



Genetic structure of *Doronicum austriacum* (Asteraceae) in the Carpathians and adjacent areas: toward a comparative phylogeographical analysis of tall-herb species

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Abstract

Tall-herb communities play an important role in the vegetation of the European mountains. They are developed in humid habitats with nutrient-rich soil, from submontane to subalpine zones. As its contemporary flora consists of different geographical elements, their history, especially in relation to the climatic oscillations throughout the Pleistocene, is not straightforward. We analyze the phylogeography of *Doronicum austriacum*, one of the main species building Central European tall-herb communities. We additionally discuss these new data in the context of earlier phylogeographical studies of key species of tall-herb communities to compare the major patterns of their lineage distributions. The study was based on AFLP fingerprinting and DNA sequencing of sampled populations from the Carpathians and adjacent lowland, Alps, Sudetes, and Balkan Peninsula. Our results confirm the phylogeographical break between the Western and South-Eastern Carpathians as a major regional biogeographical feature. Close affinity of the Western Carpathian and Sudetes populations was also confirmed as a significant feature in common for studied tall-herb species. In contrast to the phylogeographical structure of other tall-herb species, a divergence of *D. austriacum* populations from the Balkan Peninsula was observed supporting the presence of divergent and taxonomically distinct lineage in the latter area. The general phylogeographical pattern indicates past survival in several distinct areas but only partly common features for the community level emerge. Finally, the history of low-elevation populations of *D. austriacum* at the northern margin of the distribution range remains unclear but mainly their close affinity to the forest Western Carpathian populations is suggested.

Keywords AFLP · cpDNA · European mountains · ITS · Refugia · Subalpine species

Introduction

The research on plant Quaternary biogeography serves a better understanding of the evolutionary history of individual species and plant assemblages in the past, and is also central

for forecasting of future climate-driven changes in vegetation. It was substantially stimulated by the advent of phylogeography and molecular tools allowing a fine-scale analysis of intraspecific genetic lineages. The European mountainous flora was among those which gained the most insight thanks to this approach because of inherent problems with other information sources, for instance, scarcity of available palaeobotanical record and its limited taxonomical resolution.

Among the plant species in Europe, much attention has been focused especially on high-mountain taxa, and this group is one of the best explored in terms of their genetic diversity, potential refugia, and ongoing microevolutionary processes (e.g., Kadereit et al. 2004; Ronikier et al. 2008; Alvarez et al. 2009; Thiel-Egenter et al. 2011; Pachschwöll et al. 2015). While, geographically, the Quaternary history of the Alps has mainly been addressed, recently the attention of researchers has also been directed to the Carpathian range (reviewed in Ronikier 2011 and Mráz and Ronikier 2016). The Carpathians are seen as a biodiversity hot spot

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Contribution to “Biogeography of the Carpathians”

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due to species richness (e.g., Tassenkevich 1998), plant communities' diversity (e.g., Pawłowski 1966; Coldea 1991; Stachurska-Swakoń 2009a, b; Chytrý et al. 2015), endemism (e.g., Pawłowski 1970; Hurdu et al. 2016; Mráz et al. 2016) and area of taxa speciation (e.g., Cieślak et al. 2000; Španiel et al. 2011; Mitka et al. 2015a, 2016; Sutkowska et al. 2017).

