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Pleistocene distribution range shifts were accompanied by breeding system divergence within *Hornungia alpina* (Brassicaceae) in the Alps ☆

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ABSTRACT

Impact of glacial history on the phylogeography of silicate-dwelling plants of the European Alps has been particularly well studied, whereas virtually no data are available for species growing on different bedrock types, as for *Hornungia alpina*. Bayesian clustering of AFLP data only partly support the distinction of three subspecies as morphologically defined. Whereas the phylogeographical N-group corresponds to subsp. *alpina*, the congruence of the SW-group and SE-group with subsp. *brevicaulis*, and subsp. *austroalpina*, respectively, is limited. High levels of rarity and genetic diversity in the N-group suggest Pleistocene survival along the outer margin of the Alpine arc. For subsp. *brevicaulis* we suggest a single origin from a refugium in the Southwestern Alps, whereas subsp. *austroalpina* might have originated twice in the Southern and Southeastern Alps. Different levels of genetic diversity and partitioning of genetic variation indicate a divergence in breeding system, which is corroborated by pollinator exclusion experiments revealing self-incompatibility in the N-group and autonomous selfing in the SE-group.

1. Introduction

The Quaternary ice ages comprised cold stages coupled with advances of ice sheets, and warmer interglacials (Imbrie et al., 1993). During glacial as well as interglacial periods, dramatic climatic oscillations occurred with sharp changes in temperature over only a few decades (GRIP members, 1993). For lowland organisms, these oscillations resulted in large-scale latitudinal range shifts whereas mountain biota mainly responded with altitudinal migrations. Both mechanisms have massively shaped the geographical distribution and genetic structure of present-day biota (Hewitt, 1996, 2000, 2004; Comes and Kadereit, 1998, 2003; Taberlet et al., 1998; Schmitt, 2007).

During the Last Glacial Maximum the North Atlantic ice sheet reached south to 52° N while the major European mountain ranges were covered by ice to a varying extent (van Husen, 1987, 1997; Frenzel et al., 1992; Lundqvist and Saarnisto, 1995). In the European Alps, survival of high-altitude plants was mainly possible in unglaciated areas at the western, southern and eastern periphery of the Alpine arc; more controversial is survival on summits and ridges protruding from the ice sheet (Schönswetter et al., 2005). Most alpine plants show a strong affinity to either calcareous or siliceous bedrock (Ozenda, 1988), thus constraining glacial survival to areas with suitable bedrock type (Tribsch and Schönswetter, 2003). Geologically, the Eastern Alps consist of a central siliceous core flanked by peripheral limestone ranges; in the Western Alps this structure gives way to general predominance of limestone and silicates along the outer and inner peripheries, respectively, of the Alpine arc (Voges, 1995). Consequently, for calcicole species peripheral refugial areas were available around most of the Eastern Alps and along the outer margin of the Western Alps, whereas those for silicicole species were restricted to a few, mostly small, disjoint areas along the eastern, southern and southwestern border (Schönswetter et al., 2005).

☆ *Author contributions:* A.T., T.E., and IBD-C. designed the study and managed the project; M.W., P.S., O.P., and IBD-C. sampled and gathered the data and/or conducted AFLP analysis. T.E. was responsible for determination of the subspecies based on morphology. M.W., O.P., P.S., and A.T. analysed the data; M.W., P.S., O.P., T.E., and A.T. discussed the results and drafts of the manuscript; M.W. and P.S. wrote and revised the paper.

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[1] http://www.intrabiodiv.eu/IMG/pdf/IntraBioDiv_Consortium.pdf.

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The impact of glacial history on the phylogeography of silicicole plants of the European Alps has been particularly well studied. Based on emerging phylogeographical patterns, Schönswetter et al. (2005) confirmed major areas as refugia of silicicole plants arranged in a west-east sequence. Their borderlines correspond to well-known biogeographical boundaries based on floristic evidence, e.g., the Aosta valley in northern Italy or the border between the Western and Eastern Alps (Ozenda, 1988). In comparison to silicicole Alpine plants, only limited information is available for calcicole species of the Alps (but see Ehrich et al., 2007; Parisod and Christin, 2008; Paun et al., 2008; Reisch, 2008) which does not yet allow for a synopsis.

Here, we explore the phylogeographical structure of the perennial, diploid (Polatschek, 1983) herbaceous mountain plant *Hornungia alpina* (L.) O. Appel (syn. *Pritzelago alpina* (L.) Kuntze, *Hutchinsia alpina* R.Br. in Aiton; see Appel and al-Shehbaz (1997) for higher level taxonomy). Apart from a widely disjunct locality in the Moroccan Rif mountains, the species is widely distributed throughout the European Alpine system from the central and northwestern Iberian mountain ranges over the Pyrenees, Alps and Apennines to the Carpathians and the mountain ranges of the Balkan Peninsula (Jalas et al., 1996). Its preferred habitats are moist scree slopes with extended snow cover in the subalpine to nivale zone (Braun-Blanquet and Rübel, 1933; Englisch, 1999). According to Jalas et al. (1996) *H. alpina* comprises three subspecies in the Alps that can be morphologically discriminated based on differences in various floral (e.g., flower size and petal shape, length of anthers) and fruit characters (Englisch, 1995). Subspecies *alpina* and *austroalpina* (Trpin) O. Appel are restricted to limestone; in contrast subsp. *brevicaulis* (Spreng.) O. Appel has a broader ecological amplitude, mainly occurring on calciferous schists but also tolerating limestone and acidic silicates (Braun-Blanquet and Rübel, 1933; Gams, 1942; Zollitsch, 1969). Due to the late description of subsp. *austroalpina* (Trpin, 1974), most literature does not mention this taxon or distinguishes it only as var. *drexlerae* Markgr. (described under *Hutchinsia*) of subsp. *brevicaulis* (e.g., Melchers, 1932; Markgraf, 1962).

A previous molecular study on the evolutionary history of *H. alpina* by Kropf et al. (2003) did not find support for the division between subsp. *alpina* and *brevicaulis* (subsp. *austroalpina* was not mentioned) but rather proposed that subsp. *brevicaulis* which occurs on a wide range of substrates has arisen multiple times from strictly calcicole subsp. *alpina*. Using nuclear ribosomal Internal Transcribed Spacer (nrITS) sequences and Amplified Fragment Length Polymorphism (AFLP) fingerprinting data, Kropf et al. (2003) further suggested that the origin of the species predates the Quaternary. The Alpine accessions fell into a western and a northeastern group, the latter including also samples from the Carpathians. The study focused on the species' diversification pattern across the entire southern European mountain system, and the sampling in the Alps was therefore fairly scarce. Kropf et al. (2003) pointed out, therefore, that a more detailed study with a denser sampling scheme in the Alps was needed.

Recently, Alvarez et al. (2009) demonstrated that large-scale genetic structure correlates with substrate requirement in 27 Alpine species, including also the data set presented here. An additional synthetic analysis (Thiel-Egenter et al., 2009) tested the influence of various species traits on genetic diversity patterns of 22 Alpine species, including the dataset presented below. However, both these multi-species analyses did not touch on any idiosyncrasies of individual species' phylogeography.

Here, we explore phylogeographical history and intraspecific taxonomy of *H. alpina* s. l. in the Alps and — based on a few populations only — the southern Carpathians within the overall framework of the European project IntraBioDiv (for a general introduction to the project see Gugerli et al., 2008). This project focussed on the Alps and Carpathians; hence we were unable to cover the species' total distribution area. Because of the limited DNA-sequence variation encountered in nrITS within *H. alpina* (Kropf et al., 2003), the present study was exclusively based on highly polymorphic AFLPs. On the other hand, we significantly increased the density of sampled populations in the Alps and employed a Bayesian clustering approach (Pritchard et al., 2000) that allowed for detecting weak differentiation among gene pools. In particular, we addressed the following questions. (1) Is the phylogeographical pattern compatible with glacial survival in refugia on both calcareous and siliceous bedrock and are there other factors playing a role in the evolutionary history of *H. alpina*? (2) Do genetic data support the three subspecies and their geographical distributions as presented by Aeschimann et al. (2004) in the most recent account? (3) If yes, is there support for the hypothesis that subsp. *brevicaulis* has originated recurrently from *H. alpina* subsp. *alpina* as suggested by Kropf et al. (2003)?

