Arctic-alpine and serpentine differentiation in polyploid *Potentilla crantzii*

Arkto-alpinská a hadcová diferenciace polyploidního druhu *Potentilla crantzii*

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Paule J., Kolář F. & Dobeš C. (2015): Arctic-alpine and serpentine differentiation in polyploid *Potentilla crantzii*. – Preslia 87: 195–215

The link between polyploidy and the disjunct arctic-alpine European distribution of *Potentilla crantzii* was studied with particular reference to the role of serpentine habitats. Flow cytometry, AFLPs and cpDNA sequencing provided insights into ploidy level variation and the genetic structure of European populations. We recorded a ploidy differentiated arctic-alpine disjunction with tetraploids limited to the central- and southern-European mountain chains and hexaploids dominating in the Subarctic. Two lowland serpentine populations in the Czech Republic and Austria exhibited contrasting genetic patterns suggesting different evolutionary histories, with the tetraploid Czech population showing a conspicuously high genetic diversity. Finally, our genetic and cytological data did not support a distinct taxonomic status for the serpentine populations that were traditionally differentiated as *P. crantzii* subsp. *serpentini*.

K e y w o r d s: AFLP, cpDNA, polyploidy, *Potentilla*, *Rosaceae,* serpentine

Introduction

Pleistocene glacial cycles had a major effect on the distribution of the European flora (Hewitt 1999, Médail & Diadema 2009), especially on taxa adapted to high altitudes (Tribsch 2004) or latitudes (Wolfe 1980). Glacial periods increased habitat connectivity for these taxa and promoted wider distributions in a broad zone called the periglacial belt. During the interglacial periods cold-adapted taxa retreated towards the Arctic and high mountains, leading to the currently observed arctic-alpine disjunctions (e.g. *Arabis alpina*, *Veronica alpina*, *Ranunculus glacialis*; Schmitt et al. 2009).

Polyploidy is assumed to have played an important role in plant diversification during Pleistocene glacial cycles. It is usually associated with alterations in the genetic and genomic properties inherited from diploid ancestors (Soltis & Soltis 2000, 2009, Wendel 2000, Osborn et al. 2003, Kearney 2005) which potentially lead to physiological, developmental, anatomical and/or morphological changes of ecological relevance (Levin 1983, te Beest et al. 2011). Polyploids were thus reported to have spread more efficiently

than diploids into the new environments available after glacial retreat, which is thought to have led to the abundance of polyploids in both arctic and alpine floras (e.g. Löve 1953, Brochmann et al. 2004).

The commonly observed diploid-polyploid ecogeographic contrasts could have been further altered by environmental conditions unaffected by climatic oscillations, such as extreme edaphic conditions. A good example are nearly toxic serpentine soils (poor in certain nutrients, rich in heavy metals and with an extremely low Ca/Mg ratio; Brady et al. 2005, Kazakou et al. 2008), which are geographically widespread and have strong ecological effects on plant life. Serpentines can influence plant evolution in two principal ways: (i) as an ecological filter allowing only (pre)adapted genotypes to enter, possibly enhancing selective pressures for development of reproductive barriers and, finally, triggering peripatric speciation (e.g. McNeily & Bradshaw 1968, Macnair & Gardner 1998, Rajakaruna 2004) and (ii) as a refugium providing a suitable environment for many plants with reduced competition (e.g. *Cerastium alsinifolium*; Vít et al. 2014, or many representatives of Californian serpentine flora; Anacker et al. 2011). Despite increasing numbers of studies on serpentine ecology and biogeography published in the last decade, information on the role of serpentine refugia in shaping the genetic structure of European plants is extremely scarce. The few studies available have shown that serpentine sites can either harbour unexpected cryptic taxonomic diversity (distinct serpentine lineages of *Knautia arvensis*, Kolář et al. 2012), host locally adapted ecotypes differentiated from the surrounding populations (*Cerastium alpinum*; Nyberg Berglund et al. 2004), or genetically non-differentiated populations of a preadapted plant species (*Silene dioica*; Westerbergh & Saura 1992).

A species exhibiting an alpine-arctic distribution with additional serpentine occurrences is *Potentilla crantzii* (Crantz) Beck ex Fritsch. *Potentilla crantzii* is widely distributed in the alpine zone of the Eurasian mountains (the Pyrenees, the Alps, the Carpathians, Caucasus, the Urals, and Central-Asian mountains) and the Subarctic (northern Europe, eastern Canada and Greenland: Meusel et al. 1965), and exhibits conspicuous ploidy variation (e.g. Müntzing 1958, Skalinska & Czapik 1958, Smith 1963, Smith et al. 1971, Dobeš & Vitek 2000; see Electronic Appendix 1) with hexaploids ($x = 7$; $2n = 6x = 42$) dominating in Scandinavia and Scotland and tetraploids ($2n = 4x = 28$) in central Europe. In addition, hexaploids are reported in the Pyrenees, the Alps and the Tatra Mts (Walters 1968, Asker 1985, Delgado et al. 2000; see Electronic Appendix 1). Ploidy variation appears to be accompanied by a differentiation in reproductive mode: pseudogamous apomixis (i.e. asexuality requiring pollination to initiate seed formation) is documented for hexaploids in northern Europe (Håkansson 1946, Müntzing 1958, Smith 1963), whereas tetraploids from the Tatra Mts reproduce via regular sexuality (involving female meiosis and a sexual origin of the embryo; Czapik 1961, 1962).

Two geographically isolated local occurrences in the lowlands of central Europe are reported for *P. crantzii*, namely from serpentine outcrops close to Bernatice in the central part of the Czech Republic and from the large serpentine body around Bernstein in Austria (e.g. Neumayer 1930, Soják 1960). In contrast to the alpine-arctic populations inhabiting alpine grassland and open tundra, these populations grow in the understory of open pine forests together with numerous plant species that are considered relicts from low-competitive environments of the glacial and early Holocene periods (Kobrle 1964, Punz et al. 2010). Based on the ecological distinctness and peculiar morphological traits (narrow

stipule auricules, small flowers and frequent presence of glandular hairs), the serpentine populations were distinguished as *P. crantzii* subsp. *serpentini* (Borbás) Hayek (Soják 1960, 1995).

Focusing on European populations we investigated the geographic distribution of *P. crantzii* cytotypes on a continental scale and estimated the role of serpentine habitats as refugia of distinct postglacial diversity. In addition, we discuss the taxonomic status of serpentine populations. For that purpose we assessed ploidy variation and the genetic diversity as well as differentiation of European populations inferred from amplified fragment length polymorphisms (AFLP) and chloroplast DNA sequences.

Material and methods

Plant material

Plant material was collected from 38 populations in order to cover European distribution of *P. crantzii*. In addition, one population was obtained through the Index Seminum seed exchange, one from the Botanical Garden in Tatranská Lomnica and five individuals from the herbarium MA. The individuals sampled were at a distance of at least 5 m from each other. A total of 186 individuals from 45 populations were investigated, with 1–10, but mostly 4–5 individuals per population (Electronic Appendix 2). Herbarium vouchers are deposited in HEID, W and PRC herbaria (Electronic Appendix 2). ArcGIS v10.1 (ESRI, USA) was used for construction of the distribution maps.

