GENETIC DIVERSITY OF *GALIUM CRACOVIENSE*, *G. OELANDICUM* AND *G. SUDETICUM* (RUBIACEAE) – NARROW ENDEMIC SPECIES OF *GALIUM* SECT. *LEPTOGALIUM* IN NORTHEASTERN EUROPE

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ABSTRACT

Galium sect. *Leptogalium* Lange in NE Europe is represented by few, local endemic species which occur in the area covered by the continental ice sheet in the Pleistocene period. They are: *G. cracoviense* Ehrend. in S Poland, *G. oelandicum* (Sterner & Hyl.) Ehrend. in SE Sweden and *G. sudeticum* Tausch in SW Poland and N Czech Republic. 55 individuals from five populations of these species were analysed using AFLP markers. A total of 193 AFLP bands were detected using three combinations of primers; out of them 159 proved polymorphic (82.4%). The lowest values of Shannon's index and Nei's gene diversity were noted for *G. oelandicum* and the highest ones for *G. sudeticum*. The results indicate a relatively high level of genetic variability in each of endemic species in spite of that the studied species occupy very small areas and are represented by a low number of populations. We conclude that additional, demographic and genetic studies are necessary to monitor potential decrease of populations' size resulting mainly from the mechanical destruction of plants and their habitats caused by intense tourism or other human activities (as agriculture, e.g. grazing).

KEY WORDS: AFLP, endemics, *Galium* sect. *Leptogalium*, genetic variability.

INTRODUCTION

In recent years, the interest in rare and narrow endemic species has turned to urgency, as more and more species dwindle toward extinction (Gitzendanner and Soltis 2000).

Species with narrow ranges, often belong to a group of species with very high risk of extinction and therefore they are of great conservation concern. In this case, molecular tools can be a valuable means for investigating the pattern of genetic diversity in threatened species, and clarifying demographic and ecological issues early in species management in order to plan long-term conservation or restoration projects (Kim et al. 2005). In last decades, genetic issues have gone from relative obscurity to a significant emphasis in conservation research as modern molecular techniques revolutionized our ability to delineate relationships among individuals, populations, and species. Despite some researchers have questioned the relative importance of genetic information, stating that ecological or demographic issues may be more pressing (e.g. Lande 1988; Schemske et al. 1994; Avise 2008), molecular markers have become part of a repertoire of tools needed to assess the amount of genetic variation in populations of endangered species and to address the ever-increasing loss of biodiversity.

In addition, for this group of species predicting the amount of genetic variability in plant species on the basis of their distribution is often not reliable, since some endemic species exhibit equivalent or higher levels of diversity compared to their more widely distributed congeners (Gitzendanner and Soltis 2000).

This study was made an attempt to examine genetic diversity of three narrow endemic species of *Galium* sect. *Leptogalium* Lange which occur on the isolated localities at north-eastern border of the section's range (Fig. 1). *Galium cracoviense* Ehrend. is an endemic species of the Polish flora growing on Jurassic limestone rocks in vicinity of Olsztyn village near Częstochowa (S Poland). *G. oelandicum* (Sterner & Hyl.) Ehrend. is an endemic species of the Öland Island in the Baltic Sea (SE Sweden), where it grows on Ordovician limestone rocks, and *G. sudeticum* Tausch is an subendemic species of the Karkonosze Mountains (the border of S Poland and N Czech Republic), which grows e.g. in basalt rock crevices in the Mały Śnieżny Kocioł glacial cirque in the subalpine belt. *G. sudeticum* is also known from the Slavkovský les hills,

where it grows on serpentine rocks at an altitude of 600-900 m a.s.l. Krahulcová and Štěpánková (1998) have recently studied morphological variability of the plants recorded there.

Galium cracoviense and *G. oelandicum* are diploids (2n=22) while *G. sudeticum* is a tetraploid (2n=44) (Piotrowicz 1958, Ehrendorfer 1960, Krahulcová and Štěpánková 1998). The studied species are perennials, forming more or less dense carpets of numerous vegetative and generative shoots. They flower and fruit abundantly. The multiyear observations carried out by the authors on *G. cracoviense* and *G. sudeticum* allowed to establish that both these species propagate generatively. Vegetative propagation is also possible by division of the mattes due to e.g. base rock destruction. All species considered in our study belong to *Galium* sect. *Leptogalium* that, as redefined by Ehrendorfer (1960), comprises ca. 18 species with centre of its range in the mountainous areas of SW Europe (Ehrendorfer 1976). In NE Europe the section is represented only by few species that occur in small areas (Ehrendorfer 1962).

