamen purpurascens* s.l. together with *C. africanum* Boiss. et Reut., *C. colchicum* (Alboff) Alboff, *C. confusum* (Grey-Wilson) Culham, Jope et P. Moore and *C. hederifolium* belong to the subgenus *Cyclamen* (Compton et al. 2004, Culham et al. 2009). From a karyological viewpoint, all members of the subgenus *Cyclamen* are characterized by the basic chromosome number $x = 17$, with *Cyclamen purpurascens* s.l. reported to be exclusively diploid with $2n = 2x = 34$ (Hrabětová et al. 1983, Bennett & Grimshaw 1991, Anderberg 1994, Grey-Wilson 2003).

Cyclamen purpurascens s.l. is a widespread species occurring on calcareous mountain ranges from southeastern France, through the Alps towards the eastern Dinarides in the south and to the Western Carpathians in the north. Its flowers are pendant, fragrant, pink to dark purple and its leaves are cordate with variable silver-grey pigment ornamentation on the upper surface (Meikle & Sinnott 1972, Grey-Wilson 2003). In addition to their prevailing self-incompatibility, a certain level of selfing is recorded in Western Carpathian populations of *C. purpurascens* s.l. (Turis 2008, 2009). Flowers are pollinated by various unspecialized insects, especially thrips (Thysanoptera) (Turis 2008, 2009). It was believed that its seeds are dispersed by ants, similar to the other members of *Cyclamen* (e.g. Grey-Wilson 2003). However, in the Western Carpathians the seed of *C. purpurascens* s.l. is dispersed more by water from melting snow or a heavy rainfall than by ants (Turis 2008, 2009).

Table 1. – List of populations of *C. purpurascens* subsp. *purpurascens* (CP) and *Cyclamen purpurascens* subsp. *immaculatum* (CF) included in the present study. Localities: AT – Austria, BA – Bosnia and Herzegovina, CH – Switzerland, CZ – Czech Republic, DE – Germany, FR – France, HR – Croatia, HU – Hungary, IT – Italy, RS – Serbia, SI – Slovenia, SK – Slovakia. AFLP: n/n_{gen} – number of plants analysed for AFLPs/number of AFLP genotypes; MM – number of plants analysed for multivariate morphometrics.

Population code, locality	Latitude (N), longitude (E)	AFLP n/$n_{gen.}$	MM
Cyclamen purpurascens* subsp. *immaculatum			
CF1 – SK, Nízke Tatry Mts, Bukovská dolina valley, 760 m	48°51'13" 19°10'40"	6/6	21
CF2 – SK, Veľká Fatra Mts, Prašnica settlement, 694 m	48°52'22" 19°07'36"	6/6	16
CF3 – SK, Nízke Tatry Mts, Motyčky village, 691 m	48°51'39" 19°10'03"	6/6	26
CF6 – SK, Veľká Fatra Mts, Suchá dolina valley, 760 m	48°54'06" 19°09'25"	6/6	33
CF8 – SK, Veľká Fatra Mts, Belianska dolina valley, 620 m	48°58'38" 19°03'42"	6/2	22
CF9 – SK, Veľká Fatra Mts, Necpalská dolina valley, 613 m	48°58'59" 19°03'42"	6/6	19
CF10 – SK, Veľká Fatra Mts, Mošovce village, loc. Bôriny, 629 m	48°54'32" 18°55'36"	6/6	19
CF11 – SK, Veľká Fatra Mts, Rakša village, dolina Hrádky valley, 565 m	48°52'41" 18°54'31"	3/3	15
CF12 – SK, Veľká Fatra Mts, Rakša village, dolina Mača valley, 588 m, type locality of *C. fatrense*	48°53'06" 18°55'31"	6/6	17
Cyclamen purpurascens* subsp. *purpurascens			
CP2 – HU, Soproni-hegység Mts, Brennbergbánya village, 420 m	47°39'04" 16°29'05"	6/6	19
CP5 – CZ, Moravské Podhůří Vysočiny, Javůrek village, Šmelcovna settlement, 350 m	49°15'53" 16°21'43"	6/6	21
CP7 – CZ, Moravské Podhůří Vysočiny, Mt Klučovská hora, 595 m	49°10'13" 15°55'29"	6/6	21
CP10 – CZ, Znojemsko-brněnská pahorkatina, Vranov nad Dyjí town, valley of Dyje river, 320 m	48°53'32" 15°49'30"	6/6	20
CP11 – AT, Wienerwald, Sulz im Wienerwald village, Mt Weinberg, 407 m	48°05'20" 16°08'49"	6/6	21
CP14 – AT, Oberöstereichische Voralpen, Klaus an der Pyhrnbahn village, 595 m	47°49'41" 14°09'11"	5/4	21
CP16 – DE, Berchtesgadener Alpen, Unterjettenberg village, 620 m	47°40'04" 12°50'01"	6/6	18
CP19 – AT, Bucklige Welt, near Wartenstein castle, 767 m	47°38'47" 15°55'13"	6/6	21
CP21 – SI, Slovenske Gorice Mts, Spodnji Ivanjci village, 260 m	46°35'01" 15°56'48"	6/6	19
CP23 – SI, Kamniške Alpe, Nazarje village, 371 m	46°18'45" 14°57'01"	6/6	20
CP25 – SI, Karavanke, Podljubelj village, 765 m	46°24'52" 14°16'14"	6/6	19
CP26 – SI, Julijske Alpe, near Laški Rovi village, 525 m	46°16'06" 13°53'55"	–	–
CP27 – SI, Julijske Alpe, near Aljažev dom cottage, 992 m	46°27'48" 13°50'51"	6/6	20
CP30 – SI, Planinsko polje plateau, Grčarevec village, 527 m	45°51'54" 14°13'11"	6/6	20
CP31 – SI, Goteniška Gora Mts, Hrib-Loški potok village, 859 m	45°42'42" 14°35'25"	6/6	20
CP34 – IT, Alpi Carniche orientali, Pioverno village, 280 m	46°19'57" 13°07'24"	6/6	20
CP37 – IT, Alpe del Garda, Monte Baldo, 1594 m	45°46'59" 10°51'53"	6/6	20
CP38 – IT, Alpi dell'Adamello e della Presanella, Gardone Val Trompia village, 364 m	45°41'52" 10°12'07"	6/6	20
CP39 – IT, Alpi Orobie, Pasturo village, 625 m	45°56'12" 09°27'04"	6/6	19
CP41 – AT, Rätikon, near Schattenburg castle, 494 m	47°14'14" 09°36'05"	6/5	19
CP43 – AT, Karawanken, Arnoldstein village, 589 m	46°22'58" 13°43'39"	6/6	19
CP45 – AT, Grazer Bergland, Peggau village, 454 m	47°12'05" 15°21'08"	6/6	20
CP46 – Serbia, Tara Mts, Perućac village, 285 m	43°57'35" 19°23'47"	6/6	20
CP47 – BA, Varda Mts, Meremišlje village, 420 m	43°44'36" 19°13'17"	6/6	20
CP48 – BA, Volujak Mts, valley of Bijela reka river near Tarčin village, 639 m	43°48'12" 18°05'15"	6/6	21
CP51 – BA, Bandera Mts, Bočačka Klisura gorge, 320 m	44°25'04" 17°15'31"	6/6	20
CP52 – BA, Čvrsnica Mts, valley of Doljanka river, 315 m	43°39'47" 17°42'39"	4/4	20
CP53 – HR, Velebit Mts, Vratnik saddle, 695 m	44°58'43" 14°59'10"	6/6	15

CP55 – HR, Ćićarija Mts, valley of Mirna river below Sovinjak village, 34 m	45°23'13" 13°55'30"	5/5	20
CP58 – HR, Mala Kapela Mts, Lička Jesenica village, 658 m	44°59'14" 15°25'02"	6/6	20
CP60 – HR, Moravačka Gora Mts, Podgarić village, 360 m	45°37'28" 16°44'13"	5/5	10
CP62 – HR, Medvednica Mts, Pila village, 266 m	45°56'26" 15°56'50"	6/6	19
CP65 – HU, Zalai-dombság hills, Kisfernekág village, 224 m	46°50'17" 16°35'20"	6/6	21
CP66 – DE, Bayerischer Wald, Jochenstein village, 292 m	48°31'50" 13°40'59"	6/6	19
CP71 – CH, Jura Mts, Cornaux village, 536 m	47°02'37" 07°01'11"	6/4	20
CP72 – FR, Jura Mts, Cascade de la Billaude waterfalls, 609 m	46°40'54" 05°56'13"	6/2	17
CP74 – FR, Chartreuse Mts, Cascade d'Alloix waterfalls, 734 m	45°23'35" 05°56'40"	6/2	19
CP75 – IT, Prealpi Bellunesi, Seren del Grappa village, 381 m	45°58'18" 11°50'11"	6/6	18