When considering the mountain plant cover, few studies concerned the phylogeography of species forming subalpine tall-herb communities in Central Europe. These communities are built by perennial plants which require moist and fertile habitats (Pawłowski 1966; Karner and Mucina 1993; Kliment et al. 2007; Stachurska-Swakoń 2009a). They are important for the ecology and functional diversity of the subalpine zone, and their altitudinal and geographical range can be broad (Stachurska-Swakoń 2009c, 2011). According to previous studies of subalpine species, the Carpathian range plays an important role in shaping and preserving their extant genetic diversity. Available studies indicate that their phylogeographical structure across the European distribution range is shallower than that of the most alpine species, and they are representative for the history of the mountain species with larger elevational amplitude (Després et al. 2002; Michl et al. 2010; Stachurska-Swakoń et al. 2011, 2012, 2013a, b; Ekrtořová et al. 2012; Frajman et al. 2016; Sutkowska et al. 2017). However, phylogeographical studies on two key components of tall-herb communities, *Cicerbita alpina* (L.) Wallr. (syn. *Lactuca alpina* (L.) Gray) and *Ranunculus platanifolius* L., revealed a barrier between the Western and the South-Eastern Carpathians supporting a distinct glacial history of these areas (Stachurska-Swakoń et al. 2011, 2012, 2013a). They also pointed at the genetic similarity between the Southern Carpathians and northern Balkan populations that demonstrated the past connection between these two geographically distinct areas. The difference in genetic variation across the geographical range between these two species, lower in *C. alpina* and higher in *R. platanifolius*, was hypothesized to be influenced by different dispersal mode: anemochorous in *C. alpina* and barochorous in *R. platanifolius*. However, in both species, the isolated geographical regions were mirrored in genetic diversity due to inferred local refugia.

In the present study, we have chosen *Doronicum austriacum* Jacq., one of the species building tall-herb communities (Stachurska-Swakoń 2009b) but with a broader ecological amplitude (Stachurska-Swakoń and Kuř 2011). The species represents the genus that has relatives in Asia, but it is suggested that its important diversification took place in Europe (Álvarez Fernández et al. 2001; Skof et al. 2019). The main distribution range of *D. austriacum* covers the Carpathians, Eastern Alps, Eastern Sudetes, mountains of the northern part of the Balkan Peninsula; it becomes rare in the western part of Europe: the southwestern Alps, eastern Pyrenees and northern Apennines (Meusel and Jäger 1992). The species

has also detached, isolated localities north of the Western Carpathians but not exceeding the Góry Świętokrzyskie Mts. to the North (Zajac and Zajac 2001) and north of the Eastern Alps (Hegi 1987).

Taking into consideration previous studies on phylogeography of subalpine species, we approached the following questions: (i) does the genetic structure of *D. austriacum* in the Western and South-Eastern Carpathians reflect the different Quaternary history of these regions, (ii) what are the relationships between the Carpathians and neighboring major mountain ranges inferred from the genetic pattern of *D. austriacum* (including a possible divergence of the southern populations). As a separate form of the species was proposed in the Polish lowland (Piech 1924), we would also like to check (iii) whether the northernmost low-elevation populations are genetically diversified from those in the main range. Finally, we attempt to compare large-scale phylogeographical patterns of three tall-herb species to assess the congruence of their late Quaternary history.

Materials and methods

Study species

Doronicum austriacum (Asteraceae, Tubuliflorae, Senecioneae) is one of 26 species of the genus distributed in the northern hemisphere and having its center of diversity in Europe, where it is mainly distributed in the mountains (Álvarez Fernández 2003). According to the phylogenetic study of the genus, its early diversification took place in the Mediterranean basin and *D. austriacum* is a sister species to a large clade comprising seven European taxa, five southwestern Asian taxa, and one central Asian taxon (Álvarez Fernández et al. 2001). On the other hand, there was no barrier in sexual reproduction and outcrossing was experimentally proven for *D. austriacum* and *D. grandiflorum*, representing the latter clade (Wcisło 1952). Although no extended genetic studies are available for this species, in the phylogeny of the genus *Doronicum*, a divergence between northern- and southernmost populations of *D. austriacum* was apparent (Álvarez Fernández et al. 2001). Recently, the presence of a separate evolutionary lineage in South-Eastern Europe was supported by Skof et al. (2019) who discussed its possible correspondence to a separate taxon described from this area and partly neglected, *D. orphanidis* Boiss. (*D. austriacum* Jacq. subsp. *giganteum* (Griseb.) Stoj. & Stef.).