Chromosome counts and DNA ploidy

The DNA ploidy was determined using flow cytometry and the Partec Ploidy Analyser (Partec, Münster, Germany) in the IPK, Gatersleben, and at the Department of Pharmacognosy, University of Vienna, the Partec Ploidy Analyser II in the Department of Botany, University of South Bohemia, České Budějovice, and the Partec ML equipped with UV-LED lamp in the Department of Botany, Charles University, Prague. One fresh petiole or part of a silicagel-dried leaf blade was prepared according to the two-step (Otto) protocol (Doležel et al. 2007) with the internal standard (*Lycopersicon esculentum* L. cv. 'Stupické polní tyčkové rané': Doležel & Bartoš 2005). The fluorescence of at least 3000 particles was scored. Sample/standard fluorescence ratios were calculated from the means of the sample and standard fluorescence histograms, and only those with coefficients of variation (CVs) < 5% for the G_0/G_1 peak of the sample were considered. Three individuals of different ploidy levels for which the chromosome numbers had been counted (Dobeš et al. 2012, Table 1, Electronic Appendix 2) served as a reference for estimating DNA ploidy. The DNA ploidy was attributed based on the regression of sample/standard fluorescence ratios against the ratios of individuals whose chromosome number was known. In order to assure repeatability several replicate measurements of fresh and silica-gel dried samples were performed in different laboratories.

DNA extraction, cpDNA amplification and sequencing

Total DNA was isolated from silica gel-dried leaf tissue as well as from five herbarium specimens using the procedure described in Dobeš & Paule (2010). The plastid

*trn*H(gug)-*psb*A intergenic spacer was amplified using the primers *trn*H(gug) 5'-CGC GCA TGG TGG ATT CAC AAT CC-3' and *psb*A 5'-GTT ATG CAT GAA CGT AAT GCT C-3' (Shaw et al. 2005). PCR reactions were performed as described in Paule et al. (2011). The cycle sequencing was accomplished on both strands. All sequences were edited and a consensus of forward and reverse sequences was made using the software SeqMan v4.0 (DNASTAR, USA).

AFLP analysis

A subset of 66 individuals from 13 populations covering the Balkans, central Europe, Alps and Subarctic was investigated by means of AFLP using 3–5, but mostly 5 individuals per population (see Table 1, Electronic Appendix 2). The AFLP analyses were performed using the protocol established by Vos et al. (1995) with the few modifications described in Paule et al. (2011). Approximately 300 ng of DNA was digested and ligated using MseI and EcoRI enzymes (New England Biolabs, USA), and EcoRI- and MseI-adapters. The restriction-ligation product was amplified in a pre-selective PCR using EcoRI-A primer (5'-GAC TGC GTA CCA ATT CA-A-3') and MseI-C primer (5'-GAT GAG TCC TGA GTA AC-C-3') with final selective amplification of the pre-selective product using three combinations of differentially fluorescence-labelled primers [EcoRI-AGG (TET)/MseI-CTC, EcoRI-AAC (6-FAM)/MseI-CTT, EcoRI-AGC (HEX)/MseICTG]. Three differentially fluorescent labelled PCR products of the same sample were multiplexed and diluted and the fragments separated on a MegaBase 500 DNA capillary-sequencer together with an ET-ROX 550 size standard (Amersham Biosciences, USA). In each run, a total of 48 samples were analysed, including one standard sample applied to each run, one negative control, one repeat within the run and several additional repeats (altogether 6% of replicated samples). Raw data were visualized and the fragments manually scored using GeneMarker v1.8 (SoftGenetics, USA). Processed data were exported as a presence/absence matrix.

Data analyses

The DNA sequences were aligned using ClustalX v1.83 (Thompson et al. 1997) and the alignments manually refined using GeneDoc v2.7 (Nicholas et al. 1997). Indels were manually coded for presence and absence. Phylogenetic relationships among the cpDNA haplotypes were reconstructed using the haplotype network analysis as implemented in TCS v1.2 (Clement et al. 2000) with a default connection limit of 95%. Haplotype diversity (h; Nei & Tajima 1983) and nucleotide diversity (π; Nei 1987) of populations were calculated using DnaSP v5.10.01 (Librado & Rozas 2009). Indels were treated as single polymorphic sites.

For the AFLP analyses the following measures were computed using the R-script AFLPdat [Ehrich 2006; R v2.13.0 environment (R Development Core Team 2011)] for the whole dataset, the majority cytotypes and geographic groups: total number of fragments, proportion of polymorphic fragments, number of private fragments and proportion of shared fragments.

The effective number of AFLP genotypes (Nb_{eff}) and Nei's genotypic diversity (D_g) (Nei 1987) was established for each population using the software GenoDive v2.0b25 (Meirmans & van Tienderen 2004). The software allows entering a threshold/error rate, defined as the number of band differences between two individuals as estimated from the replicates (i.e. technical difference rate).

Table 1. – Overview and collection history of the 45 populations of *Potentilla crantzii* studied. Substrate: serpentine (S) or non-serpentine (NS), Nb: number of individuals analysed for DNA ploidy/AFLP/cpDNA, 2n: somatic chromosome number with the number of individuals examined in parentheses.

Population	Locality	Coordinates	Substrate	DNA ploidy	Nb	2n
Pop076	AUT, Nordtirol, Nördliche Kalkalpen, along the ascent from Lähn to Daniel, at Hebertaljoch, CD, 13. 7. 2006	N 47.4372, E 10.8569	NS	7x	5/0/5	–
Pop079	AUT, Nordtirol, Ötztaler Alpen, Ötztal, Zwieselstein, meadows E of the village, CD, 14. 7. 2006	N 46.9388, E 11.0313	NS	6x	1/0/0	–
Pop080	AUT, Nordtirol, Ötztaler Alpen, Rotmoostal S of Obergurgl, CD, 14. 7. 2006	N 46.8384, E 11.0326	NS	–	0/0/5	–
Pop082	ITA, Südtirol, Tuxer Alps, between Riederbergalm and Rollspitze, CD, 15. 7. 2006	N 46.9336, E 11.4813	NS	6x, 7x	7/0/2	42 (1)
Pop109	ITA, Lombardia, at the road in Passo del Vivione, CD, 22. 7. 2006	N 46.0422, E 10.218	NS	4x	1/0/4	–
Pop110	ITA, Lombardia, Presolana, Passo della Presolana, CD, 24. 7. 2006	N 45.9303, E 10.1	NS	4x	1/0/5	–
Pop130	NOR, Troms, Kafjord, Ankerlia, R. Hiltunen et al., 15. 9. 2005 (Index Seminum ex BG Univ. Oulu)	N 69.4333, E 20.9667	NS	6x	4/3/2	–
Pop184	SLO, Julijske Alpe, Mangart saddle, below Mangart summit, JP, 7. 9. 2006	N 46.4452, E 13.6418	NS	4x	6/0/4	28 (1)
Pop209	ITA, Marche, Monte Catria, Fossato – Cagli, CD, 2. 6. 2007	N 43.4678, E 12.7031	NS	–	0/0/5	–
Pop215	ITA, Abruzzo, along ascent from cable car station to Campo Imperatore at Monte Portella, CD, 4. 6. 2007	N 42.4479, E 13.5539	NS	4x	1/0/5	–
Pop244	FRA, Department Alpes-de-Haute-Provence, Between Grand Coyer and Sommet du Carton, CD, 13. 7. 2007	N 44.1143, E 6.693	NS	4x	6/5/5	–
Pop251	FRA, Department Hautes-Alpes, Queyras, next to the road at Col d'Agnel, CD, 16. 7. 2007	N 44.6981, E 6.9495	NS	4x	1/0/5	–
Pop255	FRA, Department Hautes-Alpes, Plateau d'Emparis, between Lac Noir und Lac Lerié, CD, 17. 7. 2007	N 45.0474, E 6.2302	NS	5x, 8x	6/5/5	35 (2)
Pop259	NOR, Svalbard, Svalbard, Ny Allesund, Ossian Sarsfjellet, area around the bird cliff towards the north along the coast, A. Tribsch, 17. 8. 2007	N 78.9283, E 12.4481	NS	6x	1/5/5	–
Pop295	ISL, Iceland, Akureyri, Krókárgerdisfjall, K. B. Westergaard & T. Dahl, 20. 7. 2007	N 65.4167, W 18.8833	NS	6x	2/5/4	–
Pop315	CHE, Graubünden, Engadin, Silvretta, Muot da l'Horn, on a ridge SE of Alp Laret, CD, 16. 6. 2008	N 46.8197, E 10.2178	NS	6x	10/0/0	–
Pop326	CHE, Uri, SE of Klausenpass, CD, 4. 7. 2009	N 46.8517, E 8.8601	NS	4x	9/0/0	–
Pop373	AUT, Burgenland, Bernstein, serpentine forest along the road from Kalkgraben at the southern margin of the town, 0.5 km SE of the church in Bernstein, open pine forest, FK, 23. 6. 2008	N 47.4014, E 16.2629	S	4x	5/5/9	–