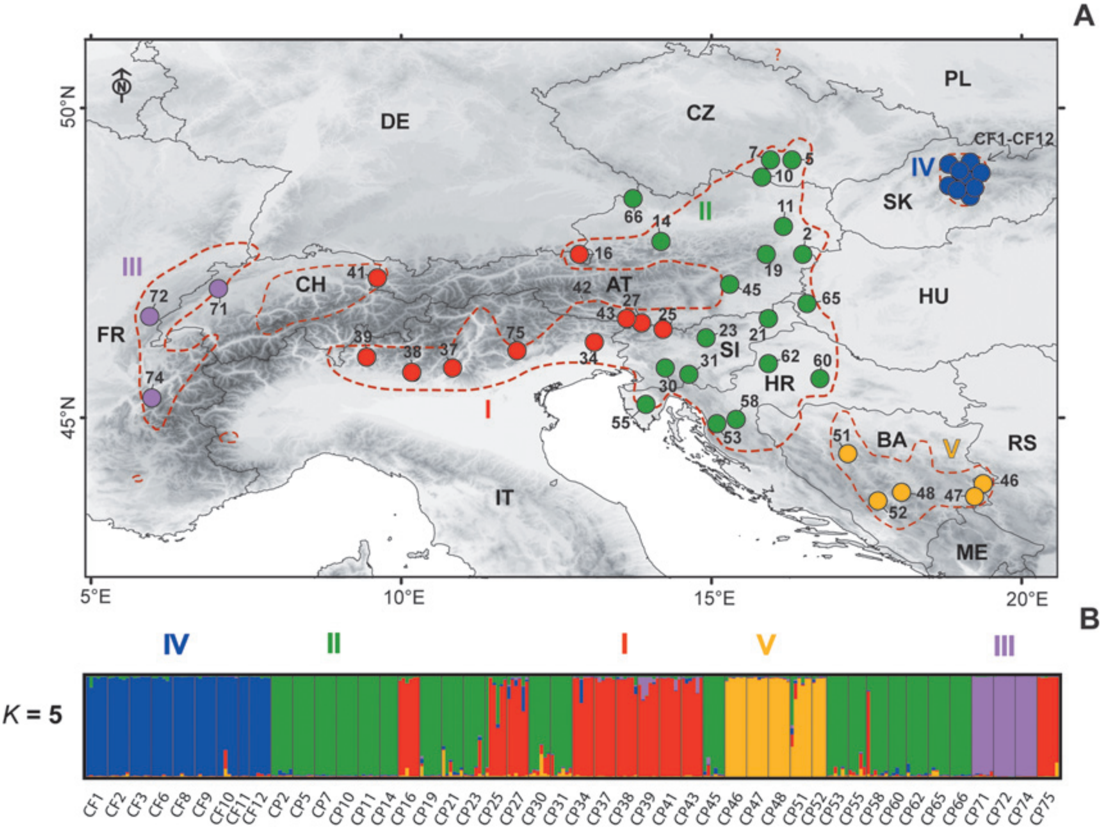

Fig. 1. – (A) Map showing the geographic location of the populations of *C. pupurascens* s.l. sampled (population numbers and country abbreviations see Table 1). Different colours indicate genetic groupings as resolved by Bayesian analysis at K = 5: red – phylogroup I, green – phylogroup II, pink – phylogroup III, blue – phylogroup IV and yellow – phylogroup V. The dashed red outlines indicate the indigenous distribution range of this species based on literature sources and herbarium specimens. (B) Genetic structure of *Cyclamen purpurascens* s.l. (286 individuals from 46 populations), as resolved by the Bayesian clustering (STRUCTURE software) of AFLP genotypes at K = 5 (denoted as phylogroups I–V). Each individual is represented by a vertical bar, coloured proportionally according to the cluster assignment. The population abbreviations follow those in Table 1.

Plant material

We analysed a total of 46 indigenous populations of *C. purpurascens* s.l., for which both AFLP and morphological data were available (Table 1). The populations sampled cover the entire distribution area and included all previously detected genetic lineages of *C. purpurascens* s.l. (Fig. 1). AFLP data were taken from our previous study, which encompassed 68 populations and 399 individuals (Slovák et al. 2012b), but in the current study we utilized the reduced AFLP dataset of 46 populations and 268 individuals and compared it with the morphological data of 903 individuals (Table 1). One fully developed leaf and one flower from 10 to 33 individuals per population were sampled and analysed using multivariate morphometric analysis (Table 1). Karyological analysis, DNA ploidy level and genome size estimation were performed on 34 individuals from 29 populations (Electronic Appendix 1). The voucher specimens are deposited in the herbarium of the Institute of Botany, Slovak Academy of Sciences (SAV) (Holmgren et al. 1990).

AFLP data analysis

The details of AFLP data generation are presented in Slovák et al. (2012b). The Bayesian analysis of the reduced dataset using the Markov chain Monte Carlo (MCMC) algorithm was conducted using STRUCTURE 2.2.3 (Falush et al. 2007). The settings for the analysis were the same as those used in Slovák et al. (2012b) and the analysis was computed at Bioportal of the University of Oslo (http://www.bioportal.uio.no). The R-script Structure-sum-2009 (part of AFLPdat; Ehrich 2006) was used to compute statistical parameters (see Evanno et al. 2005). The CLUMPP ver. 1.1.1 (Jakobsson & Rosenberg 2007) and DISTRUCT (Rosenberg 2004) software was used to display the results graphically.

Morphometric analysis

Morphological characters were selected on the basis of field observations, taxonomic literature and a study of herbarium specimen (BP, BPU, BRA, BRNM, BRNU, GRM, PR, PRC, SAV, SLO, W, WU, ZA, ZAHO; Holmgren et al. 1990). The leaves were dried and stored as herbarium specimens until use, and fresh flowers were cut with a razor blade and each flower part (petal, sepal, stamen and pistil) was attached to paper by translucent adhesive tape. In this way the original character parameters were preserved. The flower parts attached to paper were scanned using a Microtek ScanMaker 9800XL scanner and measured using Carnoy 2.0 (Schols et al. 2002), and the quantitative characters of the lamina of leaves were measured using a ruler.

A total of 21 morphological characters were measured or scored. Twelve of these were quantitative, one was binary and eight were derived ratios. As listed in Table 2, only 16 characters were included in the final data matrices; of these seven are quantitative, one binary and eight derived ratios. Only four primary characters (the maximum length and width of the leaf lamina, the length from the apex of the leaf to the petiole, the length from the apex of the leaf to its widest part and the petal length from the apex to its widest part) were used in ratio calculations. Those measured on leaf laminas were excluded from analyses because leaf size is strongly influenced by the ontogenetic stage and ecological factors. Since this is true only to a lesser extent for the primary characters of maximum petal, sepal and stamen length and width, and the pistil length, these were included in the analysis.

Although the colour of ornamentation on the upper leaf surface is rather invariable, its pattern varies considerably at the population level (authors' personal observation). Therefore, only the presence or absence of ornamentation was scored. The multivariate morphometric approach was used to elucidate the overall morphological variation in *C. purpurascens* s.l. In the first step, we computed correlation coefficients suitable for detection of highly correlated pairs of morphological characters, which could distort the computations in further multivariate analysis. Since most analysed characters showed deviation from normal distribution, the nonparametric Spearman correlation coefficient was chosen (Legendre & Legendre 1998). Subsequently, principal component analysis (PCA), based on a correlation matrix, was used as a hypothesis generating method (Sneath & Sokal 1973, Krzanowski 1990). In addition, canonical discriminant analysis (CDA), k nearest-neighbour non-parametric classification discriminant analysis (DA) and also simple linear classification functions were performed for hypothesis testing (Klecka 1980, Krzanowski 1990). Several different data matrices based on individuals and populations as operational taxonomic units were generated and used in multivariate analysis. Although the binary character presence of ornamentation on the upper leaf surface (POL) has been considered the most important diagnostic character for *C. fatrense*, we decided to precisely specify its contribution to discrimination between assumed taxa. Therefore, the majority of the discriminant analyses were run alternatively with this character included or excluded.

Table 2. – List of morphological characters used in the multivariate morphometric analyses.

Character code	Character explanation
SL	shape of leaf expressed as ratio of total length of leaf and width of leaf at widest part of the lamina
PWPL	position of widest part of leaf lamina expressed as ratio of total width of leaf and length from the apex of leaf to its widest part
SAL	shape of the leaf apex expressed as the angle of the leaf apex
DLI	depth of the lamina incision expressed as ratio of total length of leaf and length from apex of leaf to leaf petiole
POL	presence or absence of ornamentation on the upper leaf surface (0 – absent, 1 – present)
MLPe	maximum length of the longest petal (mm)
MWPe	maximum width of the widest petal (mm)
SPe	shape of petal expressed as ratio of maximum length of petal and maximum width of petal
PWPPe	position of widest part of petal expressed as ratio of maximum length of petal to length of petal from apex to widest part of petal
MLSe	maximum length of the longest sepal (mm)
MWSe	maximum width of the widest sepal (mm)
SSe	shape of sepal expressed as ratio of total length of sepal and maximum width of sepal at its widest part
MLSt	maximum length of longest stamen (mm)
MWSt	maximum width of widest stamen (mm)
SSt	shape of stamen expressed as ratio of maximum length of stamen and maximum width of stamen
LP	length of pistil (mm)

Three PCA were utilized: (i) PCA1 for 46 populations and 16 characters was performed to determine the overall morphological variation in the dataset analysed. Since the CP72 population in the Jura Mts in France was placed in a conspicuously outlying position compared to the other populations in the first PCA1 (see Results), it was decided not to include this population in the analysis, so (ii) PCA2 was computed for 45 populations and 16 characters, omitting the outlying CP72 population and (iii) PCA3 for 886 individuals and 16 characters to reveal morphological differentiation at the individual level.