2. Material and methods

2.1. Sampling

Hornungia alpina was sampled across the Alps from a total of 117 localities, and three additional localities from the Carpathians (Fig. 1, Table 1 and Appendix 1). Following the strategy adopted for the project IntraBioDiv, leaf samples were collected in every second 12′ latitude × 20′ longitude regular grid cell (Gugerli et al., 2008; Fig. 1). Eight additional grid cells in between (populations 103, 105, 107, 110, 113, 117, 120) were sampled. Three individuals per sampling locality (named hereafter population) were collected randomly at a distance of at least 10 m, and dried in silica gel. In eleven grid cells in the Alps, additional populations were sampled. Voucher specimens were assigned to subspecies according to Englisch (1995) based on differences in various floral (e.g., flower size and petal shape, length of anthers) and fruit characters (Appendix 2). Specimens were deposited at the herbarium of the University of Vienna (WU).

2.2. DNA extraction and AFLP fingerprinting

Total genomic DNA was extracted from similar amounts of dried tissue (ca. 10 mg) with the DNeasy 96 plant mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. AFLP profiles were generated following established procedures (Vos et al., 1995) with minor modifications (Gugerli et al., 2008). Genomic DNA (c. 200 ng) was digested with 1 U *Mse*I (New England BioLabs, Ipswich, USA) and 5 U *Eco*RI (Promega, Madison, USA) and ligated (with 1.2 U of T4 DNA-Ligase; Promega) to double-stranded adapters in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA, USA) for 2.5 h at 37 °C. Preselective amplification was performed using primer pairs with a single selective nucleotide. Three selective primer combinations were chosen after a primer trial with twelve primer combinations (fluorescent dye in brackets): *Eco*RI ACG (VIC)-*Mse*I CA; *Eco*RI ACA (6-FAM)-*Mse*I CA; *Eco*RI AAC (NED)-*Mse*I CA. For each individual, 1.2 μl 6-FAM, 2 μl VIC and 3 μl NED labelled, selective PCR products were precipitated with ethanol and sodium acetate, combined with 0.2 μl GeneScan ROX 500 (PE Applied Biosystems) internal size standard and 9.8 μl formamide, and separated on a capillary sequencer ABI 3170 (Applied Biosystems).

Blind samples were included to test for contamination. Fifteen plants (from nine populations) were extracted twice to test the reproducibility of AFLP fingerprinting (Bonin et al., 2004). Two samples were used as replicates between PCR plates, and were replicated more than twice, resulting in a total of 26 replicates.

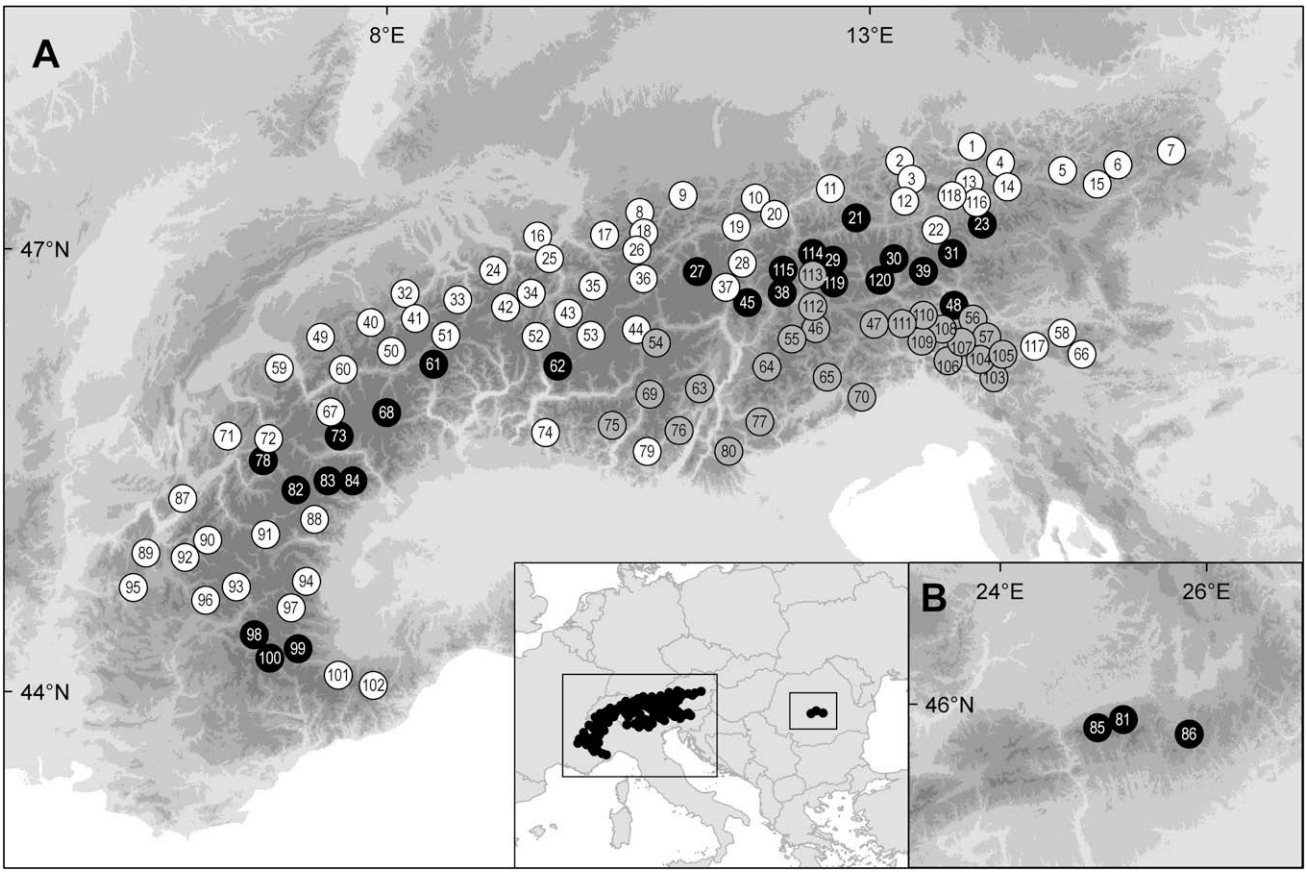

Fig. 1. Geographical location of the 120 sampled populations of *Hornungia alpina* and their assignment to subspecies according to morphological characters. (A) European Alps; (B) Southern Carpathians. White symbols, subsp. *alpina*; light grey symbols, subsp. *austroalpina*; black symbols, subsp. *brevicaulis*. Numbers in circles are population numbers. More details to the investigated populations are given in Table 1 and Appendix 1.

Fragments in the range 65–500 bp were aligned with ABI Prism GeneScan 3.7.1 Analysis Software (PE Applied Biosystems) and visualized, scored and exported as binary presence/absence matrix using Genographer 1.6 (available from http://hordeum.msu.montana.edu/genographer/).

2.3. Analysis of AFLP data

Statistical analyses were computed with R 2.7.0 (R Development Core Team, 2008) unless stated otherwise. Analysing data from only three individuals per sampling locality was counterbalanced by using a large number of genetic markers (Nei, 1987) and populations. All monomorphic fragments and the ones present/absent in all but one individual were removed from the data set to avoid biased parameter estimates (Bonin et al., 2004). We calculated Nei's gene diversity (π_n, in the following termed 'genetic diversity') for each population using the equation

$$D = \left(\frac{n}{n-1}\right) \quad \left[1 - \left(freq_1^2 + freq_0^2\right)\right]$$

for each marker and then taking the average; n is the number of individuals and $freq_1$ and $freq_0$ indicate the number of presences and absences of a particular marker in a population (Nei, 1987). In order to quantify rare markers in populations without setting an arbitrary threshold, the frequency-down-weighted marker value (in the following termed 'rarity') for individual x was calculated according to Schönswetter and Tribsch (2005).

$$R_x = \sum_{i=1}^{n} \frac{s_{ix}}{\sum_{j=1}^{k} s_{ij}}$$

where n is the number of markers, s_{ix} is the state of the ith marker in individual x (either 1 or 0 in AFLPs), and k is the total number of individuals in the data set. In the denominator the number of occurrences of the ith marker in the total data set is calculated. Population values (R_{pop}) are estimated as the average of individual values. Calculations were carried out using AFLPdat (function "DW" — rarity 1, Version 01.02. 2008; Ehrich, 2006, available from http://www.intrabiodiv.eu/spip.php?article77). To facilitate the interpretation, rarity values were standardised around zero:

$$R_{pop_{st}} = \frac{R_{pop} - \overline{R_x}}{\overline{R_x}}$$

Analyses of molecular variance (AMOVAs) were calculated with ARLEQUIN 3.11 (Excoffier et al., 2005), available at http://cmpg.unibe.ch/software/arlequin3/.