Population	Locality	Coordinates	Substrate	DNA ploidy	Nb	2n
Pop374	AUT, Oberösterreich, Ebensee, along tourist path Offensee - Wildensee, 2 km NNW from the Wildensee lake, 10 km S of Ebensee, alpine rocky outcrops, FK, 20. 6. 2009	N 47.7278, E 13.8442	NS	4x	6/5/6	–
Pop375	CZE, Středočeský kraj, Bernatice, pine forest next to the highway bridge, 2 km NW of the village, serpentine soil, open pine forest, FK, 4. 8. 2008	N 49.6888, E 15.1034	S	4x	5/5/9	–
Pop376	DEU, Bayern, Berchtesgaden, rocks along the tourist path to Hoher Goll peak, 0.5 km SSW of Purtscheller Haus mountain hut, alpine rocky outcrops, FK, 26. 6. 2008	N 47.6052, E 13.0697	NS	4x	2/4/6	–
Pop377	FRA, Hautes-Alpes, Col D'Izoard, 1 km N of the saddle, alpine rocky outcrops, FK, 23. 7. 2009	N 44.8236, E 6.739	NS	4x	5/5/5	–
Pop378	FRA, Haut-Rhin, Vosges, Hohneck mountain, glacial cirque SE of the top, glacial cirque, JP, 2009	N 48.0357, E 7.0184	NS	4x	1/0/3	–
Pop379	FRA, Hautes-Alpes, Villar D'Aréne, Romanche valley, 5 km SE of the village, alpine rocky outcrops, FK, 20. 7. 2009	N 44.9900, E 6.3800	NS	7x	1/0/0	–
Pop380	ISL, Eskifjörður, old calcite mine at Helgustadir, arctic tundra, FK, 7. 7. 2012	N 65.0333, W 13.8529	NS	6x	1/0/0	–
Pop381	ISL, Hvammstangi, along road 711, N of the town, arctic tundra, FK, 10. 7. 2012	N 65.5826, W 20.9238	NS	6x	1/0/0	–
Pop382	ISL, Hveragerði, along the path to the thermal brook, N of the town, arctic tundra, FK, 4. 7. 2012	N 64.0571, W 21.2186	NS	6x	1/0/0	–
Pop383	MNE, Durmitor, Žabljak, polje of the Zeleni Vir lake, 0.5 km SE of the Bobotov Kuk peak, alpine rocky outcrops, FK, 23. 8. 2008	N 43.1192, E 19.0374	NS	4x	2/5/5	–
Pop384	MNE, Prokletije, Gusinje, calcareous rocks at the saddle 0.5 km SW of mountain Sapica, 7 km SE of the town, alpine rocky outcrops, FK, 20. 8. 2008	N 42.5049, E 19.8952	NS	4x	5/5/5	–
Pop385	NOR, Dovrefjell, Dombas, tourist path 1 km E Kongsvoll, rocky outcrops, FK, 17. 7. 2008	N 62.2978, E 9.6052	NS	6x	2/4/6	–
Pop386	NOR, Hardangervidda, Hardangervidda, rocks at the W margin of Hellevasdallen valley, rocky outcrops in tundra, FK, 22. 7. 2008	N 59.998, E 7.1296	NS	6x	3/5/6	–
Pop387	NOR, Sor-Trondelag, Trollheimen, rocky outcrop above the saddle N of the western end of Gjevillvatnet lake, mountain tundra, FK, 15. 8. 2011	N 62.7329, E 9.1808	NS	6x	1/0/0	–
Pop388	NOR, Oppland, Jotunheimen, Øvre Leirungen valley 4 km SE of Gjendesheim chalet, open rocky grassland in tundra, FK, 10. 8. 2013	N 61.4782, E 8.7447	NS	6x	1/0/0	–
Pop389	GBE, Scotland, Tullybannocher, small gorge N of Loch Lednoch reservoir, alpine rocky outcrops, FK, 25. 7. 2011	N 56.4431, W 4.0653	NS	7x	1/0/0	–

Population	Locality	Coordinates	Substrate	DNA ploidy	Nb	2n
Pop392	ITA, Südtirol, Wolkenstein, 0.2 km S of castle ruins, rocks, walls, CD, 4. 9. 2010	N 46.5613, E 11.7679	NS	4x	1/0/0	–
Pop393	ITA, Südtirol, Dolomites, Col Raiser, close to the Regensburger Hütte, Semperviretum, CD, 4. 9. 2010	N 46.5875, E 11.7583	NS	4x	1/0/0	–
Pop396	SVK, Prešovský kraj, Botanical Garden, Tatranská Lomnica ex Belianske Tatry, Tristarská dolina, JP, 18. 7. 2007	N 49.2500, E 20.2000	NS	7x	2/0/3	–
Pop397	ESP; Palencia, Piedrasluengas, herb. MA631874	N 43.0367, W 4.4579	NS	–	0/0/1	–
Pop398	AND; Orillas del rio Valira, Bordas de Envalira, herb. MA514288	N 42.5529, E 1.6845	NS	–	0/0/1	–
Pop399	ESP; Gerona, Tosses, Niu d'Iiga, herb. MA529127	N 42.3208, E 1.8835	NS	–	0/0/1	–
Pop400	ESP; Huesca, Barranco de Montinier, herb. MA544750	N 42.6158, E 0.1835	NS	–	0/0/1	–
Pop401	ESP; Navarra, Ochagavía, Orhy desde Puerte Larraun, herb. MA544776	N 42.9752, W 1.001	NS	–	0/0/1	–
Ptl3716	ITA, Südtirol, Sanntaler Alpen, along the way from Penserjoch to Täschspitze, CD, 16.7.2006	N 46.8077, E 11.4746	NS	6x	1/0/0	–
Ptl3718	ITA, Südtirol, 0.3 km SW of Peitlerscharte/Forcela de Pütia (St. Martin in Thurn), A. Hilpold, 15. 7. 2006	N 46.6467, E 11.8097	NS	6x	1/0/0	–
Ptl4390	ITA, Lombardia, Pizzo Camino, at the ascent from Schilpario to Passo Ezendola, CD, 23. 7. 2006	N 45.9992, E 10.1876	NS	4x	1/0/0	–

Phylogenetic relationships among genotypes (in the sense of AFLP phenotype as used in the following) were visualized using Neighbor-Net analysis implemented in SplitsTree4 v4.11.3 (Huson & Bryant 2006) based on Jaccard genetic distances. In addition, main trends in genetic variation among individual genotypes were visualized by principal coordinate analysis (PCoA, based on Jaccard distances) calculated using PAST v2.7 software (Hammer et al. 2001).