Subsequently a series of CDA were used to determine whether genetic AFLP phylogroups revealed by Bayesian clustering at K = 5 can also be defined morphologically. Thus, CDA1A was for 45 populations and 16 characters and CDA1B for 45 populations and 15 characters, based on five predefined groups (K = 5), which did not include the morphologically anomalous CP72 population. The morphological variation of individuals in the five predefined groups was determined by CDA2A (886 individuals and 16 characters) and CDA2B (886 individuals and 15 characters). We explored whether individuals from the Western Carpathians are distinct from those in the other areas included in CDA3A (886 individuals and 16 characters) and CDA3B (886 individuals and 15 characters). The k nearest-neighbour non-parametric classificatory discriminant analysis was performed on 886 individuals and 15 to 16 morphological characters.

The DA1A and DA1B were used to determine the percentage of correctly classified individuals in the five genetic phylogroups, while DA2A and DA2B were initiated to discriminate between Western Carpathian plants and those from the Alps, Dinarides, Jura Mts, southwestern Pannonia and southern Moravia. In order to obtain a simple criterion that could be utilized for determining the difference between the Western Carpathian population and those from the rest of the distribution area, linear classification functions were also derived using parametric discriminant analysis.

The mean values, standard deviations, medians, 5% and 95% percentiles were then calculated for all five phylogroups and recognized units. All analytical calculations were done using the SAS 8.2 package (SAS Institute 2000).

Chromosome number

Chromosome numbers were counted in root tip cells of plants cultivated in a greenhouse at the Institute of Botany of the Slovak Academy of Sciences in Bratislava (Electronic Appendix 1). The fresh root tips were pre-treated with 0.002 M hydroxyquinoline solution overnight at 4 °C to accumulate metaphases, and then fixed in a mixture of 96% ethanol and 100% acetic acid (3:1) for up to one hour at 4 °C. Pre-treated roots were washed with distilled water and hydrolyzed using a mixture of 35% hydrochloric acid and 96% ethanol (1:1) for 3 to 5 minutes and rewashed with distilled water. Squashes were made under cellophane squares following the method of Murín (1960) and stained in a 10% solution of Giemsa in 0.2 M Sörensen phosphate buffer for one hour. Slides were examined under an Olympus BX 61 light microscope fitted with an oil immersion objective.

Genome size and DNA ploidy level

Individuals with known chromosome numbers (one accession for *C. purpurascens* and *C. fatrense*, Electronic Appendix 1) were subsequently used as reference points in the flow cytometry in order to relate fluorescent intensity to DNA ploidy level.

The absolute DNA content (C-values sensu Greilhuber et al. 2005) and DNA ploidy levels (Suda et al. 2006) of the plants analysed were estimated using flow cytometry. This analysis was performed on a CyFlow SL cytometer (Partec GmbH, Münster, Germany) equipped with a green solid-state laser (Cobolt Samba 532 nm, 100 mW; Cobolt, Stockholm, Sweden) as the excitation source. The plants of known chromosome numbers (Electronic Appendix 1) were simultaneously analysed with the most appropriate primary reference standard, which proved to be *Pisum sativum* L. 'Ctirad' (2C = 9.09 pg; Doležel et al. 1998), and the ratios of their G1 peaks were recorded. These same plants then served as reference material for DNA ploidy level inference in further analysis. As the initial analysis performed on leaf tissues posed a problem due to secondary metabolites in the leaves, flower petal tissues were used in subsequent analyses. The samples were prepared in a two-step procedure with Otto buffers and propidium iodide (PI), which was the fluorescent dye used to stain the material. Small pieces up to 0.5 cm^2 of both the internal reference standard and fresh flower tissue from the plants analysed were sliced with a sharp razor blade in 1 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20; Otto 1990). The resultant suspension was filtered through a 42 μm nylon mesh and incubated at room temperature for at least 10 min. After this, the nuclei were stained in 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$), supplemented with 50 μg/ml of propidium iodide (PI) and 50 μg/ml of RNase IIA and 2-mercaptoethanol (2 μl/ml), and incubated for at least 10 minutes at laboratory temperature. Each sample was measured independently at least three times on different days in order to minimize potential random instrumental drift, and the fluorescence intensity of 5000 particles was then recorded for each analysis. Differences in the values measured on different days were recorded as the standard errors of means, and expressed as a percentage of the mean value. Analyses that differed by more than 2% were discarded and reanalysed. Histograms containing a symmetrical peak and a coefficient of variance (CV) of both standard and sample G1 peaks below 3.5% were evaluated using Partec FloMax software (v. 2.7d; Partec GmbH Münster, Germany).

Results

AFLPs

The STRUCTURE analysis of the reduced dataset revealed an almost identical genetic partition to the one inferred previously from the complete dataset (see Slovák et al. 2012b). In order to investigate the correlation between genetic and morphological variation within *C. purpurascens* s.l., we decided to accept the results of Bayesian clustering at K = 5 (Fig. 1B), which delimited five genetic groups (phylogroups), and the geographic delimitation as proposed by Slovák et al. (2012b).

Morphometric analysis

Since the correlation coefficients between pairs of morphological characters did not exceed 0.7, none of the characters measured were excluded from further analyses. The highest correlations of between 0.5 and 0.7 were recorded for the following pairs of characters: (i) the shape of the leaf and the position of the leaf's widest part, (ii) the maximum width of the widest stamen and stamen shape, (iii) the maximum width of a petal, and petal

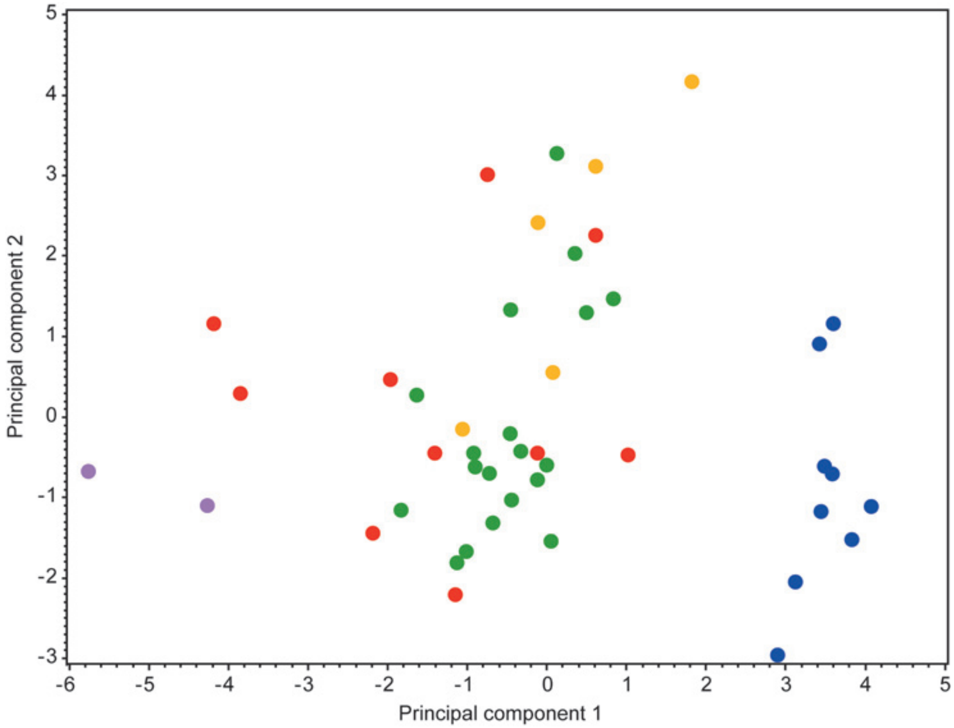

Fig. 2. – Principal component analysis (PCA) of populations of *C. purpurascens* s.l. Different colours indicate genetic groupings as resolved by Bayesian analysis at K = 5. PCA2 based on 45 populations and 16 morphological characters. The first two ordination axes explain 34.2% and 17.3% of total variation, respectively.

shape, (iv) the maximum width of the broadest sepal and sepal shape and (v) the maximum length of the longest sepal and sepal shape.

Although the initial PCA1 (figure not shown) based on all 46 populations analysed and 16 morphological characters indicated a certain amount of structuring in the dataset, the observed pattern within the morphological space was considerably biased by the strongly outlying position of population CP72 from the Jura Mts in France along the second axis. The morphological characters that most significantly contributed to the position of population CP72 were the maximum width of the broadest sepal, the length of the pistil and the maximum length of the longest petal. After excluding population CP72, the principal component analysis PCA2 revealed three groups of populations separated along the first axis (Fig. 2; 45 populations and 16 characters). The characters that contributed most to separation along the first ordination axis were the shape of the leaf lamina, the presence of ornamentation on the upper leaf surface, the maximum width of the broadest petal, the shape of the stamen and the position of the widest part of the leaf lamina (Table 3). The first and smallest group located on the left of the PCA diagram includes four populations, in particular one from the Jura Mts (CP71) and one from the western Alps (CP74), and in addition two from the central part of the eastern Alps (CP16 and CP43). From a genetic viewpoint, following the partition inferred by Bayesian clustering at K = 5, this group

Table 3. – The numerical output of morphometric analyses: PCA 1, PCA 2, PCA 3 – eigenvectors expressing correlations of the characters examined with the principal components (axis 1, axis 2); CDA 1A, 1B, CDA 2A, 2B, CDA 3A, 3B – total canonical structure expressing correlations of the morphological characters with the canonical axes (axis 1, axis 2). For details of the different PCA and CDA, see Material and methods. For character explanation, see Table 2. Important characters referred to in the text are in bold.

