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POPULATION GENETICS OF THE NARROW ENDEMIC *HLADNIKIA PASTINACIFOLIA* RCHB. (APIACEAE) INDICATES SURVIVAL IN SITU DURING THE PLEISTOCENE

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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 Čaven (*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 *trn*L-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 Prealps 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 km^2 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 relict 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

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TABLE 1. Field name, location (coordinates), elevation, habitat types (1 – rock crevices; 2 – stony pasture; 3 – scree), and estimated size of five studied populations of *Hladnikia pastinacifolia*

Population	Location	Elevation (m a.s.l.)	Habitat types	Estimated population size
1 Predmeja	45°56'33"N, 13°52'50"E	890	2, 3	100
2 Golobnica	45°56'33"N, 13°52'11"E	900	1, 3	850
3 Čaven	45°55'45"N, 13°51'31"E	1113	2, 3	2000
4 Poldanovec	46°0'36"N, 13°49'59"E	1299	1, 2	300
5 Zeleni rob	46°0'4"N, 13°51'24"E	1369	1	50

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 (Šajna 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

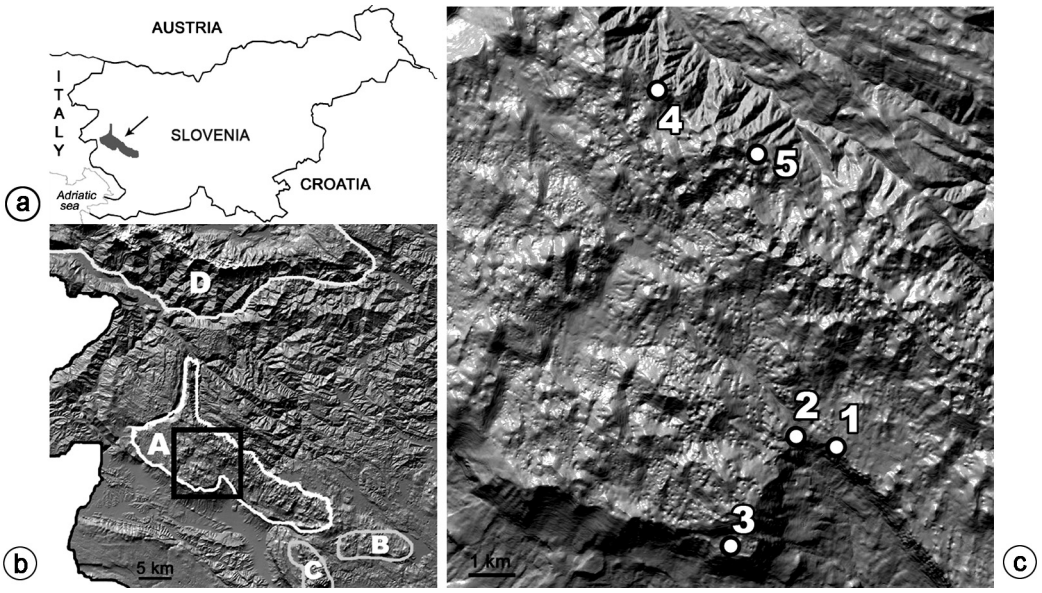

Fig. 1. (**a**) Map of Slovenia with location of Trnovski gozd karst plateau (arrow), (**b**) Trnovski gozd (A) between Julian Alps (D) and Dinaric Mts. (B – Hrušica, C – Nanos). Square indicates distribution area of *Hladnikia pastinacifolia*, (**c**) Detailed geomorphology and locations of the 5 studied populations: 1–3 in southern part, 4 and 5 in northern part of distribution area (locality numbers correspond to numbers in Table 1).

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 (^{10}log) 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-

TABLE 2. Values (mean ±SD) for each studied trait of four *Hladnikia pastinacifolia* populations. [Values with different letters differ significantly from each other (Tukey HSD test at P<0.05)]

Trait	Populations				
	Predmeja	Golobnica	Čaven	Poldanovec	F**
Plant height	20.8 ± 4.4 a	27.9 ± 5.0 b	17.2 ± 4.3 a	18.6 ± 3.8 a	31.3
Leaf length	7.8 ± 1.8 b	10.3 ± 2.1 c	6.6 ± 1.2 ab	6.1± 1.4 a	33.2
Rosette diameter *	15.1 ± 2.8 c	21.1 ± 4.3 b	12.4 ± 1.5 c	13.5 ± 2.2 ac	45.6
Peduncle height	19.1 ± 4.1 b	27.4 ± 4.7 c	17.2 ± 4.6 ab	15.0 ± 4.5 a	43.5
Umbel diameter	9.0 ± 1.7 a	10.1 ± 1.8 b	7.0 ± 1.1 c	8.2 ± 1.6 ac	15.9
No. of fruit *	216.3 ± 77.6 a	213.6 ± 60.4 a	145.5 ± 52.7 b	219.7 ± 75.3 a	6.7
Root diameter *	0.60 ± 0.17 a	0.63 ± 0.23 a	0.51 ± 0.11 a	0.83 ± 0.26 b	7.3
N	27	37	17	22	