A neighbour-joining analysis based on a matrix of Jaccard distances was generated and bootstrapped (2000 pseudo-replicates) with Paup4.0b10 (Swofford, 2002). A Principal Co-ordinate Analysis (PCoA) based on the same matrix was calculated using the modules 'SimQual', 'Dcenter' and 'Eigen' from NTSYS-pc 2.0 (Rohlf, 1997).

The software STRUCTURE v.2.2 (Pritchard et al., 2000; Falush et al., 2007) was employed to complement the distance-based analyses with a Bayesian clustering approach. We used an admixture model with uncorrelated allele frequencies and recessive alleles. Ten replicate runs for K (number of groups) ranging from 1

Table 1
Geographical location, altitude, assignment to subspecies, genetic diversity and rarity estimates of 120 populations of *Hornungia alpina*. Grid Cell, IntraBioDiv grid cell code (Gugerli et al., 2008); N_{FRAG}, number of AFLP fragments in the population; P_{POLY}, percentage of polymorphic fragments in the population; π_n, Nei's gene diversity over loci; $R_{pop_{st}}$, standardised within-population rarity of markers (negative values are below and positive values above the mean).

Code	Grid cell	Co-ordinates (*E*/*N*)	Altitude (m s. m)	Subspecies	N_{FRAG}	P_{Poly}	π_n	$R_{pop_{st}}$
1	D28	13.69/47.81	1628	*alpina*	70	13.4	0.089	−0.119
2	E27	13.01/47.72	1795	*alpina*	78	16.0	0.107	−0.135
3	E27	13.07/47.61	1860	*alpina*	87	19.5	0.130	0.454
4	E29	13.97/47.69	1693	*alpina*	74	13.0	0.087	−0.047
5	E31	14.59/47.61	1862	*alpina*	73	14.3	0.095	0.081
6	E33	15.15/47.62	2105	*alpina*	79	15.2	0.101	0.367
7	E35	15.70/47.69	1775	*alpina*	78	15.2	0.101	−0.106
8	F18	10.32/47.42	1690	*alpina*	79	16.5	0.110	0.903
9	F20	10.76/47.54	1950	*alpina*	79	16.9	0.113	0.247
10[a]	F22	11.49/47.51	2015	*alpina*	70	10.8	0.108	−0.079
11	F24	12.24/47.56	1681	*alpina*	80	16.0	0.107	0.364
12	F26	12.95/47.49	1875	*alpina*	79	16.0	0.107	0.024
13	F28	13.65/47.52	1812	*alpina*	76	15.2	0.101	0.257
14	F30	14.03/47.52	1655	*alpina*	80	19.5	0.130	0.215
15	F32	14.93/47.50	2129	*alpina*	71	15.2	0.101	−0.170
16	G15	09.29/47.23	1470	*alpina*	76	16.9	0.113	0.207
17	G17	09.97/47.27	2026	*alpina*	77	15.6	0.104	−0.020
18	G19	10.36/47.28	2210	*alpina*	72	13.9	0.092	−0.183
19	G21	11.29/47.32	2100	*alpina*	81	17.3	0.115	0.897
20	G23	11.68/47.40	1861	*alpina*	78	16.9	0.113	0.318
21	G25	12.49/47.36	2346	*brevicaulis*	63	6.9	0.046	0.634
22	G27	13.29/47.21	1978	*alpina*	76	13.9	0.092	0.135
23[a]	G29	13.75/47.27	2180	*brevicaulis*	51	0.4	0.004	−0.288
24	H14	08.86/47.02	1700	*alpina*	70	16.0	0.107	−0.274
25	H16	09.42/47.11	2075	*alpina*	81	19.0	0.127	0.214
26	H18	10.29/47.17	2322	*alpina*	81	16.9	0.113	0.521
27	H20	10.89/47.02	2414	*brevicaulis*	49	1.7	0.012	−0.041
28	H22	11.34/47.07	2864	*alpina*	74	14.7	0.098	−0.208
29	H24	12.19/47.04	2450	*brevicaulis*	48	6.9	0.046	−0.524
30[a]	H26	12.86/47.07	2490	*brevicaulis*	55	5.2	0.052	−0.366
31	H28	13.43/47.10	2165	*brevicaulis*	74	16.5	0.110	−0.018
32	I11	07.98/46.85	1907	*alpina*	73	15.6	0.104	−0.316
33	I13	08.51/46.82	1686	*alpina*	71	16.5	0.110	0.030
34	I15	09.24/46.87	2217	*alpina*	82	21.6	0.144	0.894
35	I17	09.85/46.92	2328	*alpina*	77	14.7	0.098	0.276
36	I19	10.35/46.97	2451	*alpina*	71	14.7	0.098	−0.138
37	I21	11.17/46.91	2481	*alpina*	73	15.2	0.101	−0.277
38	I23	11.67/46.90	2200	*brevicaulis*	49	1.3	0.009	−0.499
39	I27	13.15/46.97	2320	*brevicaulis*	52	0.4	0.003	0.140
40	J10	07.65/46.65	2348	*alpina*	72	16.5	0.110	0.334
41	J12	08.09/46.68	2120	*alpina*	77	19.9	0.133	0.056
42	J14	08.98/46.77	2146	*alpina*	72	14.7	0.098	−0.157
43	J16	09.61/46.73	2497	*alpina*	81	18.2	0.121	0.359
44	J18	10.28/46.62	2600	*austroalpina*	54	5.6	0.038	−0.432
45	J22	11.39/46.80	2200	*brevicaulis*	66	9.5	0.063	−0.489
46	J24	12.02/46.61	2125	*austroalpina*	53	0.4	0.003	−0.034
47	J26	12.72/46.62	2100	*austroalpina*	48	2.6	0.017	−0.252
48	J28	13.43/46.73	1900	*brevicaulis*	61	6.9	0.046	0.152
49	K09	07.16/46.53	1873	*alpina*	73	16.0	0.107	−0.273
50	K11	07.86/46.45	2100	*alpina*	78	19.0	0.127	0.617
51	K13	08.40/46.57	2050	*alpina*	68	13.4	0.089	1.359
52	K15	09.29/46.57	2160	*alpina*	77	18.2	0.121	0.199
53	K17	09.83/46.58	2434	*alpina*	79	17.7	0.118	0.227
54	K19	10.48/46.53	2240	*austroalpina*	57	5.6	0.038	0.311
55	K23	11.82/46.54	2150	*austroalpina*	51	3.9	0.026	−0.447
56	K27	13.30/46.57	1900	*austroalpina*	50	1.7	0.012	−0.205
57	K29	13.74/46.44	1610	*austroalpina*	53	2.2	0.014	−0.330
58[a]	K31	14.49/46.50	2060	*alpina*	67	9.1	0.091	0.415
59	L08	06.77/46.31	1740	*alpina*	74	13.4	0.089	0.570
60	L10	07.40/46.32	2153	*alpina*	78	17.3	0.115	0.419
61	L12	08.28/46.37	2245	*brevicaulis*	49	2.6	0.017	−0.623
62	L16	09.50/46.37	2550	*brevicaulis*	53	5.2	0.035	−0.197
63	L20	10.90/46.22	2575	*austroalpina*	53	2.6	0.017	1.043
64	L22	11.57/46.36	2600	*austroalpina*	58	7.4	0.049	0.319
65	L24	12.16/46.28	2237	*austroalpina*	50	2.2	0.014	−0.459
66	L32	14.67/46.35	1780	*alpina*	73	12.6	0.084	0.103
67	M09	07.29/46.01	2230	*alpina*	44	0.9	0.006	−0.564
68	M11	07.83/46.03	2413	*brevicaulis*	59	12.6	0.084	0.199
69	M19	10.41/46.18	2297	*austroalpina*	63	5.6	0.038	0.037
70	M25	12.49/46.13	1819	*austroalpina*	46	2.2	0.014	−0.408
71[a]	N06	06.29/45.83	1930	*alpina*	74	13.0	0.130	0.521
72	N08	06.69/45.83	2420	*alpina*	78	15.6	0.104	0.329
73	N10	07.35/45.91	2520	*brevicaulis*	49	3.0	0.020	−0.641