Genetic admixture analysis using the program Structure v2.3.3 (Pritchard et al. 2000) was applied to identify genetic clusters. Structure implements a model-based Bayesian clustering algorithm for estimating the likelihood of different numbers of genetic clusters (K). The admixture model with both independent and correlated allele frequencies and recessive allele coding, as implemented in Falush et al. (2007), was used. Polyploids were coded as proposed by Stöck et al. (2010). Each individual was coded as a formal octoploid (highest degree of ploidy present in the dataset), assigning the missing chromosome sets for other ploidy levels as "missing data" (-9). The data were tested with K ranging from 1–6, with 20 replicate runs for each K, and a burn-in period of 5×10^4 and 10×10^4 iterations. In order, to find the most probable value of K, the Structure output files were analysed using Structure Harvester v0.6.92 (Earl & von Holdt 2012) and Evanno's delta K (Evanno et al. 2005) and the outputs of the repeated runs at each K were averaged using CLUMPP v1.1.1 (Jakobsson & Rosenberg 2007). Similarity coefficients

for each pair of the Structure runs with particular K were calculated using the R-script Structure-sum v2011 (Ehrich et al. 2007).

In order to independently confirm the results of the Structure analysis, we performed a non-model approach based on replicated non-hierarchical K-means clustering (Hartigan & Wong 1979) using the R-script of Arrigo et al. (2010). We performed 5×10^4 independent runs (i.e. starting from random points) for 28 clusters and recorded the inter-group inertia of each run. We used the inter-group inertia as a proxy of clustering accuracy and calculated the delta K values (Evanno et al. 2005) using the method adopted by Arrigo et al. (2010). The partition with the highest delta K was considered to be the most probable number of groups in our dataset.

Results

Flow cytometry

The DNA-ploidy was determined for 111 individuals from 38 populations (Table 1, Electronic Appendix 2). The CVs of the G_0/G_1 sample peaks ranged from 1.89 to 4.85 (mean 2.53±0.72) and reliable results were recorded for up to four-year old silica-gel dried samples. Five distinct classes of sample/standard fluorescence ratios were detected, which corresponded to tetra-, penta-, hexa-, hepta- and octoploidy (Table 1, Electronic Appendix 2). Tetra- and hexaploids were the most common cytotypes (53.2% and 31.5%, respectively). The geographic distribution of cytotypes identified in this study complemented previously published records as shown in Fig. 1. The tetraploids are restricted to alpine habitats of central and southern-European mountains as well as lowland serpentine habitats, while the hexaploids occur throughout the study area. Minority cytotypes (penta-, hepta- and octoploids) were almost exclusively restricted to the Alps, Scotland and Pyrenees, with additional occurrences of heptaploids in the Tatra Mts and Scandinavia.

CpDNA sequence data and haplotype distribution

The cpDNA sequences were obtained for 132 individuals (see Table 1, Electronic Appendix 2). The length of the *trn*H-*psb*A IGS ranged from 356 bp to 399 bp. Twenty nucleotide substitutions, 12 indels and six repeated sequence motifs (4 poly-A/poly-T stretches, two microsatellite motifs) were detected. The length of the alignment was 482 bp. After manual coding of the indels for the presence and absence and removal of the repeated sequence motifs, the total length of the alignment was reduced to 367 bp and 32 variable and 19 parsimony informative sites were considered. The sequences are deposited in the NCBI GenBank (see Electronic Appendix 2).

Nineteen *trn*H-*psb*A cpDNA haplotypes were identified and the TCS network analysis revealed two groups of haplotypes (Fig. 2) separated from each other by seven mutations. The first group consists of the majority of the haplotypes, the second group of haplotypes ZB, ZI, ZR and Q. Only two haplotypes (ZJ, ZM) are present in the Subarctic. Haplotype and nucleotide diversities of the populations studied are summarized in Table 2. Highest haplotype diversity was recorded for the tetraploid populations in the Balkans (Pop384), SW and SE alpine populations (Pop377 and Pop184) and the lowland serpentine population in the Czech Republic (Pop375). These populations showed also

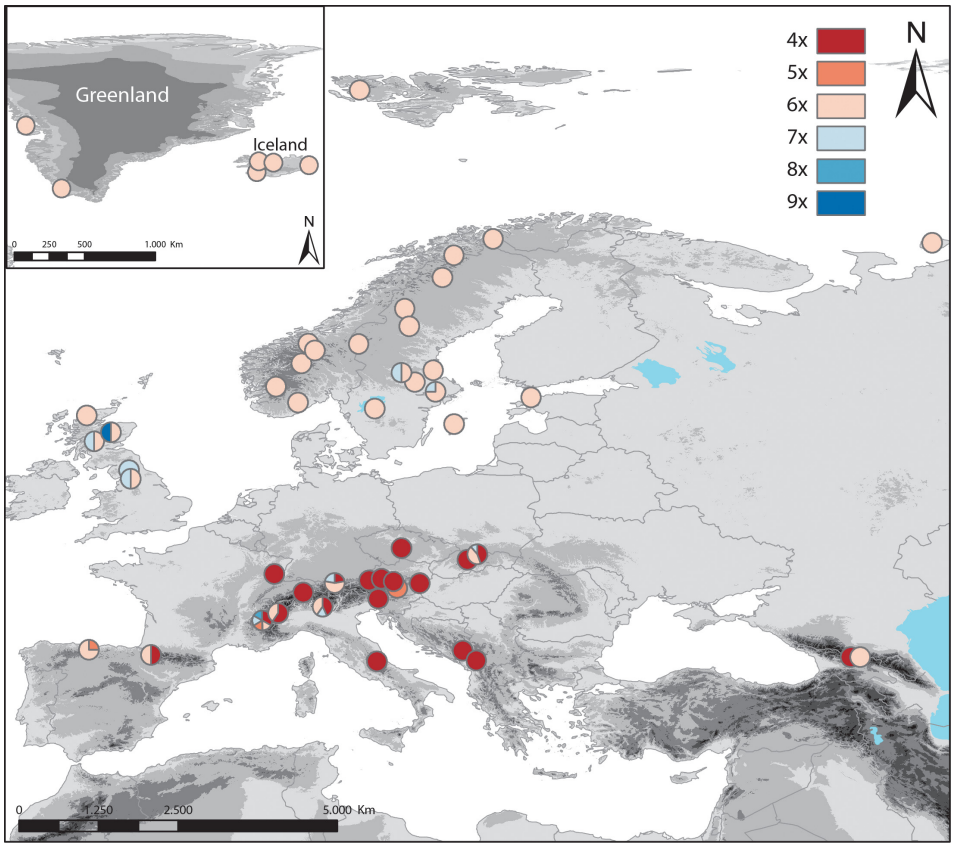

Fig. 1. – Map showing the distribution of cytotypes of *Potentilla crantzii* in Europe based on both newly acquired and previously published data (see Electronic Appendix 1 for a complete list). Pie charts are proportional to the frequency of a particular cytotype recorded in one population or in several closely adjacent populations (up to 65 km).

high levels of nucleotide diversity as did some alpine populations (Pop082, Pop110, Pop215). In contrast, zero diversity was recorded for the populations in the Subarctic except for Pop295 and Pop385, in which there are two haplotypes.

AFLP analyses

Three AFLP primer combinations resulted in 182 clearly scorable fragments sized from 63–537 bp, out of which 89.01% were polymorphic (Table 2). The repeatability (technical difference rate; Bonin et al. 2004) of replicated individuals was 95.60–98.35% (mean 96.70%).