The present study was aimed at: (i) assessment of the genetic diversity of three species with very narrow geographical range, (ii) comparison of genetic variability within a group of diploid species, (iii) comparison of genetic variability of a diploid versus tetraploid species, (iv) attempting to establish the influence of historical factors on the variability level of study species and (v) providing suggestions for effective conservation programs.

MATERIAL AND METHODS

Collection of plant material

As the species studied are very narrow range endemics, the number of samples was directly determined by the population abundance. Nevertheless a random sampling covered the whole area occupied by these species and is representative for each of them.

Samples of *Galium cracoviense* were collected on the Jurassic rock outcrops in vicinity of the village Olsztyn

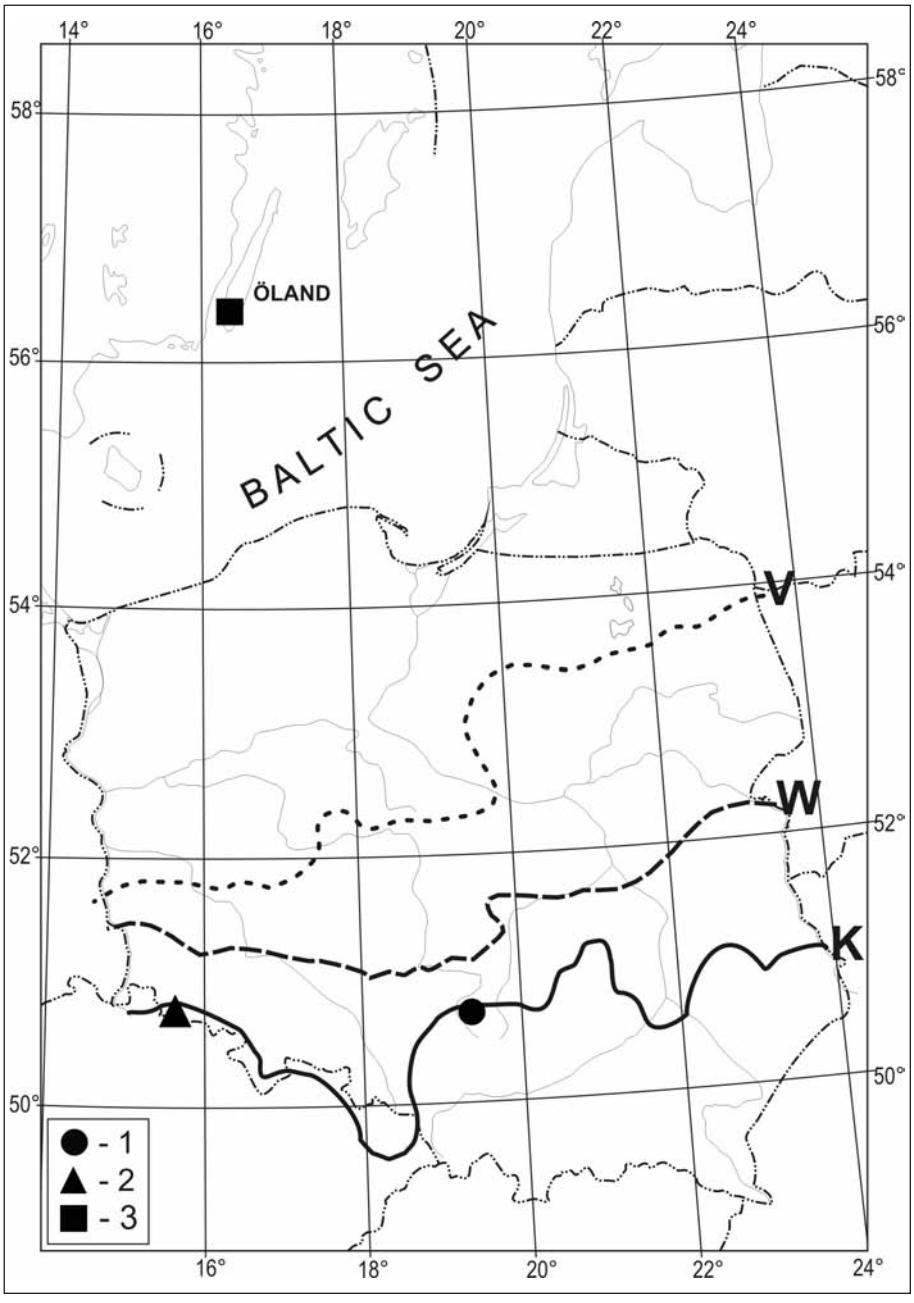

Fig. 1. Distribution of *Galium cracoviense* (1), *G. sudeticum* (2), *G. oelandicum* (3) and limits of the Pleistocene glaciations: K – Krznanian Glaciation, W – Wartanian Glaciation and V – Vistulian Glaciation (after Ber 2005, modified).

TABLE 1. Origin of plant material.

Taxon	No. of samples	Localities
G. cracoviense	17	Poland, Olsztyn village, Towarne hill (50°46'N, 19°16'E), alt. 340 m a.s.l.
G. cracoviense	5	Poland, Olsztyn village, Zamkowa hill (50°45'N, 19°16'E), alt. 350 m a.s.l.
G. cracoviense	7	Poland, Olsztyn village, Biakło hill (50°44'N, 19°16'E) alt. 360 m a.s.l.
G. oelandicum	14	Sweden, Island of Öland, Gynge alvar, Resmo (56°31'N, 16°25' E), alt. 50 m a.s.l.
G. sudeticum	11	Poland, Karkonosze Mts, Mały Śnieżny Kocioł glacial cirque (50°46'N, 15°34'E), alt. 1270-1360 m a.s.l.

near Częstochowa (S Poland) (Cieślak and Szeląg 2009). *G. oelandicum* was collected on the Öland Island (SE Sweden) and *G. sudeticum* in the Karkonosze Mountains (SW Poland; Fig. 1, Table 1). Together, 55 samples were used in the analyses. Leaf material of young living plants from randomly chosen individuals were sampled and immediately stored in plastic tubes with silica gel. Samples were afterwards maintained in the laboratory at room temperature until analysis.