Table 1 (*continued*)

Code	Grid cell	Co-ordinates (*E*/*N*)	Altitude (m s. m)	Subspecies	N_{FRAG}	P_{Poly}	π_n	$R_{pop_{st}}$
74	N16	09.39/45.92	2080	*alpina*	63	10.4	0.069	0.083
75	N18	10.04/45.97	2085	*austroalpina*	65	11.3	0.075	−0.453
76	N20	10.69/45.93	1940	*austroalpina*	69	13.0	0.087	0.440
77	N22	11.48/45.99	1974	*austroalpina*	51	3.5	0.023	0.139
78	O07	06.65/45.68	2150	*brevicaulis*	51	3.9	0.026	−0.080
79	O19	10.38/45.79	1814	*alpina*	62	10.4	0.069	−0.054
80[a]	O21	11.18/45.79	2170	*austroalpina*	62	6.5	0.065	−0.181
81	O62	24.74/45.60	2530	*brevicaulis*	50	2.6	0.017	−0.624
82	P08	06.98/45.49	2350	*brevicaulis*	44	0.9	0.006	−0.754
83	P10	07.53/45.56	2576	*brevicaulis*	58	10.0	0.066	−0.402
84	P10	07.53/45.56	2576	*brevicaulis*	54	7.4	0.049	−0.493
85	P61	24.62/45.60	2197	*brevicaulis*	61	9.1	0.061	0.089
86	P64	25.47/45.43	2400	*brevicaulis*	53	3.0	0.020	0.273
87	Q05	05.89/45.39	1050	*alpina*	76	17.3	0.115	0.417
88	Q09	07.17/45.29	2200	*brevicaulis*	46	1.3	0.009	−0.298
89	R04	05.57/45.01	2060	*alpina*	85	20.3	0.136	1.663
90	R06	06.15/45.12	2335	*alpina*	83	18.2	0.121	0.987
91	R08	06.71/45.18	2300	*alpina*	60	10.4	0.069	−0.365
92	S05	05.95/44.99	2000	*alpina*	70	9.5	0.063	0.652
93	S07	06.45/44.81	1980	*alpina*	49	3.5	0.023	0.207
94	S09	07.12/44.87	2364	*alpina*	67	13.4	0.089	0.236
95	T04	05.47/44.77	1795	*alpina*	79	18.6	0.124	0.356
96	T06	06.16/44.71	2275	*alpina*	71	13.4	0.089	0.167
97	T08	06.98/44.68	2780	*alpina*	79	14.7	0.098	1.691
98	U07	06.64/44.49	2630	*brevicaulis*	47	4.8	0.032	−0.476
99	U09	07.06/44.41	2230	*brevicaulis*	51	6.5	0.043	−0.166
100	V08	06.80/44.34	2591	*brevicaulis*	48	2.2	0.014	−0.287
101	V10	07.45/44.24	1700	*alpina*	64	8.7	0.058	0.383
102	W11	07.79/44.18	2630	*alpina*	73	19.5	0.130	−0.217
103	L29	13.18/46.23	1880	*austroalpina*	48	0.9	0.006	−0.202
104	K29	13.74/46.43	1850	*austroalpina*	51	5.2	0.035	−0.624
105	L29	13.81/46.38	1900	*austroalpina*	51	4.8	0.032	−0.569
106	L28	13.47/46.36	2120	*austroalpina*	49	1.7	0.012	−0.618
107	K28	13.56/46.42	850	*austroalpina*	52	4.8	0.032	−0.611
108	K27	13.30/46.58	1850	*austroalpina*	45	2.2	0.014	−0.491
109	K27	13.30/46.57	1880	*austroalpina*	46	3.5	0.023	−0.515
110	J27	13.16/46.69	2090	*austroalpina*	55	5.6	0.038	−0.456
111	J26	12.87/46.61	1990	*austroalpina*	47	7.4	0.049	−0.733
112	J24	12.01/46.72	1980	*austroalpina*	54	4.8	0.032	−0.434
113	I24	12.08/46.98	2450	*austroalpina*	52	5.2	0.035	−0.553
114	H24	12.13/47.03	2240	*brevicaulis*	51	6.9	0.046	−0.082
115	I23	11.82/46.94	2500	*brevicaulis*	51	5.2	0.035	−0.522
116	F29	13.69/47.44	1950	*alpina*	70	13.4	0.089	−0.267
117	K30	14.18/46.36	2050	*alpina*	72	14.7	0.098	−0.182
118	F28	13.65/47.45	2650	*alpina*	67	16.9	0.113	−0.395
119	I24	12.20/46.90	2590	*brevicaulis*	46	1.7	0.012	−0.642
120	I26	12.71/46.94	2490	*brevicaulis*	54	3.5	0.023	−0.459

[a] Only two individuals per population instead of three were analysed.

to 10 were carried out at the Bioportal of the University of Oslo (http://www.bioportal.uio.no/), using a burn-in of 100,000 iterations followed by 1,000,000 additional MCMC iterations. For comparison, we also ran a no admixture model with the same MCMC parameters. Similarity among results of different runs for the same *K* was calculated according to Nordborg et al. (2005) using the R-script Structure-sum (Ehrich, 2006) available from http://www.nhm.uio.no/forskningsamlinger/forskning/forskninggrupper/ncb/Online_publications. According to Rosenberg et al. (2002), a similarity value above 0.85 corresponds to a generally similar population structure. We identified the number of main groups as the value of *K* where the increase in likelihood started to flatten out, the result of replicate runs was similar and the clusters were non-empty. Additionally, we employed the method of Evanno et al. (2005). Replicate runs of the best *K* were then merged with CLUMPP 1.1.1. (Jakobsson and Rosenberg, 2007) using the full-search algorithm. The proportions of membership of all individuals were then averaged for each population. For AMOVA analyses, populations were assigned to the cluster with the highest cluster membership coefficient. For each group identified at the optimal *K*, a separate STRUCTURE analysis was carried out.

Additionally, population mixture analysis implemented in the program BAPS v. 5.2 (Bayesian Analysis of Populations Structure; available at http://www.abo.fi./fak/mnf/mate/jc/software/baps.html; Corander et al., 2003) was used to detect population structure. The program treats both the frequencies of the markers and the number of genetically diverged groups as random variables. Stochastic optimisation is used to infer the mode of the posterior distribution. Because AFLPs are dominant markers, only AFLP phenotypes can be analysed, but this procedure does not violate the assumptions of BAPS (Corander et al., 2004). We conducted mixture analysis of individuals with the geographical origin of the samples used as informative prior ("spatial clustering of individuals"). BAPS was run with the maximal number of groups (*K*) set to 2–10. Each run was replicated 10 times and the results were averaged according to the resultant likelihood scores. Results of the mixture analysis were used as input for population admixture analysis (Corander and Marttinen, 2006) in order to detect admixture between clusters. Admixture coefficients were estimated using 200 iterations, and the significance of these coefficients was estimated by employing the simulation strategy described by Corander and Marttinen (2006) using 50 reference individuals and 20 iterations each.