The number of fragments per individual was higher in hexaploids (72–92) than in tetraploids (65–88; Wilcoxon rank sum test: $W = 166$, $P < 0.001$). However, the total number of fragments was higher in tetraploids (169 including 47 privates) compared to

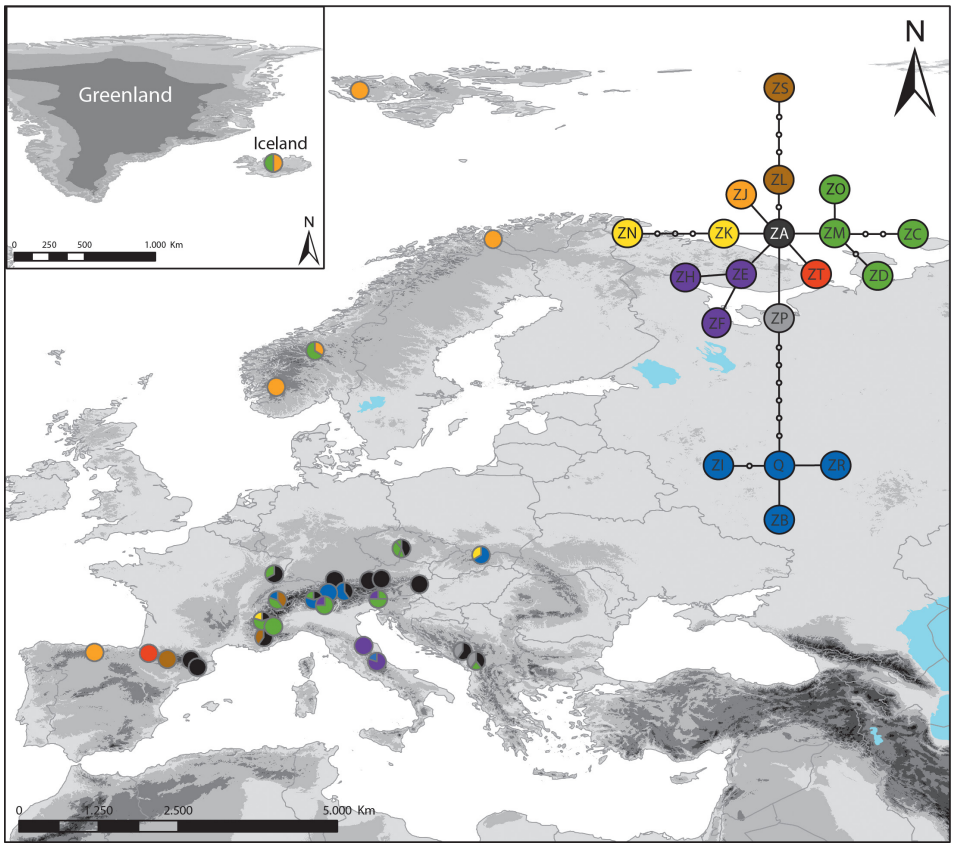

Fig. 2. – Statistical parsimony network based on *trn*H-*psb*A cpDNA sequences of *Potentilla crantzii* and distribution of the cpDNA haplotypes in Europe. Small empty circles represent haplotypes that are not present, but necessary to link all the haplotypes recorded to the network. All haplotypes are separated from the nearest haplotype by one mutation/indel.

hexaploids (133 including 11 privates). Moreover, 122 (67.78%) fragments were shared by both major haplotypes.

The major splits in the Neighbor-Net separated particular populations and identified the following geographic groups: the Balkans and central Europe (including the Austrian serpentine population Pop373), Eastern and Western Alps, Czech serpentine population and a subarctic group consisting of populations in Iceland and Norway (Fig. 3).

In the Structure analysis the highest ΔK value was assigned to K = 3 for analyses using both independent and correlated allele frequencies. Hence, those results based on only independent allele frequencies were considered further. Twenty structure runs produced nearly identical individual memberships, having pairwise similarity coefficients of above 0.98. The genetic clusters largely corresponded to: 1. subarctic populations, 2. central- and southern-European populations and 3. Pop384 in Montenegro (Fig. 4). Almost all individuals and populations assigned according to geography show no or marginal

Table 2. – Indices of haplotypic (cpDNA) and genotypic (AFLPs) diversity for populations of *Potentilla crantzii*. Nb: number of individuals, Nb_{haplo}: number of haplotypes, h: haplotype diversity (Nei & Tajima 1983) and π: nucleotide diversity (Nei 1987), FT: total number of fragments; FP: proportion of polymorphic bands; FPP: number of private fragments. Effective number of genotypes (Nb_{eff}) and Nei's genotypic diversity (D_g). *serpentine population.

Group	cpDNA					AFLP					
	Ploidy	Nb	Nb_{haplo}	h	π [%]	Nb	FT	FP [%]	FPP	Nb_{eff}	D_g
Total		132	19	0.828	0.869	66	182	89.01	–	36.92	0.98
Central- and southern-European		109	19	0.791	0.939	44	173	83.51	49	42.09	0.99
Subarctic		23	2	0.403	0.220	22	133	51.65	9	6.72	0.89
4x		75	13	0.747	0.707	39	169	82.22	47	39.00	1.00
6x Subarctic only		23	2	0.403	0.220	22	133	52.22	11	6.72	0.89
6x		28	4	0.590	0.717	22	133	52.22	11	6.72	0.89
Central- and southern-European											
Pop076	7x	5	1	0.000	0.000	–	–	–	–	–	–
Pop080		5	1	0.000	0.000	–	–	–	–	–	–
Pop082	6x/7x	5	2	0.600	1.471	–	–	–	–	–	–
Pop109	4x	4	2	0.500	0.681	–	–	–	–	–	–
Pop110	4x	5	3	0.700	1.798	–	–	–	–	–	–
Pop184	4x	4	3	0.833	1.045	–	–	–	–	–	–
Pop209		5	1	0.000	0.000	–	–	–	–	–	–
Pop215	4x	5	2	0.000	1.308	–	–	–	–	–	–
Pop244	4x	5	2	0.600	0.327	5	101	27.47	2	5.00	1.00
Pop251	4x	5	1	0.000	0.000	–	–	–	–	–	–
Pop255	5x/8x	5	3	0.700	0.708	5	119	28.57	3	3.57	0.90
Pop373*	4x	9	1	0.000	0.000	5	102	23.08	2	5.00	1.00
Pop374	4x	6	1	0.000	0.000	5	108	31.87	3	5.00	1.00
Pop375*	4x	9	4	0.750	0.333	5	108	30.77	3	5.00	1.00
Pop376	4x	6	1	0.000	0.000	4	95	22.53	2	4.00	1.00
Pop377	4x	5	3	0.800	1.471	5	105	30.22	5	5.00	1.00
Pop378	4x	3	2	0.667	0.182	–	–	–	–	–	–
Pop383	4x	5	2	0.600	0.163	5	109	32.42	3	5.00	1.00
Pop384	4x	5	3	0.800	0.272	5	113	44.51	8	5.00	1.00
Pop396	7x	3	2	0.667	1.635	–	–	–	–	–	–
Subarctic											
Pop130	6x	2	1	0.000	0.000	3	83	7.14	0	1.00	0.00
Pop259	6x	5	1	0.000	0.000	5	93	6.59	1	1.00	0.00
Pop295	6x	4	2	0.667	0.363	5	113	31.32	5	5.00	1.00
Pop385	6x	6	2	0.533	0.291	4	87	7.72	1	1.00	0.00
Pop386	6x	6	1	0.000	0.000	5	100	20.88	1	3.57	0.90

genetic admixture. However, individuals from the Icelandic population Pop295 were assigned either to the 2. cluster (Ptl7513, Ptl7514) or were admixed with the 2. and 1. cluster (10.29–47.83%). In addition, two individuals from the subarctic Pop386 exhibited a 9.94–13.90% admixture with the 2. cluster and the Czech serpentine Pop375 revealed 4.81 to 22.22% admixture with 1. cluster. Non-hierarchical K-means clustering largely supported the Structure results by confirming the northern and southern cluster (the latter including also the Pop384 in Montenegro). Only two populations included