DNA extraction and AFLP fingerprinting

Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol, using ca. 10 mg of dried tissue per plant. DNA integrity was estimated on 1.5% agarose gels and DNA concentration was determined by spectrophotometry with the Gene Quant RNA/DNA calculator (Pharmacia).

AFLP analysis was performed according to the procedure described by Vos et al. (1995). Genomic DNA was digested with two restriction enzymes: *Eco* RI and *Mse* I (New England Biolabs, Inc.). The resulting fragments were ligated to end-specific *Eco* RI and *Mse* I double strand adaptors using T4 DNA Ligase (Roche Diagnostics). Success of restriction was verified in 1.5% agarose gels. Next, samples were diluted 1:10 with deionised H_2O.

PCR amplification was carried out in two steps: preselective and selective amplification. Preselective amplification was performed using primers with single selective nucleotides: *Eco* RI + A and *Mse* I + C. PCR products were verified on 1.5% agarose gels and diluted 1:20 with deionised H_2O. Final selective amplification was performed using primers with three selective nucleotides (*Eco* RI primers were labelled with a fluorescent marker).

Amplification products were separated in POP 4 polymer with an internal size standard (GeneScan 500 Rox) on the ABI Prism 3100 Avant automated sequencer (Applied Biosystems).

Several samples were used in duplicates for each analysis as a control. Control reactions were used for the verification against possible genotyping errors.

Primer screening was conducted on three individuals of every species in a preliminary experiment. 18 primer combinations were tested, three of which were chosen for final analysis. The selection of primer combinations was based on the number of polymorphic fragments and repeatability of at least 98%.

Raw data were aligned with the size standard using the GeneScan 1.3 Analysis Software (Applied Biosystems). Data scoring was done using Genographer version 1.6 software (Montana State University; http:\\hordeum.oscs.montana.edu). Peaks (fragments) were scored in the range of 50-500 bp and coded as a binary presence (1)/absence (0) matrix for further analyses.