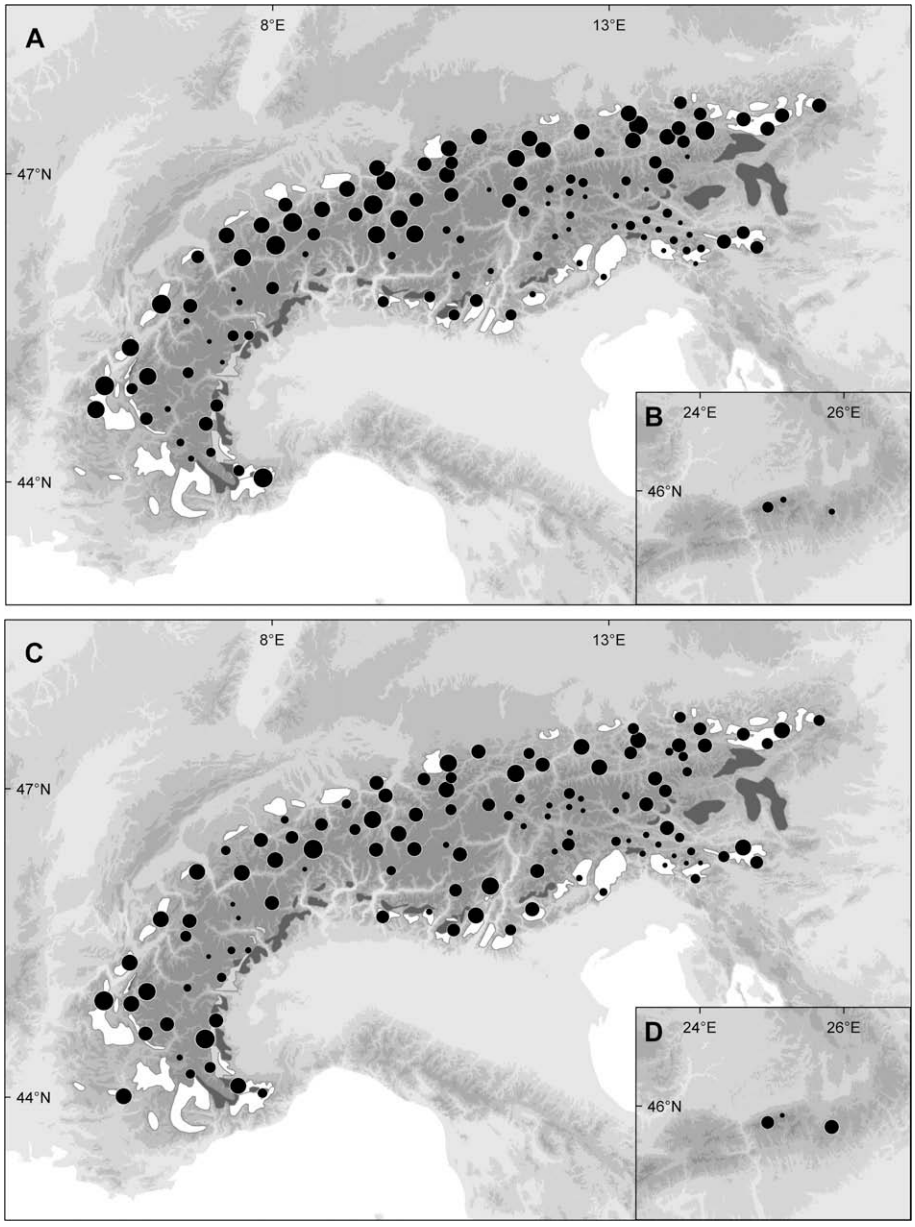

Fig. 2. Patterns of within-population AFLP genetic diversity (π_n, Nei's gene diversity over loci; A and B) and rarity (R_{POP}, within-population rarity of markers; C and D) in 120 sampled populations of *Hornungia alpina* from the Alps (A and C) and Southern Carpathians (B and D). The size of the dots is directly proportional to the amount of genetic diversity, or rarity, respectively (see also Fig. 1 and Table 1). In (A) and (C), white areas indicate presumed glacial refugia on calcareous bedrock, dark grey areas those on siliceous, and light grey areas (south-western Alps only) those on intermediate bedrock (modified from Tribsch and Schönswetter, 2003 and Schönswetter et al., 2005).

Table 2
Overview of global genetic diversity and rarity estimates of morphologically defined subspecies and groups separated by STRUCTURE at $K = 3$ of AFLP data of *Hornungia alpina*. $R_{pop_{st}}$, mean ± SD of standardised within-population rarity of markers (negative values are below and positive values above the mean); π_n, mean ± SD of Nei's gene diversity; *n*, number of sampled populations. For the STRUCTURE grouping, populations were assigned to the cluster with the highest cluster membership coefficient.

Grouping	$R_{pop_{st}}$	π_n	*n*
Subspecies			
alpina	0.221 ± 0.454	0.101 ± 0.023	64
austroalpina	−0.240 ± 0.406	0.030 ± 0.020	27
brevicaulis	−0.250 ± 0.335	0.034 ± 0.025	29
STRUCTURE at K = 3			
N-group	0.202 ± 0.395	0.105 ± 0.019	56
SE-group	−0.150 ± 0.404	0.037 ± 0.029	39
SW-group	−0.200 ± 0.580	0.039 ± 0.029	25

2.4. Breeding system

In order to test if the pronounced differences in the partitioning of genetic diversity observed among the clusters recognised by STRUCTURE (see Results) were caused by different breeding systems, we carried out pollination experiments. Fifteen individuals of *H. alpina* subsp. *austroalpina* were collected at Vršič pass (Julijske Alpe, Slovenia, 1600 m s. m.; legit Peter Schönswetter and Božo Frajman), and ten individuals of *H. alpina* subsp. *alpina* on Schneeberg (Rax-Schneeberg-Gruppe, Austria, 1850 m s. m.; legit Peter Schönswetter) in June/July 2008. *H. alpina* subsp. *brevicaulis* could not be included in the experiment. All individuals had inflorescences with buds, and were bagged with a fine-meshed tissue to exclude pollinators. Flowers were checked every third day for fruit set.

3. Results

3.1. Morphological assignment to subspecies

Sixty-four, 27 and 29 sampled populations belonged morphologically to *H. alpina* subsp. *alpina*, subsp. *austroalpina*, and subsp. *brevicaulis*, respectively (Table 1). The distribution of *H. alpina* subsp. *alpina* mainly covers the entire northern part of the Alpine arc, whereas subsp. *austroalpina* is restricted to the southeastern Alps, and subsp. *brevicaulis* occurs in the western as well as along the main chain of the middle Alps and in the Romanian Southern Carpathians (Fig. 1).

3.2. AFLP data

The three AFLP primer combinations yielded 231 clear polymorphic fragments after the removal of fourteen invariable markers. Six individuals consistently failed to produce reliable AFLP patterns and were excluded from analyses, so finally 354 individuals were included in the analysis. In the AFLP profiles from replicated samples, 179 differences were observed out of 6370 phenotypic comparisons, resulting in an error rate of 2.81%. One AFLP phenotype was represented by three individuals, and six phenotypes by two individuals, respectively. Repeated phenotypes belonged to the same or neighbouring populations. Nei's gene diversity varied from 0.003 in populations 39 and 46 to 0.144 in population 34 (Table 1). Genetic diversity followed a clear N-S distribution with highest diversity in the Northern Alps and lowest diversity in the Central and SE Alps (Fig. 2A). Mean individual rarity ($\overline{R_x}$) was 0.652. Rarity in sampled populations ($R_{pop_{st}}$) ranged from −0.754 in population 82 to 1.691 in population 97 (Table 1), with no obvious geographical trend (Fig. 2C). Values of genetic diversity and rarity calculated for subspecies and STRUCTURE groups defined below ($K = 3$) are given in Table 2. Both genetic diversity and rarity were highly congruent with subspecies distribution, with *H. alpina* subsp. *alpina* having higher values than the other two subspecies (ANOVA Tukey post-hoc test, $p < 0.001$) which did not differ significantly from each other (ANOVA Tukey post-hoc test, $p > 0.05$; Figs. 1 and 2). In the NJ analysis, none of the deeper nodes received any bootstrap support (data not shown).