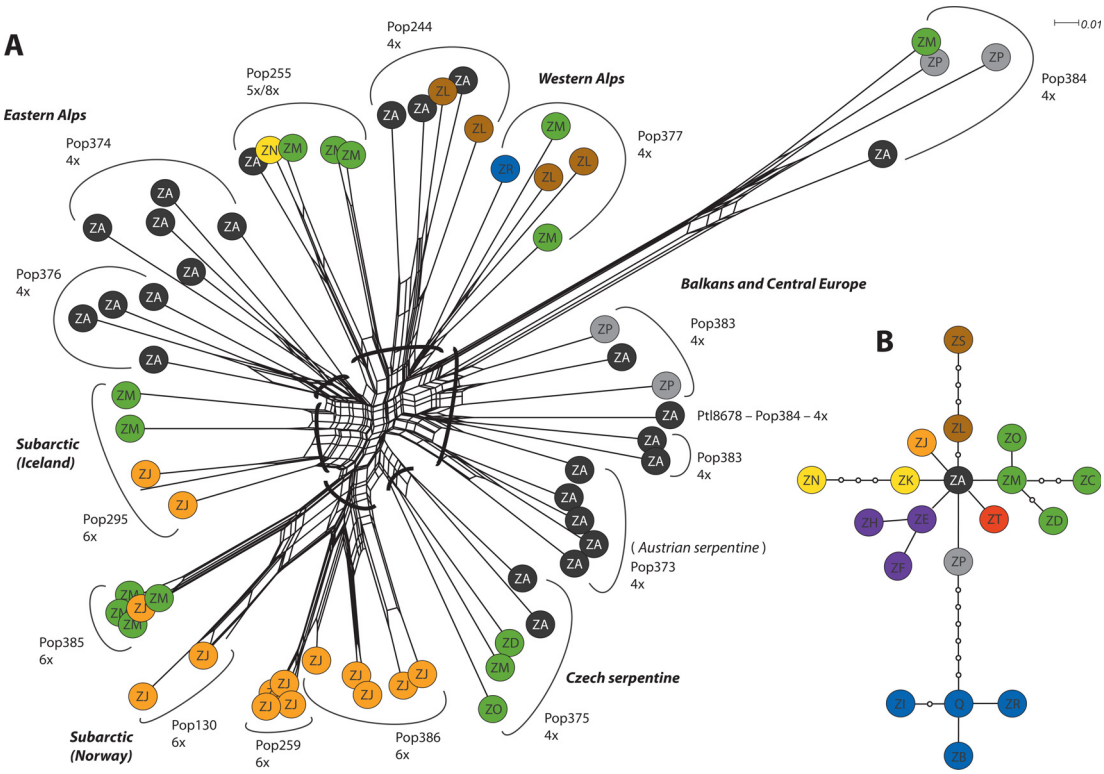

Fig. 3. – Phylogenetic relationships among individuals of *Potentilla crantzii* based on AFLP and cpDNA data. A. Neighbor-Net dendrogram based on Jaccard distances among AFLP genotypes with chloroplast haplotypes depicted on corresponding branch tips. The scale bar indicates genetic distance. Bold lines indicate geographic groups written in bold italics. B. Statistical parsimony network based on *trn*H-*psb*A cpDNA sequences.

individuals assigned to both main K-means clusters: Icelandic population Pop295 and Czech serpentine Pop375 with individuals corresponding to those strongly admixed in the Structure analysis (Fig. 4).

Due to its high genetic distinctness, Pop384 was excluded from the two-dimensional PCoA. The first axis (explaining 15% of the total variability) separated the subarctic and central- plus southern-European populations, except for the Czech serpentine population (Pop375) and Icelandic Pop295 which were intermediate between the two major geographical groups (Fig. 5).

Based on data repeatability (i.e. technical difference rate) a threshold of eight AFLP bands (i.e. 4.4% of the total number of AFLP loci) was inferred. Even though a relatively high threshold was used, the genotypic diversity of all tetraploid populations studied was high. In contrast, the genotypic diversity of the majority of the hexaploid populations was zero (Table 2), except population Pop295 (Nb_{eff} = 5.00, D_g=1.00) in Iceland and Pop386 (Nb_{eff} = 3.57, D_g= 0.90) in Norway.

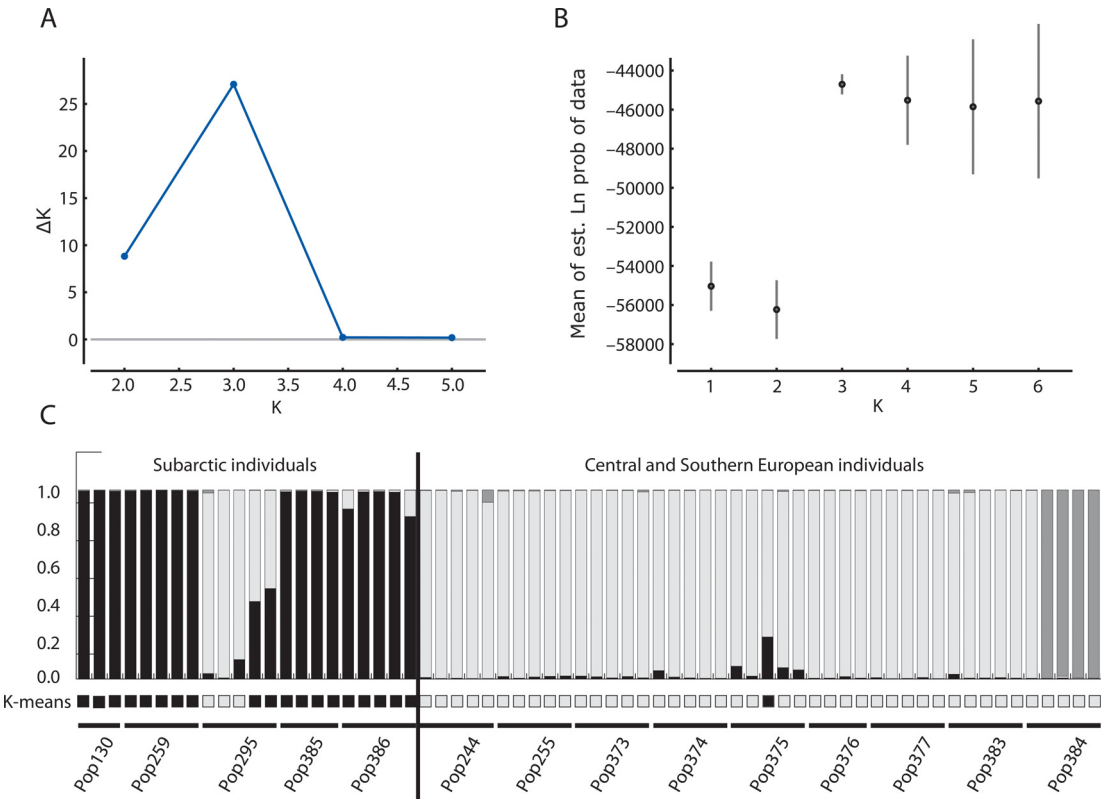

Fig. 4. – Grouping of AFLP genotypes of 66 individuals of *Potentilla crantzii* based on two independent approaches: Bayesian clustering using Structure and non-hierarchical K-means clustering. A. The ΔK values for Ks ranging from 1 to 6. B. Mean Ln likelihood of K values. C. Cluster membership of individuals for K = 3 using Structure (bars) and K = 2 using K-means clustering (squares).

