POPULATION GENETICS OF THE NARROW ENDEMIC

**Hladnikia pastinacifolia** Rchb. (Apiaceae)

INDICATES SURVIVAL IN SITU DURING THE PLEISTOCENE

**NINA ŠAJNA**¹, **TATJANA KAVAR**², **JELKA ŠUŠTAR-VOZLIČ**², and **MITJA KALIGARIĆ**¹

¹University of Maribor, Biology Department, Faculty of Natural Sciences and Mathematics, Koroška c. 160, SI – 2000 Maribor, Slovenia

²Agricultural Institute of Slovenia, Field Crop and Seed Production Department, Hacquetova 17, SI-1000 Ljubljana, Slovenia

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_Hladnikia pastinacifolia_ Rchb., a narrow endemic, has an extremely restricted distribution in Trnovski gozd (Slovenia), despite the presence of many sites with suitable habitats. We compared the morphological traits of plants from different populations and habitats. The overall pattern showed that the smallest plants, with low fruit number, are found on the locus classicus or type locality; the largest individuals, with high fruit number, grow in the Golobnica gorge. As judged by plant size and seed set, the optimal habitats are screes. We used RAPD markers to estimate genetic variation between and within populations, as well as between and within the northern and the southern parts of the distribution area. _Hladnikia_ showed only a low level of RAPD variability. AMOVA partitioned the majority of genetic diversity within selected populations. The low genetic differentiation between populations and their genetic depauperation indicates survival in situ, since the Trnovski gozd plateau most likely was a nunatak region in the southern Prealps during Pleistocene glaciations. Later range expansion of extant populations was limited by poor seed dispersal. We also analyzed the cpDNA _trnL-F_ intergenic spacer to check whether the sequence is useful for studying the phylogenetic relationships of _Hladnikia_ within the family Apiaceae (Umbelliferae). Our results support the assertion that _H. pastinacifolia_ is an old taxon.

**Key words:** Apioideae, Pleistocene, RAPD, cpDNA, nunatak, plant genetics, _Hladnikia pastinacifolia_, relict species, endemites.

INTRODUCTION

Rare species are characterized by low abundance, restricted distribution area and/or small geographical range (Gaston, 1997). The Trnovski gozd karst plateau in western Slovenia belongs to the southern Preamals and is one of the places where rare species, many of them endemic, are common. Another reason for its overall high species diversity is its biogeographical position as a meeting zone between Submediterranean, Dinaric and Alpine biomes.

One of the most remarkable species among the Pleistocene survivors found there is _Hladnikia pastinacifolia_ Rchb., the only representative of this endemic genus, with an extremely narrow distribution in a 4 km² area. The species is not a habitat specialist, however: it can be found growing in stony grassland, rock crevices and screes. _Hladnikia_ is regarded as an ancient paleoendemic genus containing a single Tertiary relic species (Mayer, 1960; Pawlowski, 1970), since paleoendemics are remnants of previously widespread taxa and their current distribution is sometimes reduced to small refugia (Kruckenberg and Rabinowitz, 1985).

Rare narrow endemic species occurring in a few small populations have to cope with random genetic drift, inbreeding, a stronger founder effect, and a greater potential for demographic bottlenecks that result in low genetic variability (Kunin and Gaston, 1997). Genetic diversity analyses of narrow endemics have generated considerable interest, especially because depauperated genetic variability is an important factor for conservation planning (Oiki et al., 2001; Jimenez et al., 2002; Cole, 2003; Torres et al., 2003; Gaudeul et al., 2004; Vilatersana et al., 2007; Mameli et al., 2008). Studies of rare species often involve comparisons with common congeners (Ayres and Ryan, 1999; Gitzendanner and Soltis, 2000; Kim and Chang, 2005). Various genetic markers have been used for...
these analyses, most often allozymes, RAPDs, AFLPs and microsatellites (SSRs).