Data analysis

The level of diversity was estimated as the percentage of polymorphic markers. Private bands unique to respective species were also noted (private bands were defined as present in all analyzed samples of a respective taxon and absent in the compared ones). The degree of AFLP polymorphism was quantified by Shannon's information index (Lewontin 1972) and gene diversity index (Nei 1978). The dendrogram representing genetic relationships between populations was constructed applying UPGMA according to genetic distance (Nei 1978) using POPGENE software version 1.32 (Kovach 1999). The matrix based on the Euclidean distance was used in principal coordinates analysis (PCoA) performed with the MVSP 3.10b software. Eigenvalues, giving the measure of variance accounted for by corresponding eigenvectors (coordinates), were given for the first three most important axes (or fewer if data points were less than four). The percentages of variance accounted for by these components are also given.

Molecular variance analysis (AMOVA) was performed for two levels: between groups (defined as species) and within populations of each of species. Data were analysed using ARLEQUIN 2.0 software (Schneider et al. 2000), in which the fixation index (F_{ST}) was also estimated. The parameters estimating genetic diversity were assuming Hardy-Weinberg equilibrium.

RESULTS

AFLP was performed on 55 individuals of the three species (five populations). It yielded informative band patterns polymorphic among individuals and between the species. A total of 193 AFLP bands were detected using three combinations of primers. The mean number of fragments per individual was 64.3 (SD=7.6). 159 bands were polymorphic, representing 82.4% of the total number of bands (mean number per individual 42.2, SD=13.3). The number of polymorphic bands was variable and depended on primer combinations. Out of the total of 159 polymorphic bands, 92 for *Galium cracoviense*, 91 for *G. sudeticum* and 73 for *G. oelandicum* were noted (Table 2). Generally, the polymorphism of *G. cracoviense* was higher than that of *G. oelandicum* and similar to that of *G. sudeticum.* The private bands were present in each taxon (Table 2). Analysis of genetic diversity of diploid species showed five bands private for *G. cracoviense* and six for *G. oelandicum*.

Comparisons between the tetraploid *Galium sudeticum* (2n=44) and each of the two diploid species, gave 10 private bands in each case (5/5 for *G. cracoviense*/*G. sudeticum* and 4/6 for *G. oelandicum*/*G. sudeticum*). When com-

