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

ELŻBIETA CIEŚLAK¹, ZBIGNIEW SZELĄG²

¹ Institute of Botany, Polish Academy of Sciences
  Lubicz 46, 31-512 Kraków, Poland
  e-mail: e.cieslak@botany.pl

² Institute of Botany, Jagiellonian University
  Kopernika 31, 31-501 Kraków, Poland

(Received: December 15, 2009. Accepted: May 11, 2010)

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).


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 congers (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 Malý 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 Štĕpánková (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 Štĕpánková 1998). The studied species are perennials, forming more or less dense carpets of numerous vegetative and generative shoots. They flower and fruit abundantly. The multi-year 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.
TABLE 1. Origin of plant material.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>No. of samples</th>
<th>Localities</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. cracoviense</em></td>
<td>17</td>
<td>Poland, Olsztyn village, Towarne hill (50°46'N, 19°16'E), alt. 340 m a.s.l.</td>
</tr>
<tr>
<td><em>G. cracoviense</em></td>
<td>5</td>
<td>Poland, Olsztyn village, Zamkowa hill (50°45'N, 19°16'E), alt. 350 m a.s.l.</td>
</tr>
<tr>
<td><em>G. cracoviense</em></td>
<td>7</td>
<td>Poland, Olsztyn village, Biakło hill (50°44'N, 19°6'E) alt. 360 m a.s.l.</td>
</tr>
<tr>
<td><em>G. oelandicum</em></td>
<td>14</td>
<td>Sweden, Island of Öland, Gynge alvar, Resmo (56°31'N, 16°25'E), alt. 50 m a.s.l.</td>
</tr>
<tr>
<td><em>G. sudeticum</em></td>
<td>11</td>
<td>Poland, Karkonosze Mts, Mały Śnieżny Kocioł glacial cirque (50°46'N, 15°34'E), alt. 1270-1360 m a.s.l.</td>
</tr>
</tbody>
</table>

**RESULTS**

AFLP analysis 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*.

**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 (FST) was also estimated. The parameters estimating genetic diversity were assuming Hardy-Weinberg equilibrium.
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*.

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

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

<table>
<thead>
<tr>
<th>Taxon</th>
<th>N</th>
<th>UB</th>
<th>T</th>
<th>P</th>
<th>G</th>
<th>h</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. cracoviense</em></td>
<td>29</td>
<td>2</td>
<td>92</td>
<td>58%</td>
<td>0.1736</td>
<td>0.1902 (±0.0867)</td>
</tr>
<tr>
<td><em>G. oelandicum</em></td>
<td>15</td>
<td>2</td>
<td>73</td>
<td>46%</td>
<td>0.1713</td>
<td>0.1742 (±0.0888)</td>
</tr>
<tr>
<td><em>G. sudeticum</em></td>
<td>11</td>
<td>3</td>
<td>91</td>
<td>57%</td>
<td>0.2383</td>
<td>0.2033 (±0.1264)</td>
</tr>
</tbody>
</table>

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

![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).](image1)

![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*.](image2)
TABLE 3. Results of molecular variance analysis (AMOVA). Level of significance tests are based on 1023 permutations.

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

The total variation was has been attributed within populations 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).

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 Galium cracoviense could have been one of them. Thus, the uninterrupted occurrence of Galium 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 (Szelag 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 Galium 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 aiation 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 autoploidyization 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 whichurae* (Buser) Steffansson, *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; Eillastrand 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).

**ACKNOWLEDGMENTS**

We are grateful to David Stählberg and Ejvind Rosén for sending material of *Galium oelandicum* from the Öland Island. We are also grateful to F. Ehrendorfer and W. Paul for useful comments on earlier drafts of the manuscript and the anonymous reviewer for helpful comments on the paper. This study was partly supported by the Polish Ministry of Sciences and Higher Education, grant no. 2 P04G 042 28.

**LITERATURE CITED**