Doronicum austriacum is a perennial species forming a short rhizome without runners and with numerous adventitious roots. The abundantly leaved stem grows up to 150 cm high (Kucowa 1971; Álvarez Fernández 2003; Stachurska-Swakoń and Kuř 2011). At the top, the stem branches into a sub-umbelliferous form. The plant produces 1 to 16 capitula

on long peduncles; capitula could differ in diameter between 2 and almost 8 cm. The yellow inflorescence consists of two flower types: female (ligulate) and bisexual (disc florets). The species bloom in late spring/early summer (May–July) depending on geographical location. The fruit is an achene, up to 2 mm long. *D. austriacum* produces two types of achenes: those developing from disc florets have pappus and stiff hairs on the ribs whereas those developing from ligulate florets are naked and without pappi. The species reveals phenotypic plasticity influenced by habitat conditions (Stachurska-Swakoń and Kuź 2011; Kostrakiewicz-Gierałt and Stachurska-Swakoń 2013). It is diploid with $2n = 60$.

Plant material and DNA isolation

Fifty-two populations of *Doronicum austriacum* (297 specimens in total) were sampled across the distribution range of the species but with a focus on the Central European range, especially the Carpathians (Table 1, Fig. 1). For the purpose of this study, we considered *D. austriacum* in its wide sense, i.e., including the populations from the Balkan Peninsula. Leaf samples from three to eight specimens (usually six per population) were collected in each population. The sampled individuals were randomly selected in each population. Fresh leaf material was placed in hermetic plastic bags or tubes with silica gel for quick drying. Dried samples were stored in hermetic plastic boxes at room temperature. The sampled specimens originated from: the Eastern Alps, Sudetes, Carpathians (western, eastern, and southern parts), isolated low-elevation populations located to the north of the Carpathians (Polish lowlands and Świętokrzyskie Mts., including the northernmost locality in the species' range), and the Balkan Peninsula: Mt. Snežnik in the Dinarides, Baba, Jakupica, Osogovska Planina, Rhodopes, Rila, and Vitosha. The vouchers for a part of populations are stored in the herbarium of the Institute of Botany, Jagiellonian University (KRA).

Total DNA was extracted from c. 15 mg of dried leaf tissue per sample using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's protocol. The quality of isolates was assessed on 1% agarose TBE gels.

Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP; Vos et al. (1995) analysis was carried out following the protocol of Paun and Schönswetter (2012), as described in detail in Migdalek et al. (2017). A preliminary screening of 14 selective primer pair combinations performed on four samples from geographically distant populations was conducted. Three pairs of selective primers were chosen on the basis of polymorphism, clarity and reproducibility of AFLP profiles:

EcoR1-AAG/Mse1-CTG, EcoR1-ACC/Mse1-CAT, and EcoR1-ACA/Mse1-CTA.

AFLP data were manually scored in Genographer v.2.1 (<http://sourceforge.net/projects/genographer>). AFLP markers were scored in the size range 50–500 bp and coded as present (1) or absent (0) in a binary data matrix.

Sequencing of cpDNA and nrDNA fragments

Based on a preliminary screening of eight plastid DNA regions, a variable, non-coding cpDNA intergenic spacer *trnL(UAA)-trnF(GAA)* (Taberlet et al. 1991) was selected and analyzed in a subset of plants (one to three per population) from 32 populations (63 samples in total; Table 1, Online Resource 1) representing all sampled geographical regions. In addition, the ITS1 + ITS2 fragment of the internal transcribed spacer region (ITS) of the nuclear ribosomal DNA (nrDNA) was analyzed for plants from 33 populations (95 samples in total; Table 1, Online Resource 1). This region was amplified using universal primers ITS1 and ITS2 (White et al. 1990; Blattner 1999).

The composition of PCR mixture and program for the *trnL-trnF* region was used according to Shaw et al. (2007), with AmpliTaq DNA Polymerase (ThermoFisher Scientific Inc.), BSA (New England Biolabs) and dNTP (Sigma-Aldrich Co.). In a part of samples, DNA was diluted 50× prior to PCR.