Bayesian clustering approaches (admixture analysis with BAPS, no admixture and admixture models in STRUCTURE) yielded similar results, and only results of the STRUCTURE admixture model are presented. Likelihood values for K reached a plateau at $K = 4$; however for $K \geqslant 4$ they differed strongly among replicate runs (see Appendix 3 for more details). As the search strategy of Evanno et al. (2005) suggested $K = 3$ as the best clustering solution, we chose $K = 3$ as optimal solution. The largest group in $K = 2$ to $K = 4$ roughly corresponded to subsp. *alpina* (Fig. 3). At $K = 3$, the remaining populations were separated along a SW/NE line between upper Valtellina (Italy, population 69) and western-most Hohe Tauern (Austria, population 119), with the southeastern group mainly comprising subsp. *austroalpina* (Fig. 3). At $K = 4$, the latter group was split into a western and an eastern subgroup along the upper Piave valley, thus separating Dolomiti from Alpi

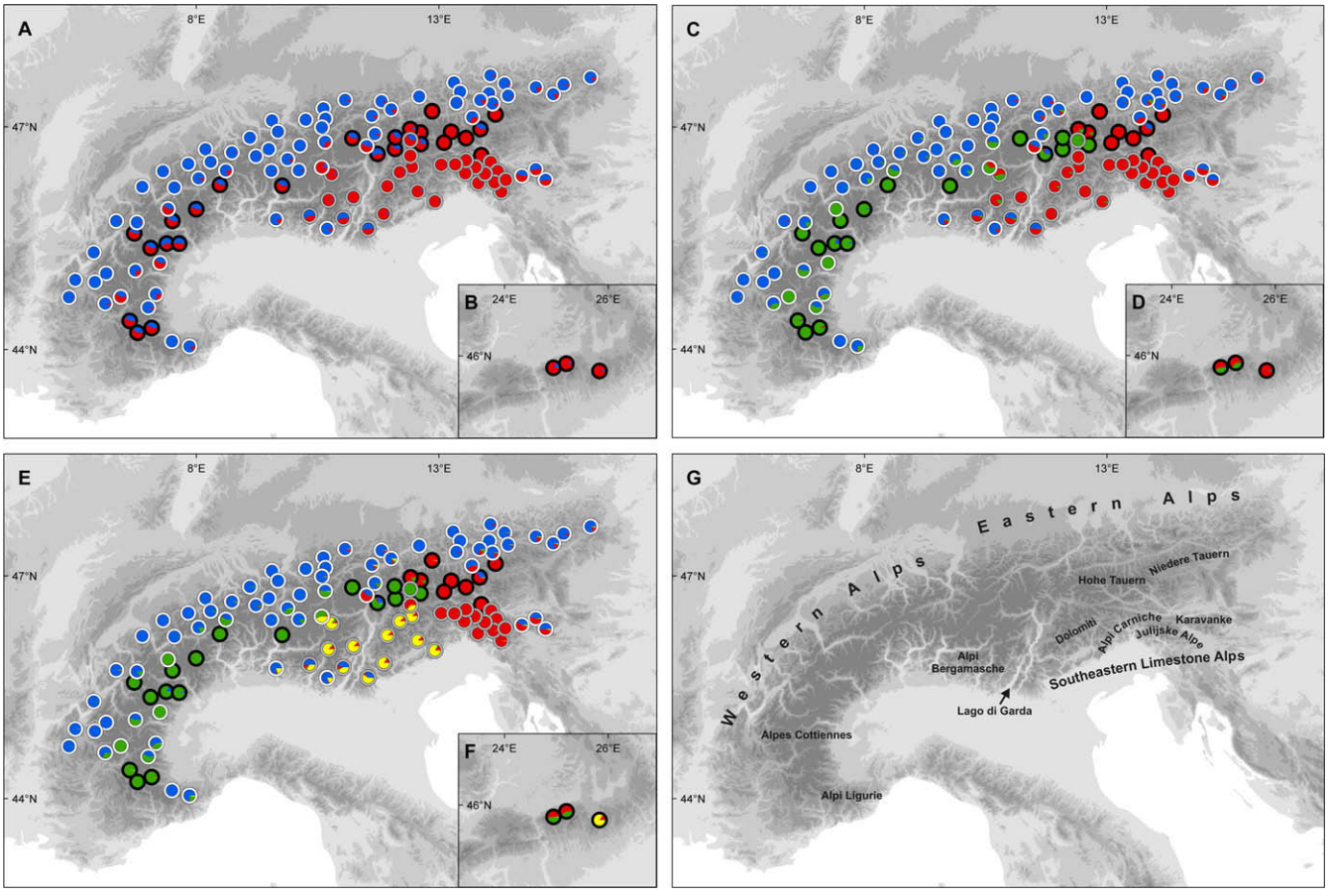

Fig. 3. Phylogeographical grouping of *Hornungia alpina* in the Alps and Carpathians according to Bayesian clustering analyses of AFLP phenotypes conducted with STRUCTURE. The number of groups, K, was set to 2–4. (A and B), $K = 2$; (C and D) $K = 3$; and (E and F) $K = 4$. Assignment to morphological subspecies is colour-coded with circles around the pie charts: white, subsp. *alpina*; light grey, subsp. *austroalpina*; black, subsp. *brevicaulis*. Toponymes used in the text are given in (G).

Carniche. Separate STRUCTURE analyses revealed no further subdivision of the SW-group, and a subdivision in two groups each of the N and SE-group. The split in the SE-group is the same as at $K = 4$ (Fig. 3C). The N-group was divided in a western subgroup which is confined to the Western Alps, and a much larger predominantly eastern subgroup. The eastern subgroup also occurs in the eastern parts of the Western Alps, where populations are admixed, and the southwestern Alps.

The two-dimensional PCoA showed a pattern compatible with the STRUCTURE grouping at $K = 3$, whereas the grouping according to subspecies fitted less well (Fig. 4). AMOVAs (Table 3) attributed 51.3% of the overall genetic variation to the among-population component. In nested AMOVA analyses, the variation between the three subspecies accounted for 13.7% and the variation among the groups indicated by STRUCTURE analyses ($K = 3$) for 19.2% of the overall variation. Separate analyses for each subspecies showed a much smaller fixation index in subsp. *alpina* than in the other two subspecies (Table 3).

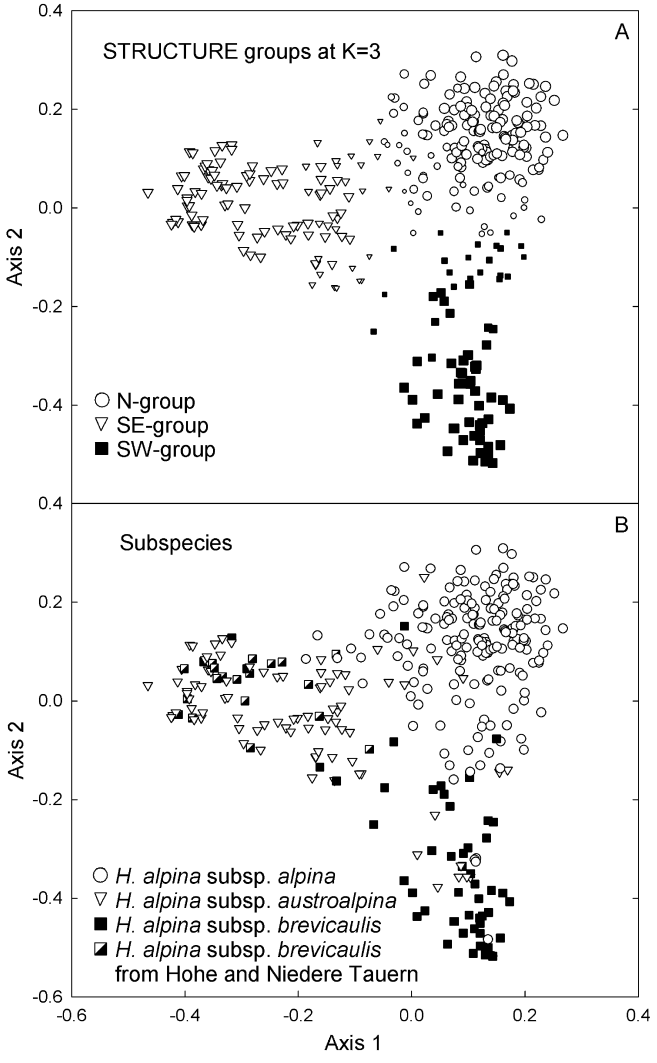

Fig. 4. Principal co-ordinate analysis of a matrix of pair-wise Jaccard distances derived from AFLP analysis of 120 populations of *Hornungia alpina*. The first ordination axis explains 14.4%, and the second 12.5% of the variation in the data matrix, respectively. (A) Groups derived from Bayesian clustering analyses conducted with STRUCTURE at $K = 3$ overlaid; symbol size corresponds to degree of admixture: the larger the symbol the less admixed is an individual. (B) Assignment to subspecies based on morphological characters overlaid; semi-filled squares indicate morphological *brevicaulis*-individuals from Hohe and Niedere Tauern which belong genetically to the SE-group (for details see text).