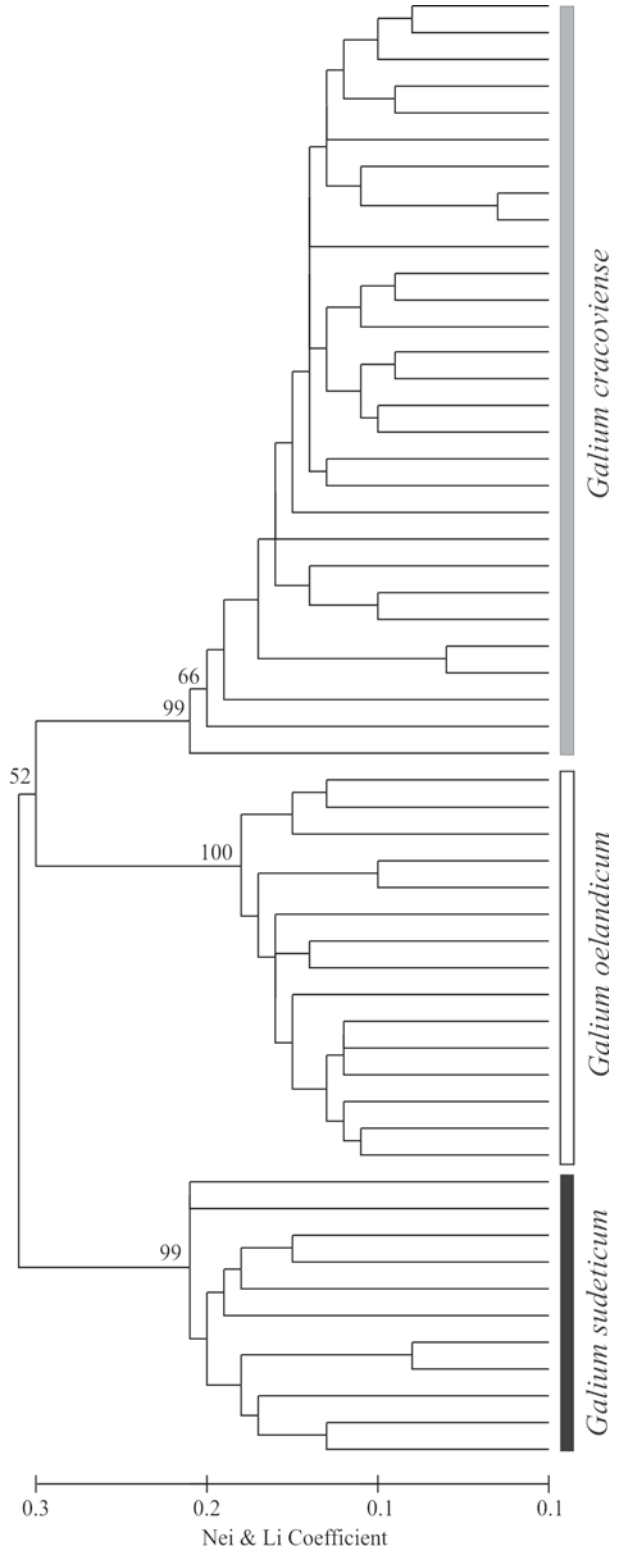

Fig. 2. UPGMA dendrogram based on Nei & Li coefficient calculated from AFLP analysis of *Galium cracoviense*, *G. oelandicum* and *G. sudeticum*. (Bootstrap values >50% are given at nodes).

paring the polyploid taxon against both diploids (counted together), three bands were found unique to the polyploid taxon whereas no bands unique to the group of diploid species were detected.

The degree of AFLP polymorphism quantified by Shannon's index (H_{SH}) was similar for *Galium cracoviense* and *G. oelandicum* (equalled 2.8 and 2.5 respectively) and was higher for *G. sudeticum*, equalling 3.03. The same proportions could be observed comparing the values of average gene diversity across loci and of Nei's gene diversity (Table 2). The lowest values of these coefficients were noted for *G. oelandicum* and the highest for *G. sudeticum*.

TABLE 2. The parameters of genetic diversity of *Galium cracoviense*, *G. oelandicum* and *G. sudeticum* based on 193 AFLP bands.

Taxon	N	UB	T	P	G	h
G. cracoviense	29	2	92	58%	0.1736	0.1902 (±0.0867)
G. oelandicum	15	2	73	46%	0.1713	0.1742 (±0.0888)
G. sudeticum	11	3	91	57%	0.2383	0.2033 (±0.1264)

Abbreviations used: N – no. of individuals; UB – no. of private bands of a given taxon; T – no. of total bands; P – percent of polimorphic bands; G – average gene diversity across loci, h – Nei's gene diversity.

The dendrogram constructed with UPGMA in cluster analysis on the basis of Nei's genetic distance proved grouping of the respective species into distinct groups. This analysis showed a closer affinity of diploid taxa – *G. cracoviense* and *G. oelandicum* to each other than to *G. sudeticum*, but this arrangement was supported by low bootstrap values (Fig. 2).

The main groups revealed by cluster analysis were also confirmed by PCoA. The three groups corresponding with the species were clearly defined by the first and second principal coordinates which represented 22.6% and 14.8% of total variation, respectively (Fig. 3). These results revealed genetic separation of the taxa, supporting their present taxonomic status. PCoA carried out separately on the infraspecific groups of *Galium cracoviense, G. sudeticum* and *G. oelandicum* showed that all the taxa were very homogenous, and no further structuring was observed within each taxon (data not shown).

Results of the AMOVA analysis showed that 54.49% of the total variation was partitioned within populations whereas 45.5% – among populations. The same partition of the total variability was shown by the analysis of differentiation of populations of diploid taxa. In this case 52.9% of

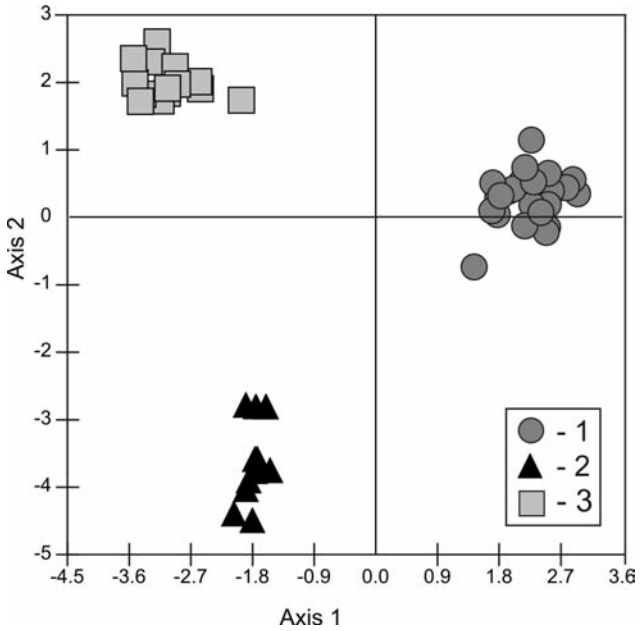

Fig. 3. Principal Coordinates Analysis (PCoA) of AFLP profiles constructed using 193 variable DNA bands from 55 individuals: 1 – *Galium cracoviense*, 2 – *G. sudeticum*, 3 – *G. oelandicum*.