Character	PCA1		PCA2		PCA3		CDA1A		CDA1B		CDA2A		CDA2B		CDA3A	CDA3B
	Axis1	Axis2	Axis1	Axis2	Axis1	Axis2	Axis1	Axis2	Axis1	Axis2	Axis1	Axis2	Axis1	Axis2	Axis	Axis
SL	–0.330	0.146	**–0.355**	0.019	**–0.353**	0.199	0.701	–0.478	0.795	–0.293	0.504	–0.468	0.687	–0.200	0.461	**0.618**
PWPL	–0.289	0.201	**–0.313**	0.071	–0.292	0.231	0.585	–0.356	0.658	–0.189	0.339	–0.276	0.451	–0.115	0.312	**0.419**
SAL	0.060	0.027	0.067	0.084	0.138	–0.116	0.107	0.523	–0.002	0.513	0.068	0.483	**–0.025**	0.630	0.110	0.147
DLI	0.269	–0.160	0.270	–0.190	0.193	–0.121	–0.532	0.285	–0.598	–0.028	–0.299	0.217	–0.409	–0.087	–0.290	–0.389
POL	–0.323	0.231	**–0.338**	0.169	**–0.308**	0.143	0.964	0.165	–	–	0.933	0.224	–	–	0.946	–
MLPe	0.240	**0.354**	0.200	0.288	0.301	0.160	–0.192	**0.598**	–0.306	**0.602**	–0.140	**0.620**	–0.304	**0.601**	–0.086	–0.115
MWPe	0.326	0.122	0.319	0.159	0.384	0.326	–0.499	0.383	–0.568	0.348	–0.355	0.367	–0.491	0.216	–0.318	**–0.427**
SPe	–0.120	0.269	–0.197	0.107	–0.157	–0.213	0.405	0.106	0.380	0.159	0.254	0.096	0.267	0.239	0.257	0.345
PWPPe	–0.292	–0.016	–0.277	0.033	–0.194	0.068	0.454	–0.377	0.527	–0.274	0.254	–0.328	0.368	–0.195	0.224	0.301
MLSe	0.240	0.245	0.215	0.256	0.291	0.056	–0.373	0.122	–0.390	0.088	–0.217	0.098	–0.280	–0.076	–0.208	–0.280
MWSe	–0.071	**0.482**	–0.111	**0.473**	0.075	**0.529**	0.383	0.033	0.397	0.420	0.184	0.030	0.216	0.274	0.197	0.265
SSe	0.259	–0.201	0.267	–0.177	0.178	**–0.377**	**–0.631**	0.039	**–0.650**	–0.304	–0.348	0.028	–0.421	–0.325	–0.353	**–0.474**
MLSt	0.296	0.289	0.276	**0.350**	0.349	0.238	–0.440	0.434	–0.511	0.519	–0.305	0.425	–0.443	0.347	–0.259	–0.347
MWSt	–0.038	0.273	–0.028	**0.443**	–0.021	**0.376**	0.139	0.073	0.150	0.413	0.057	0.075	0.061	0.253	0.076	0.102
SSt	0.327	0.002	0.314	–0.132	0.274	–0.196	**–0.610**	0.387	**–0.696**	0.086	–0.285	0.248	–0.393	0.015	–0.268	–0.360
LP	0.195	**0.394**	0.147	**0.381**	0.142	0.122	–0.194	0.163	–0.221	0.210	–0.097	0.092	–0.127	0.094	–0.084	–0.113

included populations belonging to phylogroups I and III. The largest group, located in the middle of the PCA diagram, is the most heterogeneous and tends to be divided into two subclusters along the second axis based on the following floral characters; the width of the widest sepal, the maximum width of the widest stamen, the length of the pistil and the maximum length of the longest stamen (Table 3). Both subgroups include a mixture of populations originating from a large area covering almost the entire Alps, the Dinarides, southwestern Pannonia and southern Moravia. These form part of genetic phylogroups I, II and V. Finally, the last group located on the right of the diagram includes populations exclusively from the Western Carpathians and belong to phylogroup IV. Furthermore, one apparently unpartitioned large group was revealed by PCA3 (Fig. 3), which was based on 886 individuals and 16 characters. Although the individuals belonging to phylogroups III and IV were mainly located at the peripheries of this group, they overlapped significantly with those belonging to phylogroups I, II and V.

Putative morphological differentiation of genetic phylogroups was subsequently tested by a series of canonical discriminant analyses. The CDA1A based on 45 populations and all 16 characters computed using five predetermined groups (Fig. 4A) detected three clusters that were approximately identical to the ones recorded in PCA2. The only difference was that the two populations CP 16 and CP 43 belonging to phylogroup I were in the largest heterogeneous group instead of the two populations belonging to phylogroup III. The distinction of these three groups was affected mostly by the presence of ornamentation on the upper leaf surface and to a much lesser extent the shapes of the leaf, sepal and stamen (Table 3). All three clusters were clearly separated along the first canonical axis, even when the presence of the ornamentation on the upper leaf surface was not considered

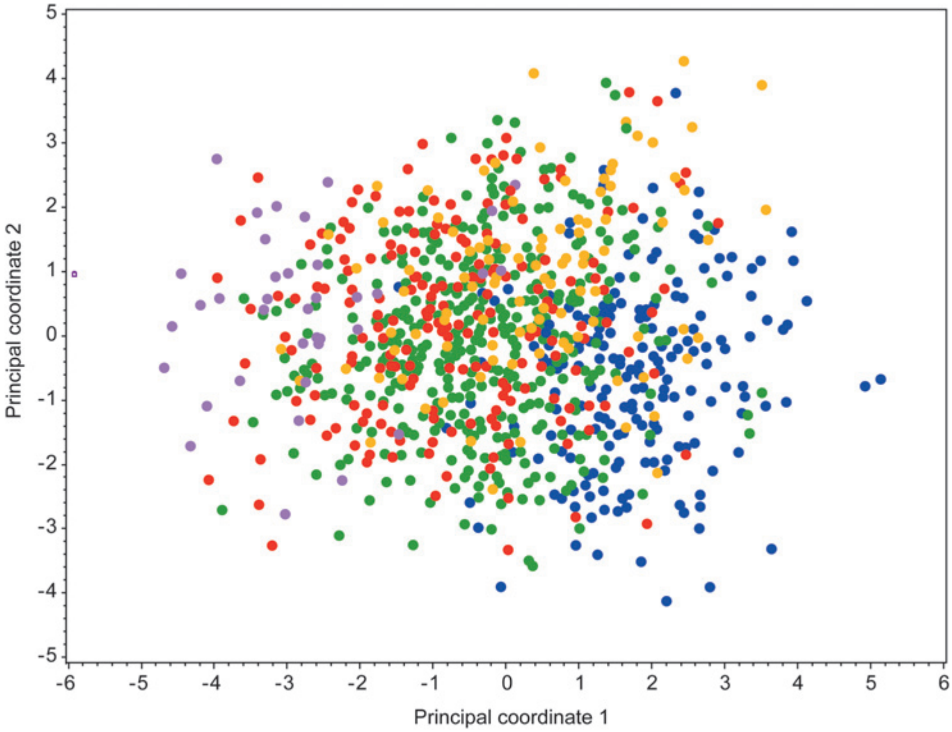

Fig. 3. – Principal component analysis (PCA) of *Cyclamen purpurascens* s.l. based on 886 individuals and 16 morphological characters. Different colours indicate genetic groupings as resolved by Bayesian analysis at K = 5. The first two ordination axes explain 18.7% and 14.5% of total variation, respectively.

(CDA1B, Fig. 4B). The four ratio characters, namely the shape of the leaf, shape of the stamen, position of widest part of the leaf lamina and shape of the sepal, repeatedly supported this division (Table 3). Somewhat unexpectedly, CDA2A (Fig. 5A) based on the 886 individuals and 16 morphological characters distinguished two well separated groups of individuals along the first canonical axis. Both groups included a mixture of individuals belonging to all five phylogroups. Hence, plants belonging to phylogroup IV in the Western Carpathians were admixed with a few individuals belonging to the other four phylogroups in the group furthest along the axis, and vice versa. This was especially the case for individuals that lacked ornamentation on the upper leaf surface. The character POL was more strongly correlated with the first canonical axis than the rest of the morphological characters (Table 3). This division into two discrete groups was obviously caused by the much stronger discrimination power of the binary character compared to those of other ratios and quantitative characters. After exclusion of the character POL, CDA2B resulted in a loose and relatively unstructured aggregation of individuals very similar to that recorded in PCA3 (Fig. 5B). There was a conspicuous overlapping of individuals belonging to the two marginal phylogroups III and IV with the core group composed of phylogroups I, II, and V. Characters with the highest correlations with canonical axes are listed in Table 3.