The numerous synonyms of Hladnikia show that has been variously considered to belong to the genera Falcaria Fabr., Carum L., Oenanthe L. or Prionitis Adans. (Sušnik, 1964; Hegi, 1975). Reichenbach (1831) recognized it as the distinct genus Hladnikia with one species H. pastinacifolia. The botanist W.D.J. Koch disagreed with the placement of this species in the genus Hladnikia. He included another monotypic endemic species from Trnovski gozd, Athamanta golaka Hacq. instead, and changed its name to Hladnikia golaka (now Grafia golaka (Hacq.) Rchb.) (Wraber, 1990). Despite the disagreement, Reichenbach's name was retained. Recently the taxonomic status of Hladnikia was studied using nrDNA ITS sequence data (Ajani et al., 2008). Those results placed H. pastinacifolia within the tribe Careae, as more closely related to the Falcaria group than to the Carum group, genera to which Hladnikia were thought to belong based on its morphology and fruit anatomy.

Hladnikia pastinacifolia was discovered in 1819 (Fleischmann, 1844), but no population studies of it have been made. In this work our first task was to compare plants from different populations and habitats morphologically. Our second was to determine the levels of genetic diversity within and between populations by RAPD analysis. The third was to analyze the cpDNA trnL-F intergenic spacer in order to use the data to elucidate the phylogenetic relationships of Hladnikia within the family Apiaceae (Umbelliferae).

MATERIALS AND METHODS

STUDY SPECIES

Hladnikia pastinacifolia is a monocarpic herbaceous perennial that develops into a flowering plant during several vegetation periods. In the early life stages it forms rosettes. The leaf area gradually increases with age, and the leaf shape develops from simple to lobate. What triggers flowering is yet unknown. The flowers are insect-pollinated. Seeds form from the end of August through September. We still lack information about the breeding system. The existence of numerous and varied pollinators, as well as protandry, support the idea that outcrossing operates. The pollen:ovule ratios for flowers have been calculated at ~4000:2 (Sajna N, unpublished data), suggesting low-efficiency pollinators which might also be palynophagous (Cruden, 1976). The fruit is a schizocarp, bearing two 4 mm mericarps with an underdeveloped embryo. The seeds have no specialized dispersal adaptations.

Hladnikia pastinacifolia is an extremely rare and strictly protected species; this limited our research. We followed the strict restrictions on sampling quantities issued by the Republic of Slovenia's Institute for Nature Conservation, which often allowed only small samples. We employed mainly non-destructive research methods.

STUDY SITE

Trnovski gozd is a large limestone plateau in a mountainous area up to 1500 m a.s.l. Together with the South Julian Alps it forms an orographic barrier between the Mediterranean and moderate continental climatic regions. This area receives one of the highest amounts of precipitation (above 3000 mm on more than 120 precipitation days) in Slovenia (Melik, 1960). Most of the area is covered with natural forest stands. Traditional farmland is distributed sparsely. The geographic range of H. pastinacifolia is limited to the southern slopes of the Trnovski gozd plateau and two isolated locations 9 km away on the northern slopes. The entire distribution area is included in the Natura 2000 Network as a Site of Community Interest. We chose

<table>
<thead>
<tr>
<th>Population</th>
<th>Location</th>
<th>Elevation (m a.s.l.)</th>
<th>Habitat types</th>
<th>Estimated population size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Predmeja</td>
<td>45°56'33&quot;N, 13°52'50&quot;E</td>
<td>890</td>
<td>2, 3</td>
<td>100</td>
</tr>
<tr>
<td>2 Golobnica</td>
<td>45°56'33&quot;N, 13°52'11&quot;E</td>
<td>900</td>
<td>1, 3</td>
<td>850</td>
</tr>
<tr>
<td>3 Čaven</td>
<td>45°55'45&quot;N, 13°51'31&quot;E</td>
<td>1113</td>
<td>2, 3</td>
<td>2000</td>
</tr>
<tr>
<td>4 Poldanovec</td>
<td>46°03'6&quot;N, 13°49'59&quot;E</td>
<td>1299</td>
<td>1, 2</td>
<td>300</td>
</tr>
<tr>
<td>5 Zeleni rob</td>
<td>46°04'7&quot;N, 13°51'24&quot;E</td>
<td>1369</td>
<td>1</td>
<td>50</td>
</tr>
</tbody>
</table>
five populations for the study: three at the southern edge, and both known locations from the northern edge (Fig. 1). The location at Predmeja is a small stony pasture with a man-made stone wall. The habitat is threatened by encroaching woody vegetation (Šajna et al., 2011). Location 2 was previously described as only a secondary occurrence at the road verge (Čušin, 2004) near the Golobnica gorge. When we made a detailed search in the field, however, we observed that plants were also present in rubble and rock crevices of the eastern rocky walls within the gorge (Šajna et al., 2009), which should therefore be classified as a primary habitat location. Location 3 is the type locality (locus classicus): stony grassland with co-occurring smaller screes. On the northern edge is a larger population stand on a small shelf overgrown with vegetation belonging to the association Primulo carniolicae-Caricetum firmae Dakskobler 2006 on the peak of Mt. Poldanovec (Dakskobler, 2006). The second northern location is a small rock formation within a beech forest where H. pastinacifolia is found on steep walls; the top of the rock is covered by a Pinus mugo stand.