TABLE 3. Results of molecular variance analysis (AMOVA). Level of significance tests are based on 1023 permutations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	F_{ST}
a) All populations					
Among populations (total)	2	535.29	14.95	45.51	
Within population	52	930.78	17.89	54.49	
Total	54	1466.07	32.85	100.00	0.45
b) Diploid (*G. cracoviense* and *G. oelandicum*) vs polyploidy (*G. sudeticum*) populations					
Among groups	1	225.30	0.33	1.00	
Among populations within groups	1	309.99	14.77	44.76	
Within populations	52	930.78	17.89	54.24	
Total	54	1466.07	33.01	100.00	0.47

the total variation was has been attributed within population and 47.1% among populations (FST=0.47). For grouping "diploid versus polyploid populations" no significant variation among groups was detected (the major part of genetic variation was within population.)

These same analyses were performed on three groups representing each of the species. For each of species AMOVA also showed that the major part of genetic variation was within population (Table 3). The values of F_{ST} obtained were the following: 0.03 – *Galium sudeticum*, 0.03 – *G. oelandicum* and 0.12 – *G. cracoviense* (Cieślak and Szeląg 2009). Variation in the AFLP markers was highly significant on all AMOVA levels (p<0.001; Table 3). The F_{ST} values among all pairs of species were highly similar, ranging from 0.44 to 0.47, with mean F_{ST} value equalling 0.45 (Table 4).

TABLE 4. Pairwise F_{ST} – values among species under study. All values are significant on the p<0.001.

	G. cracoviense	*G. oelandicum*	*G. sudeticum*
G. cracoviense	0.00000		
G. oelandicum	0.44726	0.00000	
G. sudeticum	0.47064	0.44390	0.00000

DISCUSSION

The results of molecular analysis of the three endemic species of *Galium* sect. *Leptogalium* confirmed the current taxonomic division proposed by Ehrendorfer (1960) on the basis of their morphological characters. The studied species have similar values of mean genetic diversity (FST) which is in concordance with these of other species characterized by narrow geographical range, a relatively narrow ecological scale and a natural fragmentation of population, as *Campanula thyrsoides* and *Epilobium fleischeri* (Kuss et al. 2008), *Eryngium alpinum* (Gaudeul et al. 2000). These values correspond also with these of species included in literature into the group of "long-lived perennials" (Nybom 2004), the group to which the studied *Galium* species undoubtedly belong.

Despite all our species are characterised by very small natural areas of distribution, the genetic variability revealed in each of them was high. It is often cited that one of the features characterizing geographically restricted species is a low level of their genetical variability (Ellstrand and Elam 1993) that was found in such rare plants as *Primula scotica* (Glover et Abbott 1995) or *Omphalogramma souliei* (Huang et al. 2009). More and more often, however, in the literature are reported examples of naturally rare species, that are characterized by a high genetical variability, as *Antirrhinum microphyllum* (Torres et al. 2003), *Helianthus verticillatus* (Ellis et al. 2006) and *Primula apennina* (Crema et al. 2009).

This group is largely composed of the species that on the historical time scale, seem always having been rare with isolated and often small natural populations (Holderegger 1997) as it was in case of *Saxifraga paniculata* (Reisch et al. 2003; Reisch and Poschlod 2004; Reisch 2008).

An expected increase of genetic variability with increasing degree of ploidy was indeed observed in the studied species. The difference in level of genetic variation within the diploid species may indicate a differentiated history of these taxa.