For ITS, the PCR mixture, in a total volume of 25 µL, contained: 1 U AmpliTaq DNA (Applied Biosystems, Thermo Fisher Scientific Inc.), 1×PCR Buffer supplied with the enzyme (Applied Biosystems, Thermo Fisher Scientific Inc.), 2.5 mM MgCl₂ (Applied Biosystems, Thermo Fisher Scientific Inc.), 0.3 µM of each primer, 0.12 mM dNTP (Sigma-Aldrich Co.), 0.8% BSA in concentration 1 mg/ml (New England BioLabs Inc.) and 1 µL of DNA template. The following touchdown PCR cycling profile was used: 3 min at 94 °C; 10 cycles of 30 s at 94 °C, 30 s at 60 °C (with a decrease of 1 °C per cycle) and 1 min at 72 °C; 25 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C; final extension step of 7 min at 72 °C; cooling to 4 °C. When the above conditions failed, alternatively parameters according to Sabovljević and Frahm (2011) with RedTaq DNA Polymerase (Sigma-Aldrich Co.) and DNA template diluted 50× were used.

Enzymatic purification of the PCR product was conducted using a mix of exonuclease I (10 U/µl; EURx) and alkaline phosphatase (1 U/µl; Affymetrix). Sequencing was performed using BigDye Terminator ver. 3.1 Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific Inc.) with buffer BDX64 (BigDye Enhancing Buffer; MCLAB) according to BDX64 buffer manufacturer's recommendations for 32-fold dilution of BigDye chemistry. Amplifications and incubations were performed in the GeneAmp PCR System or Veriti 96-well

Table 1 Geographical origin and genetic characteristics of the sampled populations of *Doronicum austriacum*. Country: A Austria, BG Bulgaria, MA Macedonia, PL Poland, RO Romania, SK Slovakia, SL Slovenia, UA Ukraine. *N* number of samples, *P* total number of AFLP markers, % poly percentage of polymorphic AFLP markers, *Div* Nei's gene diversity based on AFLP markers, *Shannon* value of Shannon index based on AFLP markers, *DW* frequency-downweighted marker values, *M_{priv}* private markers, *ITS* ribotype, in brackets number of individual represented, *cpDNA* plastid haplotype, in brackets number of individuals per population