MORPHOMETRIC ANALYSES

In 2006, flowering plants from populations 1–4 (Fig. 1, Tab. 1) were randomly chosen for morphometric analysis. Population 5 was excluded because very few plants flowered that season. All measurement and scoring of selected plant traits were done non-destructively. The following ten plant traits were measured from a total of 103 specimens: plant height, leaf length, leaf width, rosette diameter, central umbel diameter, peduncle height, number of umbellets of central umbel, number of fruit in central umbel, number of umbels per plant, and tap root diameter.

We compared plants from different populations or habitats using ANOVA followed by the Tukey HSD. The morphological results were log-transformed ($\log_{10}$) when necessary to obtain homogeneity of variance (Levene's test) and to achieve a normal distribution (Shapiro-Wilk's test). To a significant degree the plants of the various populations were characterized by heterogeneity of variance of leaf width, number of umbellets of central umbel, and number of umbels per plant, so these were not included in ANOVA.

For the same reason we did not use the following traits for comparison of plants growing in the three types of habitats: rosette diameter, number of umbellets of central umbel, number of fruit in central umbel, and tap root diameter. Additionally, we performed discriminant analysis to maximize between-habitat variation in relation to within-habitat variation.

RAPD ANALYSIS

For genetic analyses, leaf samples of 20 individual plants from each of five populations representing the entire distribution area were collected (Fig. 1). Total genomic DNA was extracted following a modi-
RAPD-PCR amplifications were performed in a 25 μl volume with a GeneAmp PCR System 9700 thermocycler (Perkin Elmer, U.S.A.). Each reaction contained 1x PCR buffer, 200 μM dNTPs, 1.2 pmol of each primer (Tab. 3), 2.5 mM MgCl₂, 0.7 U Taq DNA polymerase (Promega, U.S.A.) and 20 ng template DNA. The PCR profile consisted of initial denaturation at 94°C for 5 min, followed by 41 cycles of strand denaturation at 94°C for 1 min, primer annealing at 37.5°C for 1 min 40 s, DNA extension at 72°C for 2 min, and final extension at 72°C for 10 min. PCR products were separated electrophoretically in ethidium bromide-stained 1.4% agarose-TBE gels. A negative control without DNA was included to check for contamination.

Reproducible RAPD bands were scored as binary presence/absence data. Statistical analysis was done with GENALEX 6 (Peakall and Smouse, 2006). Because of the high frequency of ghost bands in the RAPD profiles of some samples, we omitted those samples from the final analysis. We believe that the presence of these bands was a consequence of a fungus infection, and had we included those results the genetic diversity would have been falsely increased. Consequently, 64 individuals were included in AMOVA.

**RESULTS**

**MORPHOLOGICAL DIFFERENCES BETWEEN POPULATIONS AND HABITATS**

The investigated plants differed significantly between populations. There was a general difference between plants from Golobnica and those from the other populations. The plants from Golobnica differed significantly in vegetative traits (plant height, leaf length, rosette diameter) and in reproductive structures (peduncle height, umbel diameter), which were bigger (Tab. 2). However, the traits associated...
with reproductive success (number of fruit on main umbel) did not differ from the other populations except in those from the type locality, Čaven, where the smallest individuals were found, with only two-thirds the number of fruit.