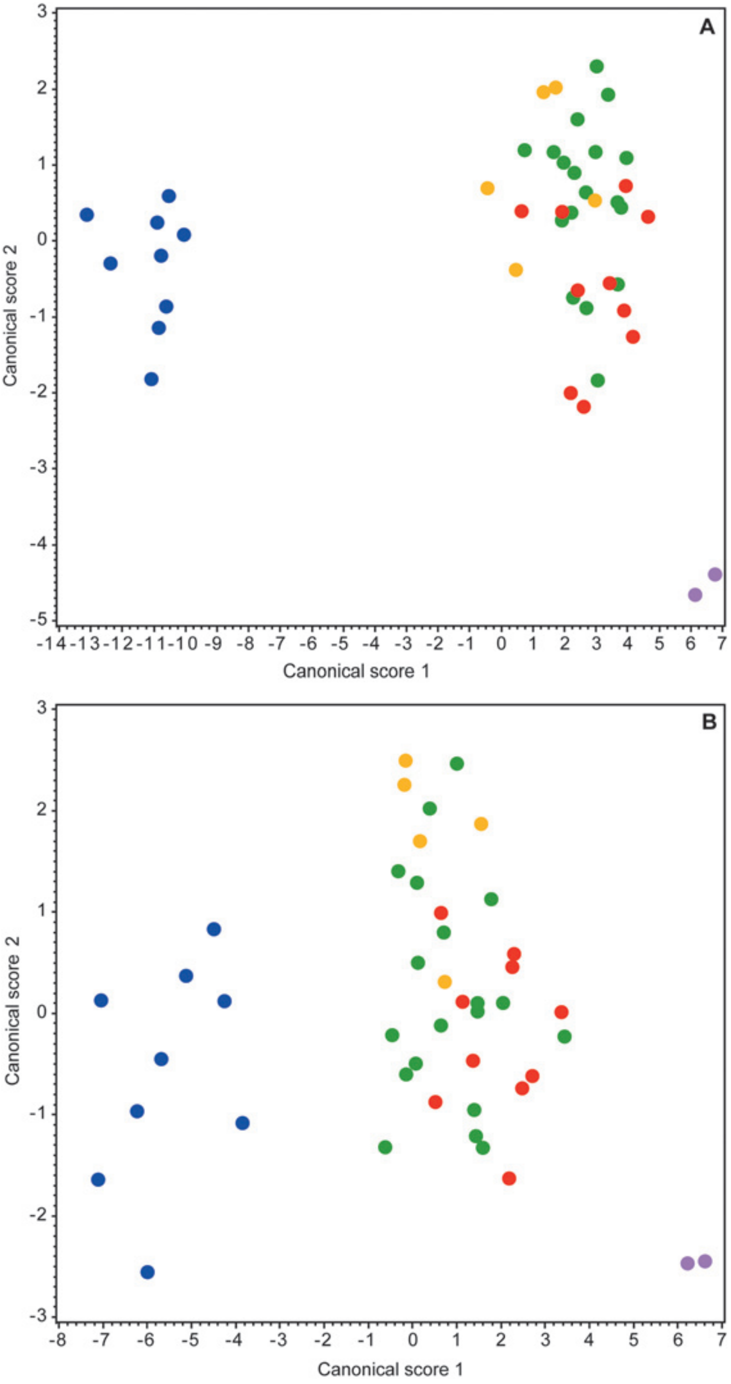

Fig. 4. – Canonical discriminant analysis (CDA) of populations of *C. purpurascens* s.l. with five predefined groups, as resolved by Bayesian clustering of AFLP data at K = 5. (A) CDA based on 45 populations and 16 morphological characters. The first two canonical axes explain 93.1% and 4.5% of total variation, respectively. (B) CDA based on 45 populations and 15 morphological characters (excluding the ornamentation on the upper leaf surface). The first two canonical axes explain 86.6% and 7.6% of total variation, respectively.

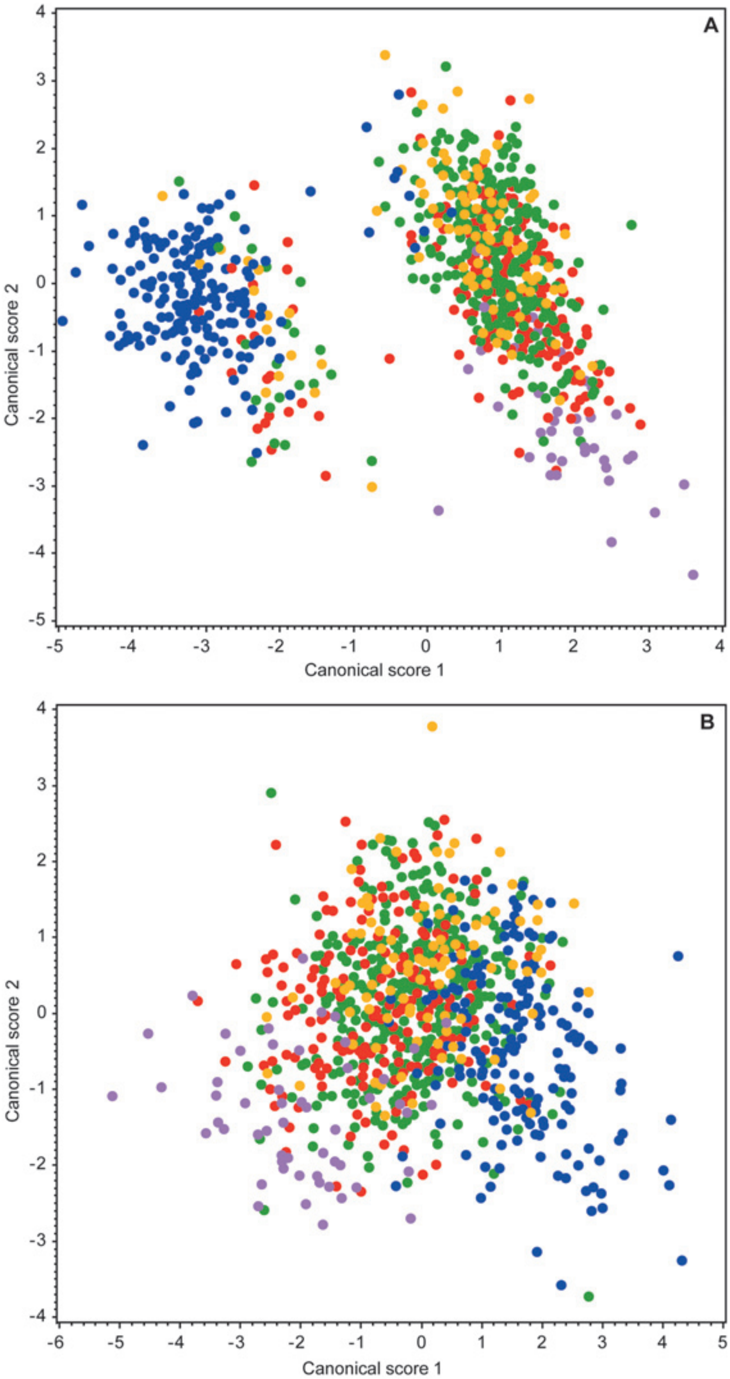

Fig. 5. – Canonical discriminant analysis (CDA) of individuals of *C. purpurascens* s.l. with five predefined groups, as resolved by Bayesian clustering of AFLP data at K = 5. (A) CDA based on 886 individuals and 16 morphological characters. The first two canonical axes explain 86.8% and 8.9% of total variation, respectively. (B) CDA based 886 individuals and 15 morphological characters (excluding the ornamentation on the upper leaf surface). The first two canonical axes explain 79.9% and 11.1% of total variation, respectively.

The classificatory DA based on 886 individuals, 16 characters and five predefined groups (k = 16; phylogroups: I – 192 plants, II – 367 plants, III – 39 plants, IV – 188 plants and V – 100 plants) resulted in a higher percentage of correctly classified individuals only for phylogroup III (82.1%) and phylogroup IV (93.1%). All the incorrect assignments belonged to phylogroups I, II and V. The percentage of misclassifications in these groups was high ranging between 55.0% and 77.1%. A second classificatory DA was performed on the same dataset (k = 15) after excluding the binary POL character. Correct classifications decreased in both phylogroups (III – 82.05% and IV –75.5%), but were still reasonably high compared to phylogroups I (29.7%), II (28.9%) and V (43%).

Finally, we specified the morphological characters that could potentially differentiate between individuals belonging to phylogroup IV from the Western Carpathians (group 1 with 188 individuals) from those belonging to phylogroups I, II, III and V from the Jura

Table 4. – The exploratory data analysis of the morphological characters of *Cyclamen purpurascens* subsp. *purpurascens* and *C. purpurascens* subsp. *immaculatum*. For explanation of abbreviations of measured or scored morphological characters see Table 2. The mean values ± standard deviations are given in the upper rows, while (1st) 5th–95th (99th) percentiles are in the lower rows. For the POL binary character, the percentage of individuals with presence of ornamentation is recorded.