Discussion

Strength and limitations of the statistics used for analysing the genetic data of polyploids

Inferring genetic structure of populations differing in ploidy level presents numerous methodological and analytical challenges (e.g. uncertain genotype inference, mixed inheritance patterns, missing population genetic models; see Dufresne et al. 2014, for a review). Nevertheless, if the potential caveats are kept in mind, a combination of several independent marker systems and different analytical approaches could provide useful insights into the evolution of polyploid complexes. We used a maternally inherited marker (plastid DNA) together with largely nuclear AFLPs, a combination commonly used in phylogeography of polyploid systems (Burnier et al. 2009, Arrigo et al. 2010, Kolář et al. 2012, 2015, Greiner et al. 2013, Marques et al. 2014). Plastid DNA markers, providing only a partial (usually maternal, Reboud & Zeyl 1994) genealogy, are not affected by ploidy level per se. The bi-parentally inherited AFLPs, despite their limited

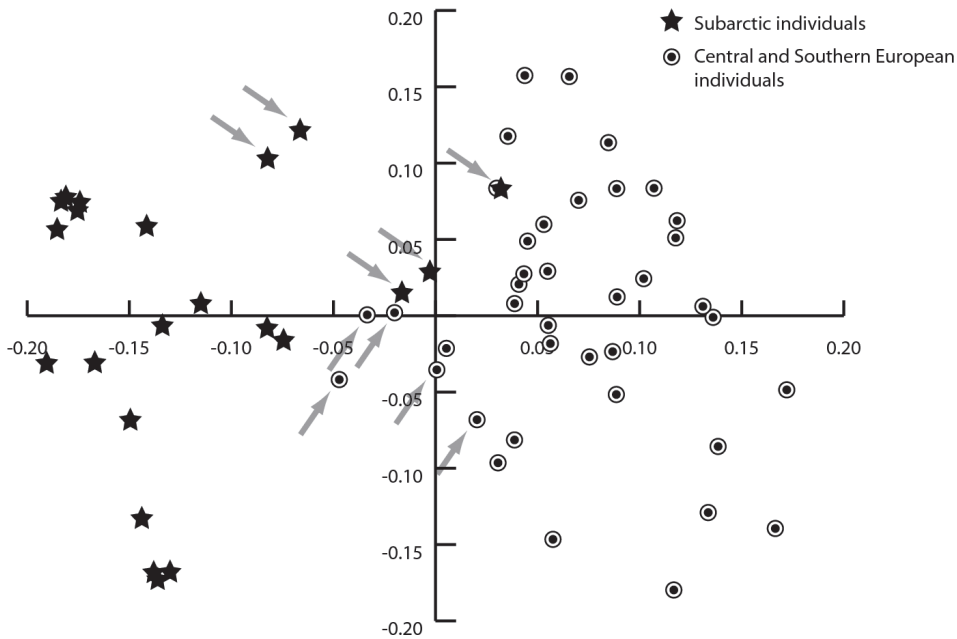

Fig. 5. – Principal co-ordinate analysis (PCoA) of AFLP genotypes of *Potentilla crantzii*. The first two axes explained 15.23% and 9.39% of the total variation, respectively. Arrows denote the lowland serpentine population in the Czech Republic (Pop375) and subarctic population in Iceland (Pop295). The genetically highly distinct tetraploid Pop384 (Montenegro) was excluded from this analysis.

informativeness due to their dominant character, are considered adequate for reconstruction of evolutionary relationships of polyploid complexes mainly because (i) they provide numerous independent markers (up to several hundreds) randomly distributed throughout the entire genome, (ii) the standard analytical tools for AFLP data do not rely on complete genotype reconstruction and (iii) the fingerprints can be simultaneously generated for individuals of different ploidy levels (Meudt & Clarke 2007, Dufresne et al. 2014).

In order to minimize the potential of a methodological bias caused by using a single analytical tool, we reconstructed the AFLP genetic relationships of the populations studied using several independent methods. Except for Structure (see below), we only used analyses that did not include assumptions of population genetics (K-means clustering, distance-based approaches). The genetic distance between individuals was calculated using the Jaccard dissimilarity index that reflects the proportion of fragments shared among individuals and is suggested for dominant polyploid data (Kosman & Leonard 2005). In addition, in the model-based Structure analysis the data were coded in a way suitable for polyploidy datasets i.e. as ambiguous genotypes according to Stöck et al. (2010) (see also Structure documentation). We further checked for the potential effect of violation of the main population genetic assumption of Structure (within-group HW equilibrium) in our data by comparing to results of the non-model-based clustering approach (non-hierarchical K-means clustering) and the grouping was very similar (Fig. 4). In fact,

all the approaches used similarly reveal a major separation among the majority of the subarctic vs the remaining (mostly alpine) populations and intermediate positions of the Icelandic (Pop295) and the Czech serpentine populations (Pop375) (see Figs 3, 4, 5). Finally, bearing in mind the potential pitfalls of population genetics of polyploids, we will further discuss only the overall genetic structure in order to provide a first outline of the relationships among the serpentine vs non-serpentine and alpine vs (sub)-arctic populations of *P. crantzii*.

Latitudinal and genetic differentiation

The distribution of *P. crantzii* indicated a ploidy differentiated arctic-alpine disjunction with tetraploids limited to central- and southern-European mountain chains and hexaploids in the Subarctic (Fig. 1). In addition, hexaploids occur in the Alps and Carpathians forming both cytologically uniform populations (Pop315) as well as populations mixed with minority cytotypes (Pop082) (Electronic Appendix 2). *Potentilla crantzii* thus has an intraspecific ploidy-disjunct distribution, which is comparatively rare (but see e.g. *Vaccinium uliginosum*, *Juncus biglumis*, *Arabis alpina*; Ehrich et al. 2007, Eidesen et al. 2007, Schönswetter et al. 2007). Interestingly, similar allopatric ploidy differentiation on smaller scale has been already observed for the closely related *P. rigoana* on the Apennine peninsula (Dobeš et al. 2013c). In contrast, of the numerous ploidy differentiated arctic-alpine disjunctions, the majority are linked to speciation and the cytotypes from the disjunct areas are recognized as separate species (e.g. *Veronica alpina* complex, *Primula*, Albach et al. 2006, Guggisberg et al. 2006).

Even though the genetic data might be biased due to the small number of populations and small population sizes studied in the Subarctic, some general patterns were found. Subarctic populations are genetically (haplotype, AFLP genotype, Table 2) and cytologically (almost exclusively hexaploid, Fig. 1) markedly impoverished. In the Subarctic there is only a subset (2/19, Fig. 2) of the haplotypes present in central- and southern-European mountain chains and just 11 private AFLP fragments (Table 2). Particular populations revealed zero haplotype, nucleotide and genotypic diversities (Table 2). Exceptions are populations Pop295 and Pop385 in which along with Pop386 there are two different haplotypes and a relatively high genotypic diversity.

In contrast, central- and southern-European populations are cytologically diverse, comprising both dominant and a number of minority cytotypes (Figure 1). The majority of private fragments (49) (Table 2) occur in central- and southern-European populations, and this pattern is supported by the genetic distinctness of both groups (PCoA, Structure and K-means clustering analyses, Fig. 4, Fig. 5) with the exception of the intermediate position of the Icelandic (Pop295) and Czech serpentine populations (Pop375) (see Figs. 3, 4, 5). Unfortunately, due to limited resources, only a subset of the populations was studied using AFLPs. However, the highest haplotype and nucleotide diversities were recorded in the Balkan populations, Pop184 and Pop384, and on the western and southern slopes of the Alps (Pop377, Pop110, Pop082). In addition, the genotypic diversity of all tetraploid populations is high even though the haplotype and nucleotide diversities of Pop373, Pop374 and Pop376 are zero (Table 2).