The plants differed significantly between habitats (AMOVA) in plant height, leaf length, diameter of central umbel, and peduncle height. Plants from stony grassland differed most, with the smallest inflorescences as well as smallest overall size. The plants found growing in screes were biggest. The bigger the plant, the higher the probability that the plant would form more prostrate branching of the stem, resulting in more inflorescences of the second or even third degree, which we observed only on plants growing in screes. The plants from rock crevices were morphologically similar to plants from the other habitat types; the biggest differences were between plants from screes and those from stony pastures (Fig. 2; discriminant analysis: $F_{16,176}=8.1507$, $P<0.05$).

**RAPD ANALYSIS**

First, 44 10-mer primers (Operon Technologies Ltd., Alameda, U.S.A.) were screened for RAPD profiles with three *H. pastinacifolia* samples in order to find the effective primers for RAPD analysis. Then the ten primers giving reproducible bands were selected for further analysis (Tab. 3). The average number of bands was 3.9 per primer, and fragment size ranged from 250 bp to 1000 bp (Fig. 3). Sixty-four individuals were included in the statistical analyses to estimate variation within and between populations (11–15 for each population).

### TABLE 3: RAPD primers used in this study, and number of amplified and scored bands

<table>
<thead>
<tr>
<th>Primer</th>
<th>Amplified bands</th>
<th>Scored bands</th>
</tr>
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<tbody>
<tr>
<td>OPU-19</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>OPR-03</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>OPX-11</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>OPW-06</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>OPO-15</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>OPO-16</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>OPO-19</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>OPP-09</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>OPQ-14</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>OPAS-13</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>56</strong></td>
<td><strong>39</strong></td>
</tr>
</tbody>
</table>

Fig. 3. Example of amplified RAPD fragments (primer OPU 19). Lanes are marked with numbers corresponding to populations: 1–21 – Čaven; 22–34 – Zeleni rob; 35–54 – Poldanovec; 55–76 – Predmeja; 77–97 – Golobnica. Nc – negative control; M – molecular weight scale (1 kb DNA ladder).
Differences between individuals were not pronounced, since many individuals from different populations shared the same RAPD profile. The data confirmed low levels of differentiation between populations. AMOVA indicated that the majority of the variation pertained to differences within populations (90%), and only 10% was attributable to variation between populations. When we grouped populations into two regions (northern or southern location of the population), AMOVA did not show any differences between regions.

Principal component analysis of RAPD data did not show genetic differentiation of populations (Fig. 4). The first three factors accounted for 66% of the total variation in the data set (32%, 19% and 15% by the first, second and third factors respectively). Ten individuals (two from each of five populations) were sequenced. All samples generated an identical 840 bp sequence, which included part of the tRNA-Leu (trnL) gene and the trnL-F intergenic spacer. No length variation or differences in the proportion of nucleotide were found. The sequence has been deposited in GenBank under Acc. No. EU514464 and used for a BLAST search against the NCBI nr database. Sequences of the first 100 BLAST hits were used for construction of the NJ tree (Appendix 1). Further analyses were limited to the dataset of 21 sequences from the group of the closest relatives of *H. pastinacifolia*. We randomly chose one sequence of each genus, and the sequence of *Apiopetalum velutinum* Baill. was selected as out-group. Phylograms were constructed by various methods (NJ, MP, ML), with gaps included or excluded. In some cases *H. pastinacifolia* clustered within the Selineae; in other cases the relationship between *Apium graveolens* L., *H. pastinacifolia* and Selineae remained unresolved. However, the majority of trees had the topology presented in Figure 5, which is, according to the bootstrap values, moderately supported. Bootstrap values ranged from 50% to 95% (average 71%), and only 10 clades were resolved having values ≥50%. The genus *Hladnikia* formed a single clade and was clustered in the group composed of the Selineae (e.g., *Angelica* L., *Cymopterus* Raf., *Zizia* W.D.J. Koch, *Aletes* Coulit. & Rose, *Lomatium* Raf.), Apiaceae incertae sedis (e.g., *Tilingia* Regel & Tiling) and *Apium* clade. These all belong to the apioid superclade (Plunkett and Downie, 1999). *Hladnikia* is also closely related to *Scandiceae* (e.g., *Daucus* L., *Myrrhis* P. Mill., *Osmorhiza* Raf.) (Fig. 5).