3.3. Breeding systems

Four individuals of *H. alpina* subsp. *austroalpina*, and two of *H. alpina* subsp. *alpina* failed to flower and were excluded from analyses. Of 299 flowers of *H. alpina* subsp. *austroalpina* included in the autogamy treatment, 177 set fruit, resulting in a mean fruit set of 0.616 (±1.87, $N = 299$ flowers/42 inflorescences/9 individuals). In contrast, none of the bagged flowers of *H. alpina* subsp. *alpina* set fruit ($N = 57/9/5$). In order to control for experimental artefacts, 14 flowers on three individuals of *H. alpina* subsp. *alpina* were manually cross-pollinated. Eight of these set fruit, resulting in a mean fruit set of 0.55 per inflorescence (±0.512 SD, $N = 14/5/3$).

4. Discussion

4.1. Phylogeography of an alpine plant tolerant of different bedrock types

Bayesian clustering analysis of AFLP data for 117 Alpine and three Southern Carpathian populations of *H. alpina* suggested a division into three phylogeographical groups as best solution (Fig. 3 and Appendix 3). This result is congruent with the PCoA (Fig. 4) and explains about one fifth of the overall genetic variation (AMOVA, Table 3). The *N-group* is distributed mainly along the northern fringe of the Alps, but comprises also some populations in the Alpi Ligurie (populations 101, 102), the southern-most Alpi Bergamasche (74, 75, 76, 79, 80) and the southeastern-most Alps (Karavanke/Karawanken and adjacent Kamniške Alpe; 58, 66, 117), all situated along the southern margin of the Alps in previously proposed Pleistocene refugia (summarised in Schönswetter et al., 2005) and being slightly to strongly admixed (i.e., being composed of more than one genepool; Fig. 3A, C and E). The second group, the *SW-group*, is most widespread in the southwestern and western Alpine arc, extending from the main chain towards the inner fringe. Additionally, it ranges eastwards along the main divide to the western-most Hohe Tauern (populations 113, 119). Finally, the *SE-group* is mainly distributed in the southeastern Limestone Alps, but also occurs in the Hohe and Niedere Tauern along the siliceous main chain and locally even reaches the northern Alps (population 21). The admixed southern Carpathian populations also belong to this group. In the suboptimal (Appendix 3) clustering solution at $K = 4$ (Fig. 3E and F), the SE-group is further divided into a more western group (SE:W-group) centred in the Dolomiti and westerly adjacent areas and a more eastern group (SE:E-group).

All three main groups overlap with areas that remained unglaciated or were only weakly glaciated during cold stages of the Pleistocene (van Husen, 1987; Voges, 1995; Fig. 2A and C) and may have acted as Pleistocene refugia (summarised in Schönswetter et al., 2005). As suggested by the high levels of rarity and genetic diversity throughout its range (Table 1), the N-group may have survived along the calcareous outer fringe of the Alpine arc, probably also in adjacent vast periglacial terraces and moraines (van Husen, 1997). The presence of the N-group along the southern margin of the Alps in the Alpi Marittime/Alpes Maritimes, the Prealpes around Lago di Garda, and the eastern part of the Karavanke/Karawanken may suggest that it was formerly more widespread. Survival in northern peripheral refugia has also been proposed for *Erinus alpinus* (Stehlik et al., 2002), *Rumex nivalis* (Stehlik, 2002), and *Biscutella laevigata* (Parisod and Besnard, 2007). However, although these studies were based on dense sampling schemes they were restricted to the Western Alps. The SE-group overlaps with major presumed refugia for limestone-dwelling plants (Dolomiti to Karavanke/Karawanken) situated along the generally weakly glaciated southern margin of the Alps. The two

Table 3
Analyses of molecular variance (AMOVAs) of AFLP phenotypes in *Hornungia alpina* for sampled populations, morphologically defined subspecies, and groups separated by STRUCTURE at $K = 3$.

Grouping	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fst*
Sampled populations	Among populations	119	3919.8	8.5	51.3	0.513
	Within populations	234	1878.0	8.0	48.7	
Subspecies	Among subspecies	2	567.1	2.4	13.7	0.539
	Among populations within subspecies	117	3352.8	7.0	40.2	
	Within populations	234	1878.0	8.0	46.1	
subsp. *alpina*	Among populations	63	1844.1	5.9	33.6	0.336
	Within populations	125	1466.7	11.7	66.4	
subsp. *austroalpina*	Among populations	26	787.9	9.1	72.1	0.721
	Within populations	53	185.5	3.5	27.9	
subsp. *brevicaulis*	Among populations	28	720.8	7.4	64.8	0.648
	Within populations	56	225.8	4.0	35.3	
STRUCTURE $K = 3$	Among groups	2	816.7	3.4	19.2	0.547
	Among populations within groups	117	3103.1	6.3	35.4	
	Within populations	234	1878.0	8.0	45.4	
N-group	Among populations	55	1549.7	5.4	30.9	0.309
	Within populations	109	1327.0	12.2	69.2	
SE-group	Among populations	38	1112.5	8.5	66.5	0.665
	Within populations	75	323.0	4.3	33.5	
SW-group	Among populations	24	440.9	4.6	50.2	0.502
	Within populations	50	228.0	4.6	49.8	

* All $p < 0.001$.

subgroups identified within the SE-group can be related to refugia in the Dolomiti for the SE:W-group and in the Julijske Alpe/Alpi Giulie eastwards to Karavanke/Karawanken for the SE:E-group. For the SW-group, which does not show any substructure, finally, we suggest a single refugium somewhere in the Western Alps, most probably in the Italian Alpes Cottiennes/Alpi Cozie, an area dominated by calciferous schists and granite and showing an increased level of rarity (Fig. 2C). From there, members of the SW-group probably migrated eastwards along the schist areas along the main chain of the Eastern Alps and finally met with the SE-group in the western-most Hohe Tauern. Migration in the opposite direction, i.e., westwards, is less likely given that populations assigned to the SW-group occur only in the central-most parts of the Eastern Alps. These areas were among the most strongly glaciated during the Last Glacial Maximum (van Husen, 1987). Survival on nunataks, i.e., exposed ridges and summits protruding from the ice sheet (Stehlik, 2000), in this area appears also highly improbable given the habitat requirements of populations assigned to the SW-group, typically scree slopes composed of fine schists with extended snow cover (Zollitsch, 1969).

The phylogeographical pattern of *H. alpina*, a species growing on limestone, calciferous schists and — more rarely — on acidic silicates (Melchers, 1932; Markgraf, 1962) differs substantially from those observed in strictly silicicolous or calcicolous species in the Alps but rather combines features of both groups. Except for the high-alpine cushion plant *Eritrichium nanum* (Boraginaceae), for which survival on ice-free summits within the Pleistocene ice sheet was suggested (Stehlik et al., 2001), other range-wide studies of widespread silicicolous plants typically revealed a west-east sequence of phylogeographical groups that could be related to refugia along the southern and eastern margins of the Alps (reviewed in Schönswetter et al., 2005). In contrast to silicicolous plants, those dwelling on limestone have received much less attention by phylogeographers. So far, only studies investigating *Arabis alpina* (Ehrich et al., 2007) and *Ranunculus alpestris* (Paun et al., 2008) were published with a sampling density and coverage comparable to the present study. In *R. alpestris*, the highest genetic diversity was found in the northern Alps. Rarity showed an inverse pattern and was highest in the southern and, to a lesser extent, in the northeastern Alps. In contrast to *H. alpina*, a southern/southwestern Alpine group was identified that only included populations close to the Alpine periphery and was overlapping with the disjoint southern Alpine occurrence of the N-group of *H. alpina* (Fig. 3C). In *R. alpestris*, the STRUCTURE group occurring in the northeastern Alps also comprised the central and parts of the southeastern Alps and the Carpathians. Again in marked contrast to *H. alpina*, a west/east differentiation existed in the northern Alps instead of the southern Alps. In *A. alpina* (Ehrich et al., 2007) seven STRUCTURE clusters were found in the Alpine populations. In the Eastern Alps the clusters showed a north-south organisation following the tectonic structure, whereas in the Western Alps the pattern was more complex. The Carpathian populations were either associated with the southeastern-most group in the Alps or formed groups on their own. Altogether, the phylogeographical pattern observed in *H. alpina* at $K = 2$ in the STRUCTURE analysis more strongly resembles the north-south structure observed in calcicolous species (Ehrich et al., 2007; Paun et al., 2008) than the east-west sequence of population groups most often encountered in silicicolous taxa (Schönswetter et al., 2005). This result reflects glacial survival at the mostly calcareous periphery of the Alps, which likely offered the least hostile growing conditions during the cold stages of the Pleistocene. However, at higher *K*'s, the north-south structure gives way to an east-west differentiation along the inner edge of the Alpine arch, very likely reflecting a series of glacial refugia along this relatively weakly glaciated area (van Husen, 1987).