*Trait was ^{10}log-transformed for ANOVA. **P <0.01

fied CTAB protocol (Šuštar-Vozlič and Javornik, 1999).

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 $MgCl_2$, 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.

cpDNA *trn*L-F SEQUENCES

A fragment of chloroplast DNA (*trn*L-F) was amplified using universal primers "c" and "f" from Taberlet et al. (1991). PCR amplification was performed in a 20 µl reaction mixture with a GeneAmp PCR System 9700 thermocycler (Perkin Elmer, U.S.A.). Each reaction contained 1x·PCR buffer, 200 µM dNTPs, 10 pmol of each primer, 2.5 mM $MgCl_2$, 0.5 U DNA polymerase (Biotools, Spain) and 50 ng template DNA. The PCR profile consisted of initial denaturation at 80°C for 5 min, followed by 35 cycles of strand denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, DNA extension at 72°C for 2 min, and final extension at 72°C for 10 min. Purified PCR fragments were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) on the ABI PRISM 310 DNA Sequencer (PE Applied Biosystems).

A search for similar sequences in the NCBI nucleotide collection (nr/nt) was performed with BLASTN programs (Altschul et al., 1997). Selected sequences were retrieved from GenBank and aligned with the sequence of *H. pastinacifolia* with ClustalX (Thompson et al., 1997) and refined manually. Phylogenetic analyses using the neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods were conducted using MEGA ver. 3.1 (Kumar et al., 2004) or the Phylip package (Felsenstein, 2004). Genetic distances were calculated using the Kimura two-parameter method (Kimura, 1980), where the transversion/transition ratio was 2:1. Gaps were either excluded or included in the dataset. When they were included, scoring of gaps was the same as for transitions (one base indel) or transversions (more than two base indels).

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

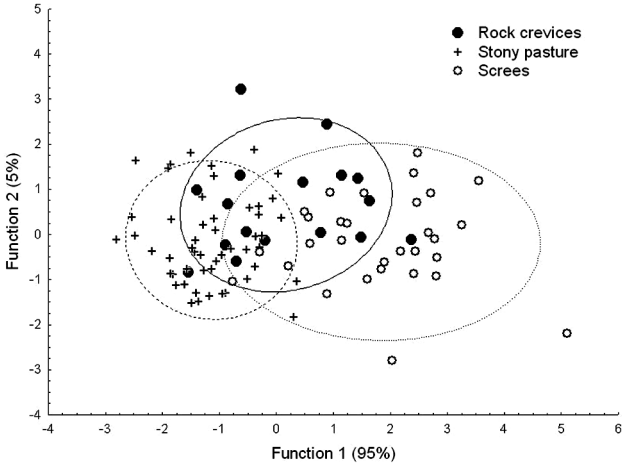

Fig. 2. Discriminant analysis of morphometric data grouped by habitat types where *Hladnikia pastinacifolia* is found. Function 1 discriminates mostly between plants from screes and those from stony pastures.

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: $F_{16,176}=8.1507$, $P< 0.05$).

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

Primer	Amplified bands	Scored bands
OPU-19	7	5
OPR-03	5	3
OPX-11	4	3
OPW-06	4	3
OPO-15	7	6
OPO-16	4	3
OPO-19	7	5
OPP-09	6	3
OPQ-14	6	6
OPAS-13	6	2
Total	56	39

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).