**DISCUSSION**

**MORPHOLOGICAL DIFFERENCES BETWEEN POPULATIONS AND HABITATS**

The plants of all the studied populations exhibited low genetic diversity but pronounced morphological differences, indicating high phenotypic plasticity of vegetative traits as well as traits of reproductive structures. The smallest plants, with low fruit number, were those from Čaven (locus classicus); the largest plants, with high fruit number, grew in the Golobnica gorge. This can be explained by the habitats of these two locations. At Čaven the plants grew in stony pastures; those plants were significantly smaller (shorter plants and peduncles, smaller leaves). Judged by plant size and seed set, the scree in the Golobnica gorge seem to be the optimal habitat.

Although it has no special habitat type preference except for preferring some degree of disturbance, and despite its high phenotypic plasticity, *H. pastinacifolia* has a very limited distribution. We suggest that it is restricted by traits related to dispersal and persistence in disturbed habitats. For example, the monocarpic life cycle of *H. pastinacifolia* is characteristic for species with transient occurrence. Also, scree slopes and similar disturbed habitats such as river banks are more or less permanent features of the landscape but they are not common and are often widely separated; dispersal within such habitats is strongly linked to the direction of gravel movement. Rock crevices represent a secondary habitat; this chasmophytic habitat seems to have become a local refuge for *H. pastinacifolia* as it presented stabler microclimatic conditions. Many chasmophytic species are believed to originate...
from before the Pleistocene (Davis, 1950; Runemark, 1969).

GENETIC DIVERSITY

The analysis of genetic diversity (RAPD) included plants from the entire distribution area of the species. We expected to find at least some genetic divergence between the southern and the northern populations, since they are divided by a continuous dense forest stand. West Dinaric fir-beech forest prevails. The stands form the geographical variant *Omphalodo-Fagetum* var. geogr. *Saxifraga cuneifolia*, further divided into two geographical subvariants: western – subvar. geogr. *Anemone trifolia*, and central-eastern – subvar. geogr. *Omphalodes verna* (Surina, 2002). Especially in the northeastern part of the Trnovski gozd plateau, where locations 4 and 5 (Fig. 1) are situated, natural black pine stands can be found in less favorable habitats such as steep rocky slopes (Dakskobler, 1999). Spruce forests are restricted to freezing ravines and cold, moist, shaded sites (Surina and Vreš, 2009). It is believed that this area has been at least partly forested since the end of the Pleistocene glaciations. The forest was not intensively exploited before the 16th century, and since then it has been managed in several ways including selective cutting (16th–18th cent.) and clearcutting in some parts in the north (Surina, 2001).

Against expectations, RAPD analysis showed that all populations express a high level of monomorphic bands and that many individuals from different populations share the same RAPD profile. Recently some authors have challenged the value of RAPD markers, citing low reproducibility of markers and suggesting that AFLP markers are preferable. Even though RAPDs show different genetic similarity ranges, as do AFLPs, they often show similar overall results (Ćwiklińska et al., 2010).

The majority of the genetic variation in *H. pastinacifolia* was partitioned within populations, and did not differentiate the southern or northern parts of the distribution area. Higher genetic diversity within than between populations has been described as a characteristic of perennial and outcrossing species (Despres et al., 2002).

The lack of differences between the northern and the southern populations can be explained in two ways: as the result of continual gene flow among populations (via pollen or seed dispersal), or as the consequence of the relatively recent establishment of extant populations from a common, genetically depauperated founder.