The occurrence range of *Galium cracoviense* was not covered by the continental ice sheet during the last two glaciations: Wartanian and Vistulian (cf. Ber 2005; Fig. 1). The front of the Wartanian and the Vistulian ice sheet stopped ca. 50 km and over 200 km north of the Olsztyn village, respectively. Even during the older and most southward advanced Krznanian (Odranian) glaciation, the Jurassic rocks around the Olsztyn village might have remained free from ice cover, establishing the local, southern border of the continental ice sheet (Marks 2005). It is thus highly probable that some saxicolous species may have survived the pleniglacials *in situ*. The biology and the types of colonised habitats let us suspect that *G. cracoviense* could have been one of them. Thus, the uninterrupted occurrence of *G. cracoviense* in the Polish Jura (The Kraków-Częstochowa Upland) may date back even to 200 000 BP. A number of dealpine species, such as *Festuca pallens* Host, *Gymnocarpium robertianum* (Hoffm.) Newman, *Hieracium bifidum* Hornem., *H. caesium* (Fries) Fries, *Polygala brachyptera* Chodat and *Saxifraga paniculata* Mill., that reach the local northern range limit on the limestone rocks in Olsztyn, grow together with *Galium cracoviense* (Szeląg 2000). The occurrence of some of them, for instance *Saxifraga paniculata*, may be as old as that of *G. cracoviense*. Among the relic species growing together with *G. cracoviense* Ehrendorfer (1962) mentions also *Sesleria varia* (Jacq.) Wettst. This species, however, has its only Polish locality in Pieniny Mts, i.e. about 200 km SSE from Olsztyn village (Zając and Zając 2001).

The plant cover on the Öland Island, housing *Galium oelandicum*, has the shortest history. The continental ice sheet retreated from southern Scandinavia after the last glaciation ca. 10 000 BP but the Öland Island did not emerge until the water level of the Ancylus lake, the present Baltic sea, went down (or rather its bottom went up) ca. 7000-9000 BP. At the time a land bridge connected Scandinavia and Central Europe, that created proper conditions for contact and migration of the flora (Lang 1994; Wysk et al. 2009).

The ablation of the Scandinavian ice sheet towards the end of the last glacial encouraged the plant colonisation of emerging areas of central and northern Poland. The conditions favoured pioneer species to which *Galium cracoviense* and *G. oelandicum* surely belong. During all the enumerated Pleistocene glaciations, the Karkonosze Mountains, house for *G. sudeticum*, remained outside the Scandinavian ice sheet; however, local mountain glaciers developed there that may have eliminated the vascular flora, at least from the high and middle altitudes.

On the other hand, presence of private bands in each species indicates their considerable genetic distinction. Additionally, the small number of polymorphic bands in the tetraploid *Galium sudeticum* suggests that the species may have been formed as a result of autopolyploidization of the genome of the diploid species (Bottini et al. 2002). It cannot be excluded, that it could have been a genome of *G. oelandicum* (but this must be confirmed by a study on a wider material). The floristic links between the Karkonosze Mountains and the northern Europe are documented by other species of vascular plants. The Karkonosze Mountains are the only Central European occurrence area of *Alchemilla wichurae* (Buser) Stefansson, *Pedicularis sudetica* Willd. and *Saxifraga nivalis* L., whose continuous range comprise the boreal and arctic area of Europe (Meusel et al. 1965, 1978; Fröhner 1990). This hypothesis however also needs further examination, despite that many molecular studies of Rubiaceae were already published (Manen et al. 1994; Natali et al. 1995).

Endemic plants of a narrow geographical area are as a rule also critically endangered species. From the point of view of the biodiversity preservation, protection of these populations is of utmost importance, the more so that genetic variation and population variability decrease both with population size and with increase of its isolation degree (Fakl and Holsinger 1991; Ellastrand and Elam 1993; Young et al. 1996; Aguilar et al. 2008).

Additionally, random variation in environmental conditions, uncertainty of demographic parameters (Lande 1988) and especially genetic processes (random changes in genetic composition due to founder effect, genetic drift or inbreeding) all lead to increased extinction probability in small and isolated populations by increasing genetic differentiation among populations, while decreasing genetic diversity within them. A population with little genetic variability or with low genetic diversity may have a reduced capacity to adapt to environmental challenges.

In the case of the three studied endemics, the high share of the intra-population diversity suggests that no decrease of it by genetical processes (as genetic drift or bottleneck effect) is observable. In the short term it is necessary to protect all existing natural populations of all three species in order to preserve as much genetic variability as possible. Extremely important is also preservation of all habitats available to these species, that are often directly influenced by man (e.g. by climbing or intensive tourism).

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