Sampling locality	Acronym	Coordinates	Elevation (m a.s.l.)	N	P	% poly	Div	Shannon	Fixed bands	DW	M _{priv}	ITS	cpDNA
Carpathians: Western Carpathians													
1	Belińskie Tatry, Śiroké Sedlo, SK	SB	N 49.14, E 20.13	1700	5	307	36.90	0.17	7.33	121	8.31	0	R4(3), H4(2), H6(1)
2	Beskid Mały, Kocierz Rychwałdzki, PL	PM	N 49.45, E 19.21	614	6	282	34.52	0.16	7.25	108	8.98	0	R3(1), R4(2), H4(1)
3	Beskid Sądecki, Krzyżówki, PL	PS	N 49.27, E 20.57	740	6	285	35.12	0.16	7.28	108	10.11	0	H4(3)
4	Beskid Sądecki, Pasma Radziejowej, PL	PR	N 49.28, E 20.34	989	6	232	12.70	0.06	5.8	168	15.37	1	R3(3), H4(1)
5	Beskid Żywiecki, Babia Góra I, PL	B	N 49.35, E 19.33	1200	7	320	45.04	0.19	7.61	93	18.25	3	R4(1), H4(1)
6	Beskid Żywiecki, Babia Góra II, PL	BA	N 49.34, E 19.31	1360	6	275	29.76	0.14	7.05	125	8.86	0	H4(2)
7	Beskid Żywiecki, Sopotnia Wielka, PL	PZ	N 49.33, E 19.17	865	6	280	52.58	0.23	7.84	15	6.32	0	H4(3)
8	Beskid Żywiecki, Wielka Racza, PL	R	N 49.24, E 19.00	1500	5	285	34.52	0.17	7.26	111	6.65	0	R4(2), R7(1), H4(3)
9	Gorce, Jaworzynka, PL	G	N 49.35, E 20.12	1180	6	261	43.65	0.19	7.61	41	6.46	0	H4(1)
10	Gorce, Bieniowe, PL	GB	N 49.33, E 20.10	1080	6	309	43.06	0.19	7.53	92	9.56	0	H4(3)
11	Mała Fatra, Chleb, SK	SM	N 49.11, E 19.03	1480	7	297	32.94	0.15	7.17	131	10.78	0	H1(1), H4(2)
12	Nízke Tatry, Chopok, SK	SN	N 48.56, E 19.35	980	7	276	34.33	0.14	7.2	103	9.98	0	H4(3)
13	Tatry, Hala Gąsienicowa, PL	TG	N 49.14, E 20.00	1500	6	249	27.38	0.12	6.91	111	8.17	0	H4(3)
14	Tatry, Kriváň, SK	SK	N 49.09, E 19.59	1580	5	294	32.14	0.16	7.17	132	8.25	1	H4(3)
15	Tatry, Morskie Oko, PL	TO	N 49.11, E 20.02	1400	6	263	28.77	0.13	7	118	7.54	0	H1(1), H4(1)
16	Tatry, Skalnate Pleso I, SK	SS	N 49.07, E 20.03	1350	6	280	31.35	0.15	7.14	122	9.65	1	H4(1)
17	Tatry, Skalnate Pleso II, SK	ST	N 49.11, E 20.13	1760	6	256	24.01	0.10	6.72	135	8.84	0	H4(1)
18	Tatry, Velická Dolina, SK	SD	N 49.14, E 20.17	1580	6	280	27.38	0.13	6.94	142	8.94	0	H4(1)
Eastern Carpathians													
19	Bieszczady, Połonina Wetlińska, PL	BW	N 49.16, E 22.53	1180	6	265	35.91	0.17	7.33	84	21.45	2	R4(2), R9(1), H4(1)
20	Bieszczady, Rozspaniec, PL	BS	N 49.06, E 22.76	1200	6	281	49.01	0.22	7.73	34	8.91	0	H4(1)
21	Chornohora, Cybulnik, UA	UY	N 48.16, E 24.50	1250	6	301	47.62	0.22	7.7	61	13.40	1	R4(3)
22	Chornohora, Howerla, UA	UH	N 48.16, E 24.9	1500	5	275	32.74	0.17	7.21	110	10.90	0	H4(1)
23	Chornohora, Zaruslyak I, UA	UC	N 48.16, E 24.53	1520	7	288	34.13	0.16	7.27	116	17.55	0	H4(1)
24	Chornohora, Zaruslyak II, UA	UZ	N 48.16, E 24.53	1385	5	321	47.62	0.23	7.71	81	8.58	0	H4(1)
25	Gorgany, Mt Berezovacky Klyvky, UA	U	N 48.46, E 24.31	1250	5	295	40.67	0.20	7.49	90	8.41	0	R4(2), R11(1), H4(1)
26	Munții Ceahlău, Ocolașul Mare, RO	RC	N 46.56, E 25.57	1860	5	307	42.26	0.21	7.54	94	9.42	1	R4(3), H4(1)
27	Munții Harghita, RO	RH	N 46.24, E 25.71	1220	6	287	43.06	0.19	7.54	70	10.49	0	R4(3), H4(2)
28	Munții Rodnei, Pietrosu Mare, RO	RY	N 47.35, E 24.38	1450	5	286	43.45	0.21	7.57	67	8.67	0	R4(3), H4(1)

Table 1 (continued)