In regard to the possibility of gene flow between populations, we observed that *H. pastinacifolia* inflorescences frequently were visited by various insects (mainly Hymenoptera, Coleoptera and...
Diptera), suggesting cross-pollination. Some pollinators can fly distances from 2.6 km up to even 9.9 km, but mostly within 1 km (Walther-Hellwig and Frankl, 2000; Kraus et al., 2009). Even with the possibility of selfing, mating would occur only between a small proportion of flowering individuals each season, thereby creating "temporal subpopulations" and delaying or reducing inbreeding depression (Lopez-Pujol et al., 2002). On the other hand, seed dispersal is limited because the seeds lack adaptations for specialized dispersal. We did not observe myrmecochory or epizoochory. There is only a small potential for endozoochory, since the seeds have a strong, unpleasant odor and taste (this is probably why we seldom observed damaged seeding umbels). Among the dispersal vectors we cannot neglect the human presence. All localities of the investigated populations experience very frequent visits as trekking or climbing destinations or as hunting spots. Wind dispersal seems the most promising vector. Most seeds remain near the maternal plant in rainy autumns, but we observed the breaking off of the entire umbel when dry conditions prevailed (personal observations in 2004–2009). A similar dispersal strategy was noted for the thermophilous *Peucedanum arenarium* subsp. *arenarium* (Šera et al., 2005). However, dry autumn conditions are rather the exception in this region; poor seed dispersal may be the reason for the clustered distribution of seedlings we observed on the microscale (Šajna N., unpublished data). The type of pollination and limited seed dispersal would indicate that gene flow, if any, is very limited. However, limited gene flow would suggest stronger genetic differentiation between the northern and southern populations, which we did not find using RAPDs.

Besides the lack of genetic differentiation between the southern and the northern parts of the distribution area, the mostly identical RAPD profiles of specimens from all populations suggest that these genetically homogeneous populations are the result of severe bottlenecks which dramatically reduced or eliminated some populations, whatever the time of colonization (before or after glaciations). Recent establishment of the extant *H. pastinacifolia* populations founded by seeds from a few nearby populations could explain the low interpopulation differentiation. Its phylogeny indicates the isolated position of *H. pastinacifolia* (Ajani et al., 2008) and does not reveal any closely related species that could result from allopatric speciation following the end of the Pleistocene glaciations. Sušnik (1964) determined the chromosome number in *H. pastinacifolia* (x=11, the most frequent number in Apiaceae) and diploid ploidy level, which are both consistent with paleoendemics (Kruckenberg and Rabinowitz, 1985). We can therefore still assume that *H. pastinacifolia* is an old taxon which lost its genetic variability at some point in the past. We lean toward the idea that the loss of genetic variation occurred during the Quaternary glaciations when the species became restricted to safe sites where it survived in situ. In the Quaternary a smaller local glacier existed in Trnovski gozd; the highest exposed peaks in the northern part were not covered by the glacier and were nunataks (Fig. 6; Perko 2001). The influence of the Adriatic Sea was reduced because the sea level was 120 m lower and the northern shoreline was at least 200 km south of the present line (Correggiari et al., 1996). The recent distribution of *H. pastinacifolia* represents only a slightly increased range in the southern part and two fragmented locations in the northern part of that distribution, separated by forest and restricted to steep overhangs (Fig. 6). As many narrow endemics found south of the Alps did not spread after the end of the glaciations and remained within refugia (Vogel et al., 1999), this seems to be the case for *H. pastinacifolia* as well. It can be additionally explained by poor seed dispersal. Paleoendemics frequently do not have the genetic variability that would allow them to increase their distribution area following a contrac-

![Fig. 6. Map showing the Trnovski gozd high karst plateau during the Quaternary (adapted after Perko, 2001). White dots indicate recent distribution of *Hladnikia pastinacifolia* (numbers of localities correspond to numbers in Table 1; white dots without numbers all belong to continuous population 3). White crosshatched area represents the nunatak region, light grey area is surface shaped by Pleistocene glaciers, and hatched areas indicate valleys, basins and karst depressions filled with periglacial debris and gravel.](image-url)
tion of the distribution. *Erinus alpinus* (Scrophulariaceae) is an interesting case of post-glacial range expansion. *Erinus* is a monotypic genus common in subalpine habitats of Southwestern and Central Europe, which survived the Pleistocene glaciations in southern refugia peripheral to the Alps, as well as in situ on nunataks in the northern Prealps (Stehlik et al., 2002). When Stehlik et al. (2002) studied genetic diversity at cpDNA level in 12 populations throughout the distribution area ranging from southern France to the Swiss-Austrian border, including nunataks like Mount Rigi, they found no variation. They identified three phylogeographic groups with AFLPs. One group was represented by a single central Swiss population on Mount Rigi. Individuals from Rigi had a significantly low number of AFLP fragments, supporting in situ survival in a nunatak region. Compared to the spatial scale of that study, our study describes a small-scale local situation. The populations we studied were close enough to each other to be regarded as one population. From this point of view the lack of cpDNA diversity is not surprising, especially when we consider the existence of a local glacier with a local nunatak where *H. pastinacifolia* might have survived in situ. In theory the cost of survival in nunataks is isolation, causing lower genetic diversity through inbreeding, as well as population shrinking accompanied by random genetic drift or recurrent bottlenecks also contributing to genetic depauperation. We can suggest that these processes of survival in situ or a combination of them are the cause of the loss of most genetic diversity as indicated by RAPD markers in *H. pastinacifolia*. The extant populations were founded by a single lineage starting from a nunatak population. In populations that have been rare for a very long period, natural selection can reduce deleterious alleles and increase species fitness (Falconer and Mackay, 1996). If populations were experiencing different selection pressures, this would result in higher genetic variability between populations, but in the habitats of *H. pastinacifolia* the ecological characteristics are similar, exerting similar selection pressure. This could help maintain low genetic differentiation among the populations.