High genetic diversity of populations in the unglaciated southern and low diversity of populations in glaciated northern Europe is a commonly observed pattern (reviewed by

Hewitt 2004), which resulted from oscillations in the Quaternary climate as a consequence of e.g. leading edge colonization by limited numbers of lineages and possibly also a strong selection for colonization ability (e.g. Ehrich et al. 2007). Interestingly, the geographic location of populations with high haplotype and nucleotide diversities is in glacial refugia (Balkans, Southern and Western Alps) identified for several other taxa (reviewed by Schönswetter et al. 2005). In addition, we also consider the Iberian Peninsula as a potential glacial refugium for *P. crantzii* as the most dominant subarctic haplotype (ZJ) was recorded in our study only in the Iberian population Pop397.

Genetic admixture, relatively high genotypic diversity (Table 2) of Pop295 and Pop386, high number of private fragments and intermediate position (Fig. 5) of Pop295 could be explained by interspecific hybridization. In the genus *Potentilla*, hybridization is common even among rather distantly related species (e.g. Paule et al. 2012, Soják 2012). Facultative apomixis was recorded in the subarctic *Potentilla* section *Niveae* whereby crosses between different taxa gave rise to higher numbers of sexually produced offspring than crosses within taxa (Nyléhn et al. 2003). Forms that are morphological intermediate with members of the *P. verna* aggregate are reported for *P. crantzii* in the Alps, Scandinavia (see Soják 1960) and Scotland (Smith et al. 1971) and possibly other hybrids exist in the Subarctic (Aiken et al. 2007). Past or present hybridization events can also explain the distinct genetic position of Balkan Pop384 and the presence of divergent haplotype group (haplotypes Q, ZB, ZI, ZR; separated by seven mutations) in central-European populations (Fig. 2, Electronic Appendix 2). Interestingly, haplotype Q was previously recorded also in *Potentilla argentea* (Paule et al. 2011). Unfortunately other taxa were not included in this study and therefore the effect of hybridization in this case is unknown.

The intraspecific ploidy differentiation and low genetic diversity in the Subarctic may coincide with reproductive differentiation. The hexaploids in northern Europe are pseudogamous apomicts (Håkansson 1946, Müntzing 1958, Smith 1963) and tetraploid Carpathian populations are sexual (Czapik 1961, 1962) as the Czech serpentine populations Pop375 [unpublished data from flow cytometric seed screen (FCSS) done using the method of Dobeš et al. 2013a]. However, for a better understanding of the link between ploidy and reproductive mode an extensive FCSS dataset is needed, covering especially the Alps, where pseudogamous apomixis is reported for *P. crantzii* individuals of unknown ploidy (Hörandl et al. 2011, Dobeš et al. 2015). This approach was successfully used in studies on *Potentilla puberula* Krašan (Dobeš et al. 2013b), for which tetraploids are mainly sexual and higher polyploids (penta- to octoploid) apomictic.

Role of serpentine populations in preserving the genetic diversity of Potentilla crantzii

The two lowland serpentine populations, for which the same number of individuals was studied, have remarkably contrasting genetic patterns, which indicate different evolutionary histories. The Austrian serpentine population (Pop373) appears to be a spatially isolated outpost of the central-European populations as it has a low haplotype diversity (only a single widespread haplotype, ZA; Fig. 3) and clusters with the central- and southern-European populations. In contrast, the Czech serpentine population (Pop375) is genetically distinct (Fig. 3). A long-term persistence in situ, at least throughout the Holocene, is indicated by a high haplotype diversity and a higher proportion of private AFLP

fragments (Table 2), which is incongruent with an alternative scenario of a recent long-distance dispersal (e.g. Tribsch et al. 2002). Long-term persistence also agrees with the relict character of the flora at this locality, including the occurrence of several other light-demanding species assumed to be relicts from the Pleistocene and/or early Holocene (e.g. *Noccaea montana*, serpentine lineage of *Knautia arvensis* agg., *Minuartia smejkalii*, *Polygala amara*, *Galium valdepilosum*, and *Sesleria coerulea*; Soják 1960, Dvořáková 1988, Kolář et al. 2012, 2014). Interestingly, the Czech population has genetic affinities with both the central- and southern-European and subarctic populations indicated by haplotype sharing (Fig. 2) and intermediate AFLP genotypes documented in all the analyses (Figs 3–5). Hence, the recorded admixture could reflect either past hybridization/introgression among the lineages (discussed above) or their common ancestry. Although we cannot quantify the role of the serpentine glacial relict population in the colonization of deglaciated areas, the results, at least, illustrate the importance of serpentine refugia for the preservation of unique genetic diversity.

Taxonomic comments

Our genetic and cytological data did not support a distinct taxonomic status for the two serpentine populations that are traditionally differentiated as *P. crantzii* subsp. *serpentini* (Borbás) Hayek. The Austrian population at Bernstein, which is the type locality of the subspecies (Neumayer 1930), is genetically merged with populations from central and south-eastern Europe. The Czech population occupies an intermediate position between the Subarctic and central- and southern-European genetic group of *P. crantzii*. The genetic differences between the two serpentine populations are in line with certain morphological differences recorded for these populations by Soják (1960). Even though this author recognizes the subspecies, he suggests repeated acquisition of the typical morphological characters and a polytopic origin for both serpentine populations. However, the distinctness of the Bernstein population was recently questioned leading to its current treatment as *P. crantzii* var. *serpentini*, possibly reflecting a mere serpentinomorphosis (Punz et al. 2010). In conclusion, we suggest treating the Czech serpentine population as *P. crantzii*, with the expectation of possible further distinction at the level of a variety.

See www.preslia.cz for Electronic Appendices 1–2

Acknowledgements

We thank A. Hilpold, A. Tribsch, K.B. Westergaard and T. Dahl for help with sampling as well as the Botanical Gardens of the University of Oulu for providing seeds under the auspices of the Index Seminum seed exchange and Botanical Garden Tatranská Lomnica for plant material. Further thanks go to T. Gregor for two unpublished chromosome counts, P. Koutecký and T. Urfus for help with the flow cytometric analyses and the staff of the Grunelius-Möllgaard Laboratory (Senckenberg Research Institute) especially H. Kappes for laboratory support. We are grateful to the DFG and FWF for funding (DO792/1-1 and FWF P 21661 granted to C.D.) as well as for the support from the SYNTHESYS Project (ES-TAF-3276) financed by the European Community Research Infrastructure Action under the FP6 "Structuring the European Research Area" Programme (granted to J.P.). The project was also supported by the Student Grant Agency of the Faculty of Science, University of South Bohemia (SGA 2008/010 granted to F.K.) and internal funds of the Senckenberg Research Institute.

Souhrn

Studie se zabývá souvislostmi mezi disjunktním arkto-alpinským rozšířením a ploidní diferenciací mochny Crantzovy (*Potentilla crantzii*), se zvláštním přihlédnutím k izolovaným populacím na reliktních hadcových stanovištích. S pomocí průtokové cytometrie, AFLP a sekvenace chloroplastové DNA přinášíme prvotní náhled na ploidní variabilitu a genetickou strukturu tohoto druhu v rámci Evropy. Arkto-alpinská disjunkce koreluje s hlavním trendem v rozšíření cytotypů, kdy jsou tetraploidní rostliny omezeny na pohoří střední a jižní Evropy zatímco hexaploidní populace obývají převážně subarktickou zónu. Dvě izolované populace z hadcových výchozů v České republice (Dolnokralovické hadce) a Rakousku (hadce u Bernsteinu) jsou geneticky relativně odlišné, což svědčí pro jejich nezávislou evoluční historii. Česká hadcová populace také vykazuje neobvykle vysokou genetickou diverzitu, kontrastující s její výrazně izolovanou polohou mezi oběma hlavními evropskými arelami druhu. Naše genetická a cytometrická data nepodporují odlišný taxonomický status hadcových populací, tradičně vylišovaných jako samostatný taxon *P. crantzii* subsp. *serpentini*.

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Received 30 May 2014
Revision received 13 February 2015
Accepted 24 February 2015