Sampling locality	Acronym	Coordinates	Elevation N (m a.s.l.)	N	P	% poly	Div	Shannon	Fixed bands	DW	Mpriv	ITS	cpDNA
Southern Carpathians													
29 Munții Făgăraș, Valea Sâmbetei, RO	RF	N 45.37, E 24.48	1300	8	316	49.01	0.20	7.65	69	13.31	1	R4(3)	H4(1)
30 Munții Făgăraș, Vârful Suru, RO	RG	N 45.36, E 24.24	1200	5	270	37.30	0.18	7.37	82	10.12	1		
31 Munții Retezat, Buta, RO	RE	N 45.31, E 22.90	1860	5	300	40.87	0.20	7.5	94	10.52	0	R4(2)	H4(2), H8(1)
32 Munții Retezat, Valea Radeșu Mare, RO	RR	N 45.21, E 22.46	1500	5	292	42.26	0.21	7.55	79	8.11	0		
33 Munții Retezat, Zlatului, RO	RZ	N 45.33, E 22.81	1450	7	272	53.57	0.23	7.84	47	14.69	0		
34 Munții Banatului, Vârful Semenec, RO	RA	N 45.16, E 21.98	1380	6	274	36.51	0.16	7.3	90	10.77	2	R4(2), R2(1)	H4(1)
Apuseni Mountains													
35 Masivul Vlădeasa, RO	RP	N 46.61, E 22.76	1500	5	317	40.67	0.20	7.49	67	6.95	1	R4(1), R5(1), R10(1)	H4(1), H5(1)
Alps													
36 North Limestone Alps, Steiermark, A	AA	N 47.28, E 14.33	1200	5	289	52.58	0.25	7.91	24	13.59	0	R2(3), R7(1)	H4(1)
Balkan Peninsula													
37 Dinariides, Snežnik, SL	AS	N 45.35, E 14.26	1640	5	284	32.94	0.17	7.21	118	13.35	0	R2(4)	
38 Jakupica, Solunska glava, MA	MJ	N 41.43, E 21.24	1800	5	282	38.69	0.20	7.47	87	18.94	1	R6(3)	H10(1)
39 Baba, Pelister, Kopaonke, MA	MP	N 41.14, E 21.12	1700	5	298	44.25	0.22	7.61	75	13.80	0	R6(3)	H2(1)
40 Osogovska Planina, Ruen, MA	MO	N 42.09, E 22.30	1800	5	294	40.28	0.20	7.48	91	15.53	0		
41 Rhodopes, Maliak Perelik, BG	BZ	N 41.36, E 24.33	2000	2	233	18.45	0.18	6.53	140	6.28	0	R1(1), R6(1)	H2(2)
42 Rila, Maljovica, BG	BR	N 42.11, E 23.22	1700	5	274	37.30	0.19	7.39	86	18.70	1		H2(1), H11(1)
43 Vitosha I, BG	BL	N 42.36, E 23.14	1800	7	286	37.90	0.16	7.33	95	25.68	0		H2(2), H12(1)
44 Vitosha II, BG	BV	N 42.35, E 23.33	2000	5	283	39.09	0.20	7.46	86	14.39	0	R1(1), R6(2)	H2(2)
Sudetes													
45 Sudety, Góry Bialskie, PL	KB	N 50.16, E 17.00	826	6	269	27.38	0.12	6.9	131	9.40	0	R4(2)	
46 Sudety, Masyw Śnieżnika, PL	KS	N 50.16, E 16.47	820	6	276	28.77	0.13	6.97	131	11.43	0	R4(3)	H4(3)
Góry Świętokrzyskie Mts. and Polish lowlands (north of Western Carpathians)													
47 Góry Świętokrzyskie, Pasma Kłownowskie, PL	XK	N 50.57, E 20.46	330	6	306	41.07	0.18	7.46	99	11.83	0	R4(3)	H3(1), H4(2)
48 Wyżyna Krakowsko-Częstochowska, Biata 1, PL	P	N 50.32, E 19.49	290	6	294	36.31	0.17	7.34	111	12.10	0	R2(2), R4(1)	H4(1)
49 Wyżyna Krakowsko-Częstochowska, Biata 2, PL	XP	N 50.32, E 19.49	290	6	260	33.33	0.14	7.19	92	9.14	0	R5(3)	H4(2)
50 Wyżyna Małopolska, Suchedniów, PL	XB	N 51.01, E 20.49	356	6	267	28.97	0.13	7.01	121	11.25	0	R4(3)	H4(2)
51 Wyżyna Śląska, Mikołów I, PL	XM	N 50.18, E 18.73	285	6	288	34.13	0.15	7.2	116	8.57	0	R4(1), R5(1), R8(1)	H3(1), H4(1), H7(1)
52 Wyżyna Śląska, Mikołów II, PL	XH	N 50.18, E 18.72	310	6	270	29.37	0.13	7.01	122	11.37	0		