Character	subsp. *immaculatum* (188 individuals)	subsp. *purpurascens* (698 individuals)
SL	0.95±0.06	1.03±0.09
	(0.82) 0.85–1.06 (1.07)	(0.84) 0.89–1.18 (1.24)
PWPL	0.56±0.08	0.62±0.09
	(0.41) 0.45–0.69 (0.75)	(0.4) 0.48–0.77 (0.84)
SAL	106.54±9.46	109.59±14.36
	(94) 108–121 (128)	(93) 108–137 (143)
DLI	1.42±0.10	1.36±0.09
	(1.21) 1.29–1.59 (1.71)	(1.15) 1.23–1.51 (1.59)
MLPe (mm)	28.68±2.91	27.83±4.04
	(22.13) 24.01–33.9 (35.61)	(18.82) 20.83–34.48 (36.62)
MWPe (mm)	9.08±1.25	8.14±1.38
	(6.67) 7.21–11.23 (11.85)	(5.35) 5.98–10.4 (12.03)
SPe	3.2±0.46	3.48±0.57
	(2.37) 2.5–3.95 (4.32)	(2.44) 2.65–4.54 (4.91)
PWPPe	3.12±0.4	3.35±0.48
	(2.45) 2.58–3.87 (4.19)	(2.49) 2.66–4.27 (4.61)
MLSe (mm)	5.23±0.77	4.93±0.65
	(3.82) 4.11–6.61 (7.38)	(3.64) 3.9–6.02 (6.45)
MWSe (mm)	3.01±0.45	3.18±0.43
	(2.09) 2.29–3.78 (4.13)	(2.28) 2.53–3.93 (4.22)
SSe	1.78±0.39	1.57±0.24
	(1.04) 1.24–2.6 (2.89)	(1.09) 1.2–2.01 (2.19)
MLSt (mm)	4.06±0.33	3.83±0.43
	(3.35) 3.52–4.56 (4.81)	(2.86) 3.15–4.57 (4.88)
MWSt (mm)	1.54±0.22	1.58±0.21
	(0.93) 1.17–1.9 (2.09)	(1.05) 1.25–1.92 (2.08)
SSt	2.68±0.38	2.46±0.37
	(1.92) 2.14–3.35 (3.8)	(1.7) 1.95–3.1 (3.5)
LP (mm)	5.78±0.59	5.65±0.66
	(3.74) 4.91–6.62 (6.82)	(2.96) 4.65–6.59 (6.94)
POL	5.86%	91.7%

Mts, the Alps, Dinarides, southwestern Pannonia and southern Moravia (group 2 with 698 individuals). The CDA3A (886 individuals and 16 characters) and CDA3B (886 individuals and 15 characters) were performed on these two pre-defined groups. Since the POL character was included in these analyses, two slightly overlapping groups were distinguished along the canonical axis (CDA3A, Fig. 6A). Here, the POL character exhibited the strongest correlation with the canonical axis (Table 3). The frequency of plants with leaves lacking ornamentation in the populations from the Western Carpathians was more than 90% and only up to 9% in the populations from other parts of the distribution (Table 4). Subsequently, CDA3B computed with ratio and quantitative characters revealed only a rather weak separation of these two groups, although some dissociation trends were apparent in the histogram (Fig. 6B). The shape of the leaves, the shape of the widest sepal, the width of the widest petal and the position of the widest part of the leaf lamina were highly correlated with the canonical axis (Tab. 3). The DA2A based on the two groups ($k = 29$), correctly classified the following percentages of individuals in terms of all 16 characters; 95.2% in group 1 and 91.4% in group 2. Following the exclusion of the POL character, correct assignment of plants to the pre-defined groups was lower at $k = 19$, with 84% for group 1 and 80.1% for group 2. The correct assignment of individuals to group 1 or 2 based on linear classification functions was similar to that obtained using DA. In particular, in the first analysis based on all 16 characters, 94.7% of individuals from the Western Carpathians were correctly classified into group 1 and 91.8% into group 2. After the exclusion of the POL character, 75% were correctly classified into group 1, 95.1% into group 2 and 1% remained unassigned.

Similarly, the multivariate analysis indicated that phylogroup III was distinct from the other four phylogroups in terms of differences in leaf shape and smaller floral parts (see Fig. 4AB, CDA1A, CDA1B; Tables 3, 4). However, a more detailed analysis of these populations was not possible because there were too few populations and individuals, and moreover, the pronounced difference in the size of this particular group would have biased the results and resulted in misleading interpretations.

Karyological analysis

The metaphase chromosome count for several individuals of *C. purpurascens* and one *C. fatrense* revealed that they were all diploids with $2n = 2x = 34$ (Electronic Appendix 1). All analyses performed using flow cytometry were of a high quality. These provided high resolution histograms and mean CVs of G_1 peaks at 2.8%, ranging from 2.07 to 3.63 for *Cyclamen* and 2.48% with a range of 1.85–3.51 for the internal reference standard. Variations in the measurements recorded on different days was very low and the standard error of the means of the three repeated measurements expressed as a percentage of the mean value reached a maximum of only 1.2%.

Estimations of the DNA ploidy level showed that all plants studied were identical DNA diploids (Electronic Appendix 1). The absolute DNA content in individuals and populations of *C. purpurascens* s.l. varied little, since the 2C values ranged between 6.63 and 6.92 pg with a maximum divergence of 4.6% (Electronic Appendix 1). Variation in genome size slightly exceeded four percent, and therefore this most likely did not represent a genuine variation in absolute DNA content, but rather the result of random instrumental drift.

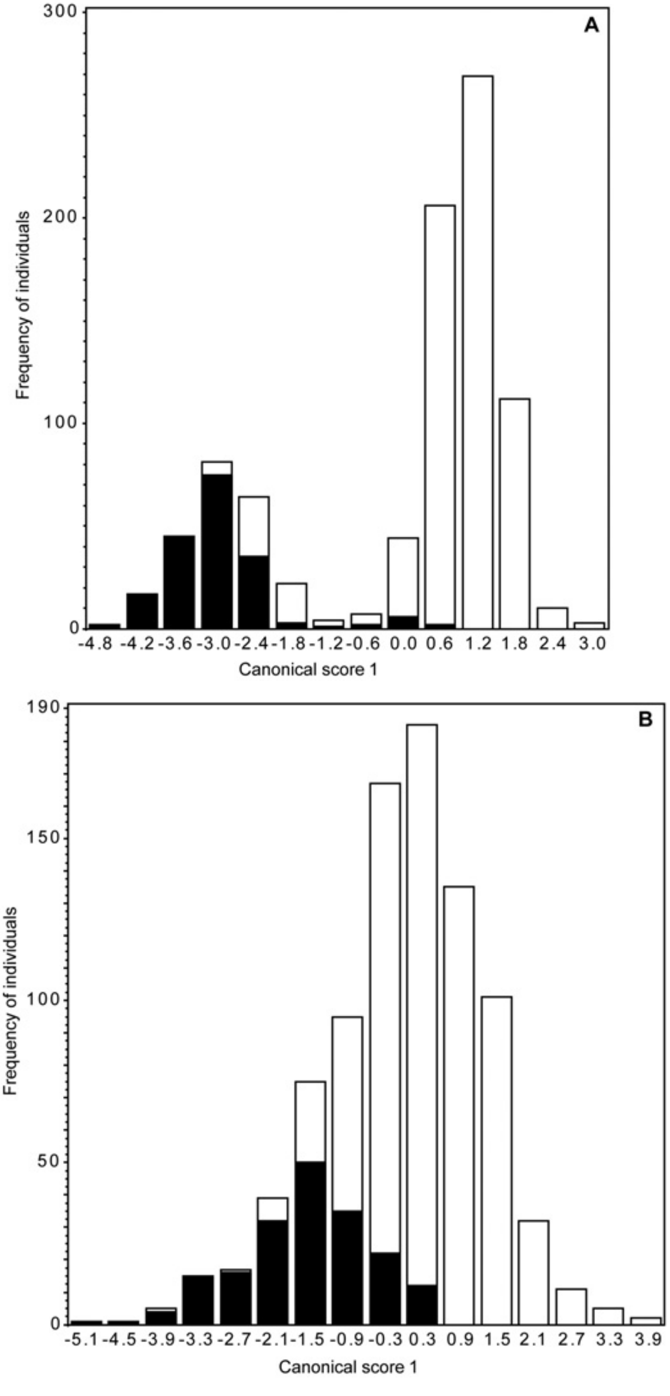

Fig. 6. – Histograms of the canonical discriminant analysis (CDA) of individuals of *Cyclamen purpurascens* subsp. *purpurascens* – phylogroups I, II, III and V (empty bars) and *C. purpurascens* subsp. *immaculatum* – phylogroup IV (solid bars). (A) CDA based on 886 individuals and 16 morphological characters. (B) CDA based on 886 individuals and 15 morphological characters (excluding the ornamentation on the upper leaf surface).