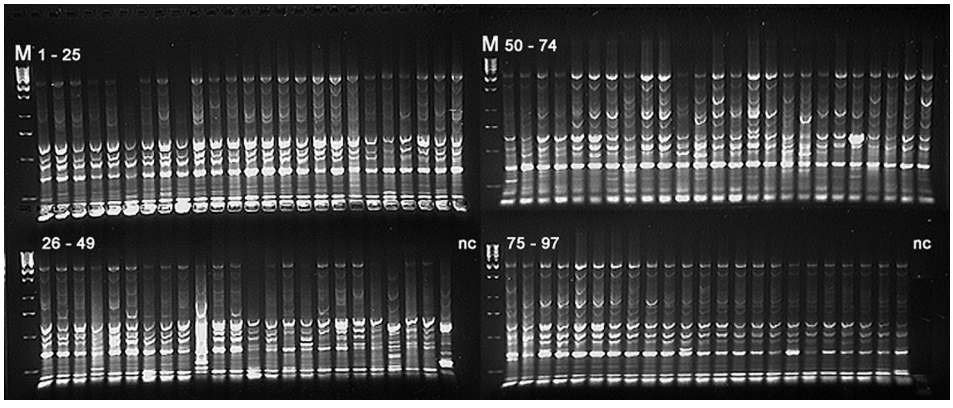

Fig. 3. Example of amplified RAPD fragments (primer OPU 19). Lanes are marked with numbers corresponding to populations: 1–21 – Čaven; 22–34 – Zeleni rob; 35–54 – Poldanovec; 55–76 – Predmeja; 77–97 – Golobnica. Nc – negative control; M – molecular weight scale (1 kb DNA ladder).

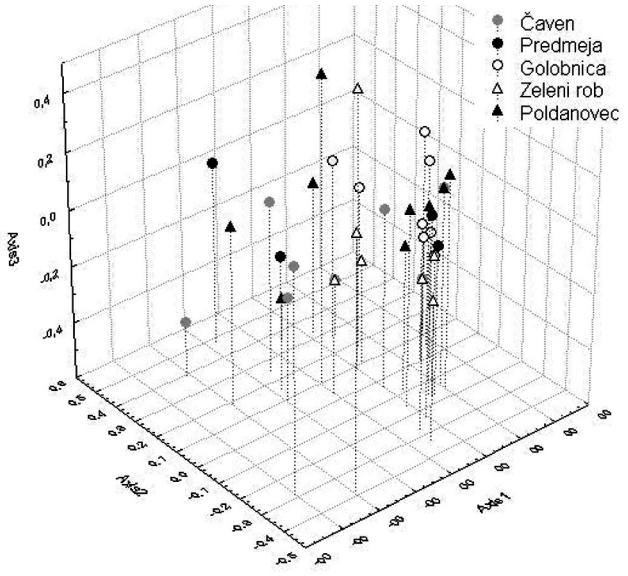

Fig. 4. Ordination of the investigated populations based on RAPDs along the first three axes extracted by principal component analysis.

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).

cpDNA *trn*L-F

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 (*trn*L) gene and the *trn*L-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* Coult. & 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 screes 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

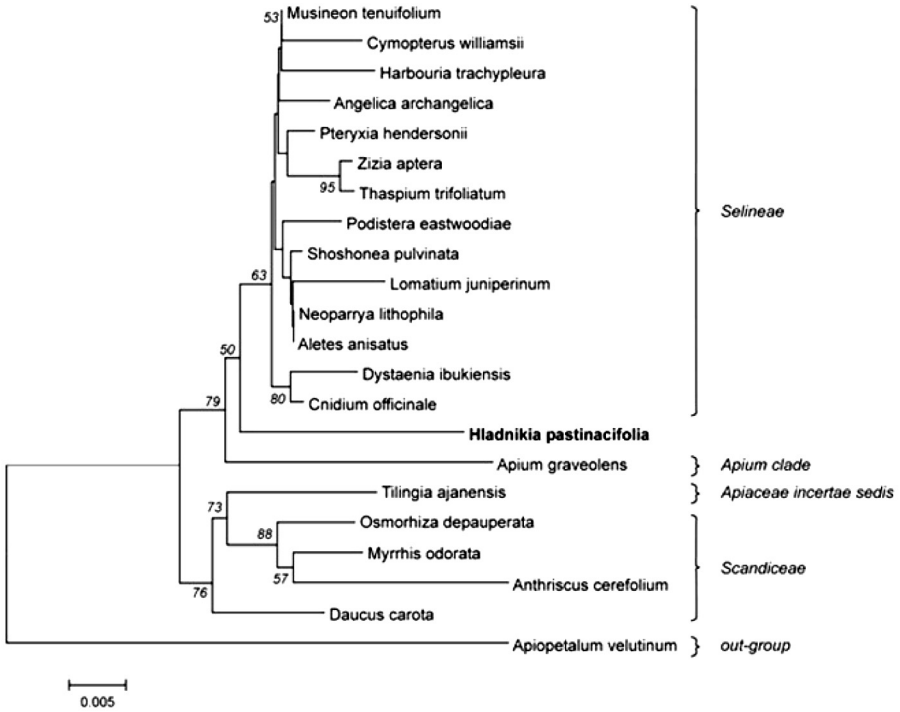

Fig. 5. Neighbor-joining tree representing the relationships between *Hladnikia pastinacifolia* and its closest relatives from the Apiaceae family. Bootstrap values higher than 50 are given at the nodes.