4.2. Are pronounced differences in genetic diversity and structure due to a divergence in breeding system?

The STRUCTURE groups identified in *H. alpina* exhibited strikingly different levels of within-population genetic diversity. Whereas the N-group was highly variable, both SW- and SE-groups were significantly less diverse (Fig. 2 and Table 2). Rarity, in contrast, did not show a geographical pattern, except for many populations with low levels of rarity in the southeastern Alps (Table 1 and Fig. 2).

Possible *ad hoc* explanations may be sought in contrasting phylogeographical histories of the groups. However, all three groups overlap with major glacial refugia rendering historical causes such as massive bottlenecks unlikely. Additional evidence against marked differences in the extent of historical bottlenecks comes from non-hierarchical AMOVAs (Table 3) conducted with

each phylogeographical group separately, which indicated contrasting structuring of genetic variation. While in the N-group only one third of the variance was assigned to the among-population component, in the SW- and the SE-groups roughly two thirds of the variance accounted for it. If the low diversity in the SW- and SE-groups were caused by glacial/postglacial bottlenecks, the pattern encountered could only be explained if each population were the independently bottlenecked descendent of a polymorphic source population. This scenario appears highly unlikely given that (1) *H. alpina* is frequent in this area, and (2) the southern margin of the Alps was only weakly glaciated and likely provided ample habitats ranging from gravelly riverbeds in the foreland of the Alps to unglaciated mountainous terrain. Furthermore, (3) independent local bottlenecks that reduced the genetic diversity of a polymorphic source populations should have led to *high* levels of within-population rarity instead of the observed pattern (Fig. 2C). In addition, comparative analyses of genetic diversity and rarity in *R. alpestris* (Paun et al., 2008) have pointed out that patterns of genetic diversity reflect recent processes (as present inbreeding in small populations or high gene flow among neighbouring populations) rather than historical bottlenecks.

The combination of different overall-levels of genetic diversity and the different partitioning of the genetic variation suggested a possible divergence in the breeding system. Accordingly initiated pollinator exclusion experiments, albeit including only one population each of the N- and the SE-groups indeed revealed self-incompatibility in the former and strong autonomous selfing in the latter group. For the SW-group, which was not included in our experiment, we also expect predominance of selfing. This assumption is based on the size of the flowers (petals are even smaller than those of the SE-group; Appendix 2) as well as on the partitioning of genetic variance (Table 3) which is similar to the SE-group.

Switches in breeding system may have been selected for reproductive reassurance one or few times during the evolutionary history of *H. alpina*. Alternatively, they may be a plastic response (even under epigenetic control; Nasrallah et al., 2007) to the decrease of insect diversity, abundance, and activity with increasing altitude (Kalin Arroyo et al., 1985; Bingham and Orthner, 1998; Charlesworth, 2006), resulting in a positive correlation of autogamy and the prevalence of harsh environmental conditions (Tate and Simpson, 2004; Busch and Schoen, 2008; but see Thiel-Egenter et al., 2009). Both populations included in the pollinator exclusion experiment were sampled at similar altitudes. In spite of the limited sampling of only two representative populations, a lineage-dependent response seems, therefore, more likely than an altitudinal response. Finally, our hypothesis of lineage-dependence of the breeding system is indirectly corroborated by the lack of correlation between altitude and genetic diversity within each of the three phylogeographical groups (data not shown).

4.3. Only partial congruence of genetic data with morphology

Our AFLP data do not fully confirm the intraspecific taxonomy of *H. alpina* as suggested by Englisch (1995) and Jalas et al. (1996) where three subspecies — subspp. *alpina*, *austroalpina*, and *brevicaulis* — were recognised in the Alps. Although a weak phylogeographical pattern was found within subsp. *alpina* and subsp. *austroalpina* (Fig. 3E and F), there is no evidence for the presence of yet unrecognised taxa.

Determination of our voucher specimens according to morphological characters (Table 2) revealed that 95.2% of the individuals of the N-group morphologically pertained to subsp. *alpina*. Importantly, the N-group was identified by STRUCTURE analysis of our AFLP data already at $K = 2$ (Fig. 3A). This finding is in congruence with an early account of Melchers (1932) who came up with a distribution map strikingly similar to ours (but combining subspp. *austroalpina* and *brevicaulis*).

In contrast to the clear morphological and genetic divergence of subsp. *alpina*, the differentiation of subspp. *austroalpina* and *brevicaulis* is comparatively weakly supported by our molecular data. Whereas the SE-group comprised 67.3% individuals that were morphologically assigned to subspp. *austroalpina* (25.0% were subsp. *brevicaulis*), only 59.0% of the SW-group was determined as subsp. *brevicaulis* (33.3% *alpina*, 7.7% subsp. *austroalpina*). With respect to subsp. *brevicaulis* the main source of incongruence relates to populations from the Hohe and Niedere Tauern in the Central Alps of Austria (populations 21, 23, 29, 30, 31, 39, 48, 114) which have been unambiguously assigned to subsp. *brevicaulis* on the basis of morphology, but genetically belong to the SE-group (SE:E-subgroup; Fig. 4B). In-depth morphometric analyses of collections from these areas are necessary to solve this discrepancy. The second source of incongruences is admixture. Most individuals assigned to subsp. *alpina* which genetically belong to the SW-group are in fact highly admixed (Fig. 4).

Our results are in contrast with previous AFLP and ITS results (Kropf et al., 2003) that did not provide a genetic basis for the differentiation of subspp. *alpina* and *brevicaulis* (subsp. *austroalpina* was not mentioned). The suggested divide between southwestern and northeastern Alps is not supported by our data, either, and probably relates to differences in the sampling density, i.e., 117 populations in our study versus 13 in Kropf et al. (2003).

Finally, a multiple origin of subsp. *brevicaulis* from geographically close populations of subsp. *alpina* was previously suggested (Kropf et al., 2003). Our analyses do not allow a straightforward testing of this hypothesis as PCoA and Bayesian clustering (Figs. 3 and 4) are non-hierarchical, and deeper nodes in the NJ analysis lack any bootstrap support (data not shown). A breeding system shift from self-incompatibility to self-compatibility is a simpler change that may more readily occur (Charlesworth, 2006) and allogamy is generally regarded as more ancestral as compared to autogamy. Hence, the divergence in breeding system may agree with a derived status of subspp. *brevicaulis* and *austroalpina* relative to subsp. *alpina* as hypothesised by Kropf et al. (2003). Our data are, however, not compatible with the hypothesis of multiple, local origin of subsp. *brevicaulis* (and subsp. *austroalpina*) but instead suggest that subsp. *brevicaulis* originated once in the southwestern Alps (see "Phylogeography of an alpine plant tolerant of different bedrock types"). Subsp. *austroalpina* likely does not have common ancestry with subsp. *brevicaulis*, and may have originated independently from two separated subsp. *alpina* genepools in Pleistocene refugia in the southern and southeastern Alps (Fig. 3E).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.08.009.

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