Discussion

Differentiation of the peripheral populations of Cyclamen purpurascens

Cyclamen purpurascens is considered to be a morphologically uniform member of the genus (Meikle & Sinnott 1972, Grey-Wilson 2003). The one exception is the plants from the Western Carpathians that lack leaf ornamentation and have a more obtuse leaf apex, which are considered to be a separate endemic species, *C. fatrense* (Marhold & Hindák 1998, Bernátová & Feráková 1999, Turis 2008, 2009, Mereďa & Hodálová 2011). The large-scale phylogeographic study using molecular markers and the morphological data gathered here indisputably revealed the existence of genetically and/or morphologically distinct entities within *C. purpurascens* s.l. (Slovák et al. 2012b). Two marginal disjunctive groups of populations assigned to the westernmost phylogroup III that occurs in the Jura Mts and Western Alps and the northernmost phylogroup IV in the Western Carpathians revealed that they are both genetically and morphologically segregated from the other populations. In contrast, the karyological study revealed uniformity in ploidy level and absolute DNA content in all the accessions and phylogroups of *C. purpurascens* s.l analysed. This invariance in chromosome numbers ($2n = 2x = 34$) and ploidy level is supported by all previous reports on this genus (see Hrabětová et al. 1983, Bennett & Grimshaw 1991, Anderberg 1994, Ishizaka 2003).

The AFLP results showed that all the accessions of *C. purpurascens* studied from the Western Carpathians formed a genetically homogenous entity distinct from all other phylogroups (Fig. 1), and also differed morphologically (Figs 2 and 4AB). They represent a unique biological entity, which because of its morphological and genetic distinctness should be treated as a separate taxon. Since Western Carpathian populations do not differ from the other populations karyologically and have the same cpDNA haplotype as phylogroups I, II and V (see Slovák et al. 2012b), and especially since their morphological distinction at the individual level is ambiguous, we considered them to be subspecies rather than species. Following the current version of the international code of botanical nomenclature (McNeill et al. 2006) the correct name at the subspecies level is *C. purpurascens* subsp. *immaculatum* (Hrabětová) Halda et Soják. In agreement with authors who have previously studied Western Carpathian populations (Hrabětová 1950, Halda & Soják 1971a, b), the most important diagnostic morphological character that separates subsp. *immaculatum* from subsp. *purpurascens* from the Alps, Dinarides, Jura Mts, Pannonia and Moravia, is the lack of ornamentation on the upper leaf surface. Although the rare occurrence of plants with ornamentation on the upper leaf surface in Western Carpathian populations is reported by Hrabětová (1950) and Turis (2008, 2009) and was also clearly apparent in our study, the frequency of such individuals in populations of *C. purpurascens* subsp. *immaculatum* is low, while it is highly prevalent in all the populations of subsp. *purpurascens* analysed (Table 4). *Cyclamen purpurascens* subsp. *immaculatum* differs from the nominate subspecies also in a combination of several other morphological traits, which have not so far been considered. These include the shape of the leaves and flower parts: subsp. *immaculatum* is characterized by more circular leaves, a lower position of the widest part of the leaf lamina, more narrowly ovate sepals and wider petals (Table 4 and Identification key below). Although the shape of the leaf apex is reported to be more obtuse in subsp. *immaculatum* than in the nominate subspecies (Hrabětová 1950, Halda & Soják 1971a, b, Mereďa & Hodálová 2011), our study shows that it is not a diagnostic character (Table 4). We suggest that the difference in the morphology of subsp. *immaculatum* indicates it has been isolated from the main distribution area for a long time. This hypothesis is favoured by the knowledge that the majority of Western Carpathian populations harbour hundreds to thousands of vigorous individuals, and several of them showed a high genetic diversity and rarity (Slovák et al. 2012b).

Genetic data indicated that the Western Carpathian populations of subsp. *immaculatum* were most likely derived from phylogroup II of *C. purpurascens* s.l. (Slovák et al. 2012b). Since they are morphologically and genetically distinct from the populations in all the other phylogroups, we suggest that their diversification did not occur recently (postglacially) but most likely prior to the last glacial maximum. If so, *C. purpurascens* subsp. *immaculatum* had to survive glaciation along with other woodland moisture-demanding organisms in a few patches of microclimatically suitable habitats located in the valleys or foothills of the Western Carpathians (see Ložek 1980, Stewart & Lister 2001, Willis & van Andel 2004, Deffontaine et al. 2005, Ložek 2006, Magri et al. 2006, Jankovská & Pokorný 2008, Kuneš et al. 2008, Willner et al. 2009). It is necessary to mention that there are several populations that morphologically and genetically clearly belong to *C. purpurascens* subsp. *purpurascens* in the Western Carpathians, however, these are of allochtonous origin (Slovák et al. 2012b).

Genetic and morphological differentiation has also occurred in a few populations at the western edge of the species' distribution, as in phylogroup III in the Jura Mts and Western Alps. All the westernmost populations studied had small flowers, with very low genetic diversity and rarity compared to Western Carpathian populations (see Table 1 and also Slovák et al. 2012b). In addition, all these populations were conspicuously small and clearly separated from one another geographically. This loss in genetic variation accompanied by a shift in morphology may have been due to a combination of factors. As hypothesized previously (Slovák et al. 2012b), stepwise postglacial colonization from other alpine refuge areas linked to repeated genetic bottlenecks and genetic drift due to isolation of small populations at the margins of this species' distribution, together with suboptimal ecological conditions and/or altered mating systems, are the most likely reasons for these changes.

The correlations between micro- and macromorphological characters and reproductive systems in four different *Cyclamen* species were studied by Affre & Thompson (1998). These species comprised *C. hederifolium* from the subgenus *Cyclamen*; *C. balearicum* Willk., *C. creticum* Hildebr. and *C. repandum* from the subgenus *Psilanthum*. These authors revealed a remarkable correlation between floral architecture and pollination ecology, the level of inbreeding and the phylogenetic relatedness of these taxa. All the parameters investigated varied significantly among the taxa studied. The corolla size, pollen and ovule production and pollen volume decreased from the most allogamous *C. hederifolium*, which is characterized by the lowest inbreeding level, to the highly inbred and almost fully autogamous *C. balearicum* (Affre & Thompson 1998). In addition, the morphological differentiation in the long-term isolated island endemic *C. balearicum* towards smaller and rather obscure white flowers, short-term floral receptivity and either a weak or complete lack of stigma-anther separation, are considered to be the most important adaptations facilitating selfing and subsequent inbreeding (Affre et al. 1995, 1997, Affre & Thompson 1997, 1998, 1999, Thompson 2005). Although the flowers of the westernmost populations of *C. purpurascens* were much smaller compared with those of other *C. purpurascens* s.l. phylogroups, no changes in corolla colour or stamen and pistil size were detected (Table 4). In contrast to the situation in Western Carpathian populations of subsp. *immaculatum* and also *C. balearicum* gleaned from morphological and genetic data, we consider that the overall distinctions of populations in the Jura Mts and Western Alps do not reflect a speciation event, but most likely result from inbreeding in small populations and a response to unfavourable eco-biological conditions.

As mentioned above, we propose to recognize two subspecies within *C. purpurascens*, namely subsp. *purpurascens* and subsp. *immaculatum*. However, it is evident that these two subspecies differ in their variation and distribution patterns. The nominate subspecies is genetically and morphologically much more diverse and occupies a larger range than subsp. *immaculatum*. More specifically, the former includes four genetic phylogroups (I–III and V) together covering most of the species' distribution area (Fig. 1), wherein phylogroup III also exhibits a trend towards morphological divergence. On the other hand, the latter subspecies is genetically (phylogroup IV) and morphologically homogeneous inhabiting only a small area within the Western Carpathians. Therefore, the most important question is whether the recognition of these two subspecies is the best taxonomic decision? Alternatives could be to recognize each phylogroup as a separate subspecies or to consider *C. purpurascens* as a single heterogeneous taxon. Combining all the evidence and arguments – the lack of morphological distinction between phylogroups I, II and V and only a tendency for phylogroup III to differ morphologically but with overlaps both at the individual and population levels (see Figs 2, 3 and 5AB), plus the genetic admixture within phylogroups I-III and V in some populations (see Slovák et al. 2012b for more details) – the recognition of five subspecies would be vague and weak. On the other hand, phylogroup IV recognized here as subsp. *immaculatum*, is a genetically homogeneous group without any admixture from the other phylogroups. It also differs from the rest morphologically, at least at the level of population means, and its distribution is geographically well delimited in that it is restricted to the Western Carpathians. Finally, we believe that the two subspecies concept best reflects the variation patterns present in the alpine violet. Several examples of taxonomically intricate taxa can be found in the recent literature in which the recognition of similarly unequal subspecies with different levels of morphological and genetic variation is advocated (Schönswetter et al. 2009, Bardy et al. 2010, Mereďa et al. 2011).