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-

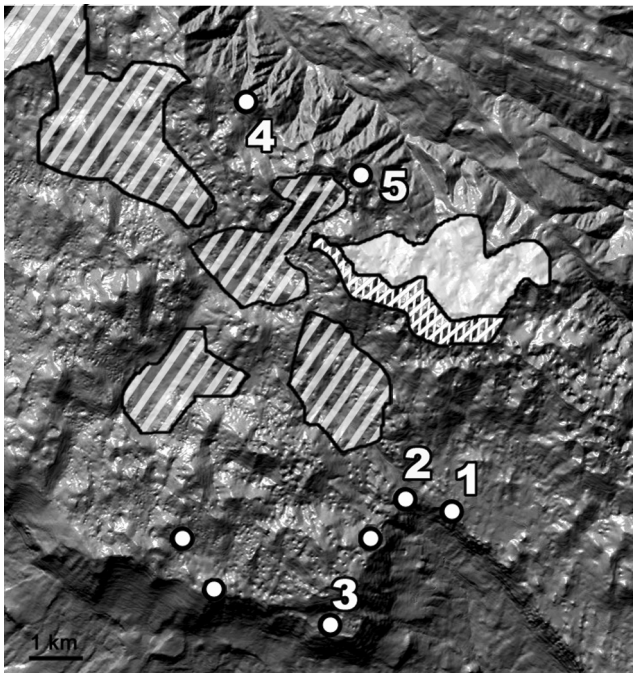

Fig. 6. Map showing the Trnovski gozd high karst plateau during the Quaternary (adapted after Perko, 2001). White dots indicate recent distribution of *Hladnikia pastinacifolia* (numbers of localities correspond to numbers in Table 1; white dots without numbers all belong to continuous population 3). 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.

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 has an important geographic position. The high level of endemism in this region is associated with the persistence of flora since the Tertiary, as Trnovski gozd is regarded as a sanctuary for species common before and at the time of the Pleistocene glaciations (Wraber, 1990). 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 *trn*L-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 (Pfosser 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 (*Polytaenia*, *Thaspium*, *Zizi*a; 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 Selinae 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 *trn*L-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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APPENDIX 1