Modern modeling studies support the existence of refugia in the southeastern Prealps (Tribsch and Schönswetter, 2003) without reference to the existence of local nunataks. Phylogenetic data indicate that potential locations of refugia may have been climatically stable for long periods of time, some even since the Tertiary (Medail and Diadema, 2009). As mentioned, Trnovski gozd also seems to be a stable region of atypical climate, since even today it is a sanctuary for many alpine and arctic plant and animal species, despite its southern location. However, it has never before been considered a sanctuary in the sense of a small nunatak region (peripheral nunatak as defined by Schönswetter et al., 2004).

Among the many molecular phylogeographic studies, seldom has nunatak survival been used to explain observed genetic patterns in temperate mountain ranges (Schneeweiss and Schönswetter, 2011); a rare exception is a study by Stehlik et al. (2002). We believe there are many similarities between the *E. alpinus* population they studied on Mount Rigi and the *H. pastinacifolia* populations in Trnovski gozd. The species most likely to retain a molecular signature of survival in situ on nunataks are those lacking the potential for rapid range expansion and recolonization, which can be identified by the current distribution pattern and/or by species ecology (Westergaard et al. 2011; Lohse et al., 2011). The dispersal characteristics of *H. pastinacifolia*, its distribution and its germination characteristics (Šajna N., unpublished data) match the description of a nunatak survivor well.

**PHYLOGENY ACCORDING TO cpDNA trnL-F**

*Hladnikia pastinacifolia* occupies an isolated position within the Apiaceae family. It seems to have shared a common ancestor with other species from the apioid superclade, although statistical confidence for such topology is not high and other explanations cannot be ruled out. The topology of the NJ tree shows that *H. pastinacifolia* forms a single clade which is sister to a clade comprising species from the genera of western and eastern North America, and from East Asia. Within this clade the North American species and Asian species are sister clades. *Dystaenia* is monophyletic and unites with Cnidium as a clade of the East Asian endemics *D. ibukiensis*, endemic to Japan, and *D. takesimana*, endemic to Ullung Island (Pflosser et al., 2006). The North American species are defined as herbaceous perennial apioid genera endemic to North America (north of Mexico; Sun and Downie, 2004). Some are polyphyletic (*Cymopterus*, *Lomatium*, *Pteryxia*; Sun and Downie, 2004), some are monophyletic (*Polytenia*, *Thaspium*, *Zizia*; Sun et al., 2004), and some are monotypic (*Neoparrya*, *Shoshonea*, *Harbouria*). *Hladnikia* and closely related endemics to some extent represent a typical disjunction pattern for Northern Hemisphere Tertiary relicts: eastern North America, western North America, East Asia, and Southeastern Europe. As said above, the statistical certainty of these conclusions is low. We must also bear in mind the sparseness of the data in
GenBank, among which members of the tribes Scandiceae and Selinaceae are over-represented. At this point the usefulness of the information obtained from the cpDNA sequence for phylogeny is open to question.

A single sequence was obtained from all analyzed samples of *H. pastinacifolia.* The level of variation within the species was generally very low or zero at the cpDNA *trnL-F* locus. *H. pastinacifolia* is an endemic and has an extremely narrow geographic distribution; there was also almost no variation detected at the nuclear level between the RAPD profiles of samples from different populations.

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