Morphological and genetic approaches have been successfully applied in studies on the taxonomic and evolutionary relationships among Mediterranean cyclamens from the subgenus *Psilanthum* (Gielly et al. 2001, Debussche & Thompson 2002, Thompson 2005). Except for the widespread *C. repandum* subsp. *repandum,* all taxa evaluated are narrow endemics, which evolved in allopatry at the peripheries of the parental species' range, as occurred in *C. purpurascens* subsp. *immaculatum* (Gielly et al. 2001, Debussche & Thompson 2002, Thompson 2005). Moreover this corroborates the theory of Yesson et al. (2009) that allopatric speciation played a crucial role in the evolutionary history of *Cyclamen*. The colouration and size of the corolla and differing colour and pattern of leaf ornamentation were shown to be the most important discriminating characters between taxa from the subgenus *Psilanthum* (Debussche & Thompson 2002). In addition to morphology, the island endemics belonging to this subgenus are also characterized by different levels of selfing and unique ecological requirements (Affre & Thompson 1999, Debussche & Thompson 2002, Thompson 2005). In contrast, corolla colour, leaf ornamentation and pattern and ecological specifications are inconsistent in the *C. purpurascens* phylogroups and therefore taxonomically unimportant.

Cyclamen purpurascens subsp. immaculatum, a 'true' Western Carpathian endemic

The Carpathians are a fascinating region harbouring a very rich biodiversity and a remarkable percentage of endemic taxa (Pawłowski 1970). The populations of *C. purpurascens* subsp. *immaculatum* have high biological and conservation value and should be considered endemics of the Western Carpathians. Recognition of *C. purpurascens* subsp. *immaculatum* as a separate taxonomic entity emphasizes the important role the Western Carpathians played in the survival and diversification of various organisms during the Pleistocene glaciations, and their great contribution to the evolution of endemic flora in Europe. Moreover, a high number of endemic taxa has already been reported and described from the Carpathians (Pawłowski 1970, Kliment 1999, Piękoś-Mirkowa & Mirek 2003, 2009, Oprea 2005, Tasenkevich 2011). The actual numbers vary considerably because authors summarize lists of endemics mainly at the national level. For instance, Kliment (1999) reevaluated the status of almost 500 taxa reported to be endemic in Slovakia, which includes the major part of the Western Carpathians, and concluded that only half of these (including apomictic microspecies of *Alchemilla* L.*, Hieracium* L.*, Pilosella* Vaill.*, Sorbus* L. and *Taraxacum* F. H. Wigg.*)* should be considered 'true' endemics. More recently, Piękoś-Mirkowa & Mirek (2003, 2009) report more than a hundred endemic and subendemic taxa in the Polish portion of the Eastern and Western Carpathians. However, the overwhelming majority of these have ranges that extend into adjacent parts of the Carpathians in Slovakia, Ukraine and Romania. In addition to this localized approach, the unproven status of the majority of the assumed Carpathian endemic and subendemic species hampers creation of a comprehensive and realistic list of endemics. A significant number of Carpathian taxa reported to be endemics may be an artefact of geographically localized studies of given taxa, which do not use the comparative analyses that have been used in studies of closely allied taxa. There are several examples in the literature of species classified as Carpathians endemics being only part of the continual variation in more widespread closely related species, and should therefore no longer be considered separate taxa (Schönswetter et al. 2009, Kolarčik et al. 2010, Španiel et al. 2011, Slovák et al. 2012a). In contrast, there is an assembly of studies that reach similar conclusions to those presented here, in which comprehensive taxonomic investigations confirmed the endemic status of Carpathian taxa (Goliašová 1985, Hodálová & Marhold 1998, Hodálová 1999, Mráz 2001, 2003, 2005, Zhang et al. 2001, Dvořáková 2003, Zhang & Kadereit 2004, Kochjarová et al. 2006, Ronikier & Szeląg 2008*,* Šingliarová & Mráz 2009, Šingliarová et al. 2011, Štěpánek et al. 2011). From a biodiversity conservation viewpoint, it is therefore necessary to rectify inadequacies in the investigations into Carpathian endemics and subendemics, by focusing on their evolutionary histories, their relationships with closely allied taxa and their population biology.

See www.preslia.cz for Electronic Appendix 1

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Souhrn

Brambořík fatranský (*Cyclamen fatrense*) byl popsán jako lokální endemit Západních Karpat, avšak díky jeho morfologické podobnosti s blízce příbuzným *C. purpurascens* nebyl dosud široce akceptován jako odlišný druh. S cílem zrevidovat taxonomický status populací *C. fatrense* a zhodnotit míru jejich odlišnosti od populací běžně rozšířeného *C. purpurascens* jsme použili kombinaci genetických (AFLP), morfometrických a karyologických analýz. Výsledky naší studie prokázaly, že západokarpatské populace jsou jak geneticky, tak morfologicky odlišné od populací *C. purpurascens*, a měly by být považovány za samostatný endemický poddruh (*C. purpurascens* subsp. *immaculatum*). Hlavní diagnostické znaky odlišující tento poddruh od nominálního poddruhu jsou absence kresby na vrchní straně listové čepele, odlišný tvar listů a kališních lístků, šířka korunních lístků a umístění nejširší části listové čepele. Všechny studované populace jsou po karyologické stránce uniformní a jsou diploidní (2n = 2*x* = 34) s minimálními rozdíly v absolutním obsahu jaderné DNA (2C = 6.63–6.92 pg).

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Appendix 1. – Taxonomic treatment and nomenclature.

Identification key to subspecies

Note: The ranges of quantitative characters correspond to the 5th and 95th percentiles, and the 1st and 99th percentiles are given in brackets. A combination of morphological characters must be used for correct determination, particularly in the classification functions mentioned below:

1a: Upper leaf surface mostly with silver-grey ornamentation: The ratio of length and width of leaf lamina (0.84–) 0.89–1.18 (–1.24); ratio of total width of leaf and length from apex of leaf to its widest part: (0.4–) 0.48–0.77 (–0.84). The ratio of length and width of sepal (1.09–) 1.2–2.01 (–2.19). Flower petals: (5.4–) 6.0–10.4 (–12.0) mm wide. .... **1. *Cyclamen purpurascens* Mill. subsp. *purpurascens***

1b: Upper leaf surface mostly without silver-grey ornamentation: The ratio of length and width of leaf lamina (0.82–) 0.85–1.06 (–1.07); ratio of total width of leaf and length from apex of leaf to its widest part (0.41–) 0.45–0.69 (–0.75): The ratio of length and width of sepal (1.04–) 1.24–2.6 (–2.89). Flower petals (6.7–) 7.2–11.2 (–11.9) mm wide. .. **2. *Cyclamen purpurascens* subsp. *immaculatum* (Hrabětová) Halda et Soják**

The following classification function was obtained for determination of *C. p.* subsp. *purpurascens* and it reads (for explanation of character codes see Table 2):
=-2012+SL1*235.37164+SL2*24.57051+SAL*1.16455+DLI*169.81694+POL*6.44379-
MLPe*34.20217+MWPe*121.76653+SPe1*293.11020+Spe2*18.94064-
MLSe*198.77850+MWSe*320.19233+Sse*613.43368-
MLSt*270.38937+MWSt*748.71692+SSt*434.52492+LP*10.80521

For determination of *C. p.* subsp. *immaculatum*:
=-2020+SL1*217.04670+SL2*24.59795+SAL*1.07357+DLI*179.18859-POL*5.76515-
MLPe*34.39945+MWpe*122.80289+SPe1*294.01472+Spe2*18.52679-
MLSe*201.52036+MWSe*323.45306+Sse*622.68484-
MLSt*272.68423+MWSt*754.29389+SSt*438.86490+LP*11.07682

These functions can be used to estimate the taxonomic assignment (particular subspecies) of an unknown individual. It is necessary to measure morphological characters with the accuracy of two decimal positions, in units as given in Table 2, and to insert their values in both formulas. Scores for each function are computed, compared and the plant individual is classified in the group (subspecies) with the higher score.

1. *Cyclamen purpurascens* Mill., Gard. Dict., ed. 8. n. 2. 1768.

Syn:

≡ *Cyclamen europaeum* L., Sp. Pl.: 145. 1753 [*Primulaceae*]., *nom. utique rej. prop.* – Ind. loc.: “Habitat in Austriae, Tatariae, Europae australis nemorosis”. – Lectotype: Herb. Burser XVII: 89 (UPS).

2. *Cyclamen purpurascens* subsp. *immaculatum* (Hrabětová) Halda et Soják, Folia Geobot. Phytotax. 6: 321–333. 1971.

Syn:

≡ *Cyclamen europaeum* subsp. *orbiculatum* var. *immaculatum* Hrabětová, Českoslov. Bot. Listy, 3: 35. 1950. – Ind. loc.: “Crescit in fagetis atque ad margines silvarum usque in pratis silvanis, solo calcareo, praecipue in partibus ad meridiem spectantibus montium Nízke Tatry et Veľká Fatra, Slovakia, ČSR.” – **Lectotype (designated here)**: “In decliv. calc. infra Motyčky, supra vicum vers. Šturec, 850 m, VIII 1950, Hrabětová” (BRNM!)

= *Cyclamen fatrense* Halda et Soják, Čas. Nár. Mus., Odd. Přír. 140: 63–64. 1971. – Ind. loc.: “Slovakia occidentalis, montes Veľká Fatra: in silvis (*Fagus*, *Picea*, *Corylus*) vallis Mača ca 2.5 km versus orientem a vico Rakša prope oppidum Turč. Teplice, ca. 500 m s.m.m.,” – Holotype: Halda et Soják, 18.7.1971 (PR!)