Trevesia sphaerocarpa tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Brassaiopsis ficifolia tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Trevesia lateospina tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Brassaiopsis hispida tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Trevesia cf. valida GMP-2003 tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Trevesia sundaica tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Brassaiopsis stellata tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Brassaiopsis simplicifolia tRNA- Leu (trnL) and trnL- trnF intergenic spacer, partial sequence; chloroplast
Brassaiopsis hispida tRNA- Leu (trnL) and trnL- trnF intergenic spacer, partial sequence; chloroplast
Macropanax undulatus tRNA- Leu (trnL) and trnL- trnF intergenic spacer, partial sequence; chloroplast
Macropanax dispermus tRNA- Leu (trnL) and trnL- trnF intergenic spacer, partial sequence; chloroplast
Macropanax dispermus tRNA- Leu (trnL) and trnL- trnF intergenic spacer, partial sequence; chloroplast
Macropanax dispermus tRNA- Leu (trnL) and trnL- trnF intergenic spacer, partial sequence; chloroplast
Metapanax davidii tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Eleutherococcus setchuenensis tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Eleutherococcus sieboldianus tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Oplopanax elatus tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Schefflera sp. Vietnam voucher Lowry 4840 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera hypoleucoides tRNA- Leu (trnL) and trnL- trnF intergenic spacer, partial sequence; chloroplast
Hedera helix subsp. hibernica chloroplast partial tRNA- Leu (UAA) gene, trnL- trnF intergenic spacer and tRNA- Phe gene (GAA) gene, specimen voucher Gornall 2.4.2000 (LTR)
Hedera helix tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Eleutherococcus sessiliflorus tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Dendropanax sessiliflorus tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera aff. jahnii Neill 12609 voucher Neill 12609 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera sp. Ecuador voucher Neill 13849 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera crassipes voucher Lowry 4797 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera gordonii voucher Lowry 5541 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera gordonii voucher Lowry 4674 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Polyscias macgillivrayi chloroplast tRNA- Leu gene and trnL- trnF intergenic spacer region, partial sequence
Schefflera moratiana voucher Lowry 4767 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera costata voucher Lowry 5512 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Pseudopanax ferox tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Pseudopanax crassifolius tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Plerandra insolita tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Pseudopanax linearis tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Schefflera plerandroides voucher Lowry 4751 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera veillonorum voucher McPherson 17801 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera veitchii voucher Lowry 4665 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera reginae voucher Lowry 4652 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera elegantissima chloroplast tRNA- Leu gene and trnL- trnF intergenic spacer region, partial sequence
Apiopetalum velutinum tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Daucus carota chloroplast, complete genome
Apium graveolens chloroplast tRNA- Leu gene for transfer RNA- Leu and partial tRNA- Phe gene for tr..
Zizia aurea tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRNA- Thr gene, partial
Zizia aptera tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRNA- Thr gene, p..
Thaspium trifoliatum tRNA- Phe gene, partial sequence; and tRNA- Leu gene, complete sequence; chloropla..
Pteryxia hendersonii tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRNA- Thr
Cymopterus montanus tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence;..
Cymopterus multinervatus tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tR...
Shoshonea pulvinata tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRNA- Thr ..
Cnidium officinale plastid partial tRNA- Leu gene and partial IGS
Seseli hippomarathrum plastid partial tRNA- Leu gene and partial IGS
Dystaenia ibukiensis plastid partial tRNA- Leu gene and partial IGS, isolate 12
Dystaenia ibukiensis plastid partial tRNA- Leu gene and partial IGS, isolate 11
Dystaenia ibukiensis plastid partial tRNA- Leu gene and partial IGS, isolate 10
Dystaenia ibukiensis plastid partial tRNA- Leu gene and partial IGS, isolate 9
Dystaenia ibukiensis plastid partial tRNA- Leu gene and partial IGS, isolate 8
Dystaenia ibukiensis plastid partial tRNA- Leu gene and partial IGS, isolate 7
Cymopterus globosus tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tR..
Harbouria trachypleura tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; a..
Podistera eastwoodiae tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and
Angelica archangelica tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and t..
Angelica sylvestris plastid partial tRNA- Leu gene and partial IGS
Musineon tenuifolium tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRNA- T...
Musineon divaricatum tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRNA..
Cymopterus williamsii tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; a...
Lomatium californicum tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRNA- ...
Cymopterus purpureus tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRN..
Lomatium nudicaule tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRNA- ...
Lomatium juniperinum tRNA- Phe gene, partial sequence; and tRNA- Leu gene, complete sequence; ..
Pteryxia terebinthina tRNA- Phe gene, partial sequence; and tRNA- Leu gene, complete sequence; chloropl..
Neoparrya lithophila tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRNA- Thr..
Aletes anisatus tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRNA- Thr gen...
Cymopterus nivalis tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRNA- Thr ..
Lomatium junceum tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and tRNA- ...
Lomatium graveolens tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; and
Lomatium dasycarpum tRNA- Phe gene, partial sequence; tRNA- Leu gene, complete sequence; a...
Hladnikia pastinacifolia
Tilingia ajanensis chloroplast trnL(UAA), trnF(GAA) genes for tRNA- Leu, tRNA- Phe, part..
Anthriscus cerefolium chloroplast tRNA- Leu (trnL) gene and trnL- trnF intergen..
Osmorhiza depauperata chloroplast tRNA- Leu (trnL) gene and trnL..
Osmorhiza berteroi x Osmorhiza occidentalis chlo..
Osmorhiza occidentalis chloroplast tRNA- Leu (tr..
Osmorhiza occidentalis chloroplast tRNA- Leu (tr..
Osmorhiza occidentalis chloroplast tRNA- Leu (tr..
Osmorhiza occidentalis chloroplast tRNA- Leu (tr..
Myrrhis odorata chloroplast tRNA- Leu (tr..
Osmorhiza depauperata chloroplast tR...
Osmorhiza mexicana subsp. b...
Osmorhiza aristata chloroplast tRNA..
Osmorhiza aristata chloroplast tRNA..
Raukaua anomalus tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Schefflera staufferana voucher McPherson 17219 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera sp. Madagascar voucher Lowry 5158 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera lukwangulensis tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Schefflera volkensii voucher Phillipson 5129 tRNA- Leu (trnL) gene, partial sequence; trnL- trnF intergenic spacer, complete sequence; and tRNA- Phe (trnF) gene, partial sequence; chloroplast
Schefflera myriantha tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Cussonia thyrsiflora tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Pentapanax fragrans tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer, partial sequence; chloroplast
Arthrophyllum mackeei chloroplast tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer region, partial sequence
Aralia nudicaulis chloroplast tRNA- Leu (trnL) gene and trnL- trnF intergenic spacer region, partial sequence