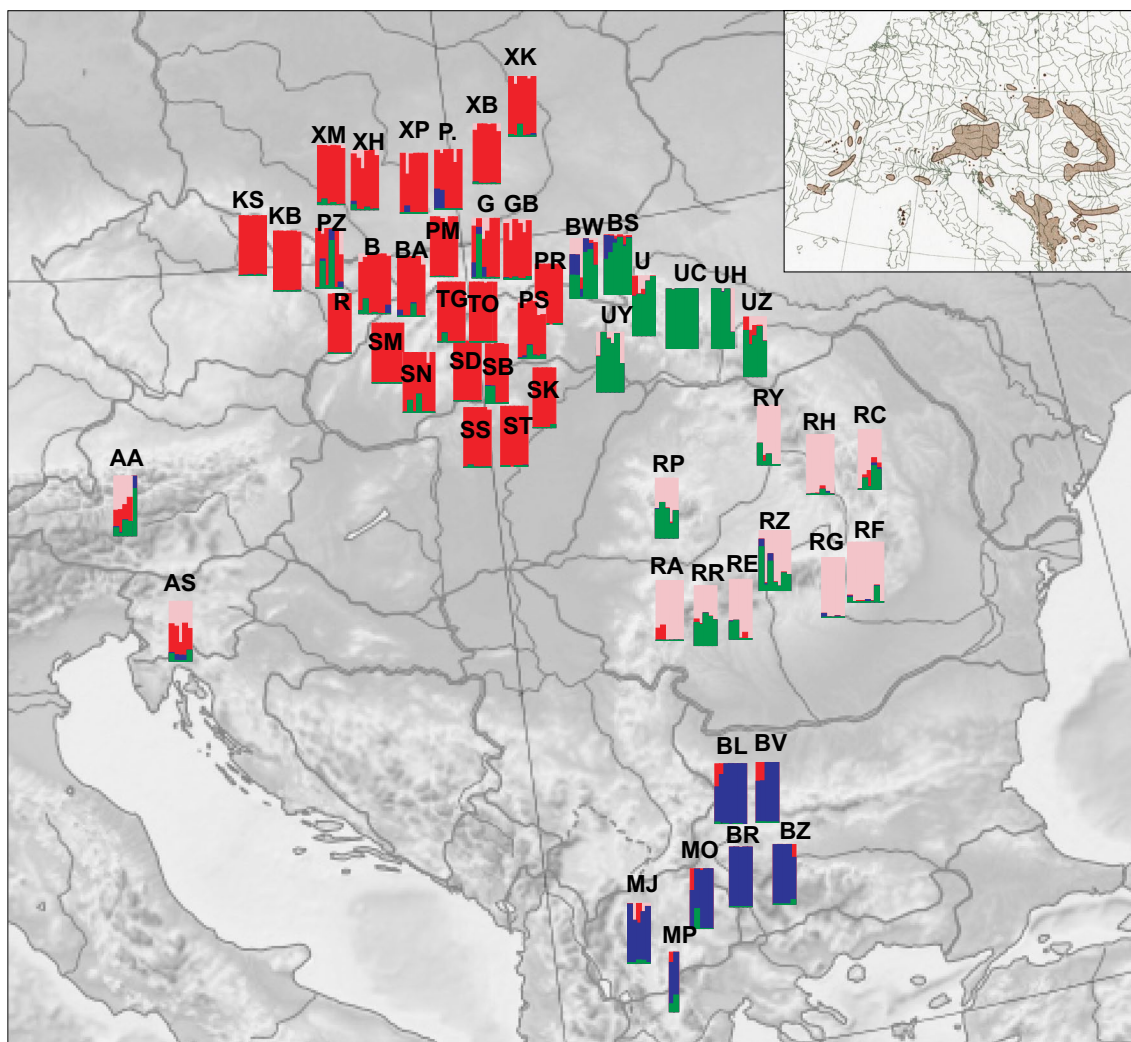


Fig. 1 Localities of sampled populations of *Doronicum austriacum*. Details of populations are given in Table 1. Charts give the proportions of the clusters present in the populations detected by the Bayes-

ian analysis (STRUCTURE software) for $K=4$ on the base of AFLP markers. The insert map shows the geographical range of the species according to Meusel and Jäger (1992)

thermocyclers (Applied Biosystems). Cycle sequencing products were purified by EDTA/ethanol precipitation, resuspended in 12 μ L formamide, and separated on an ABI 3130 Genetic Analyzer equipped with 36 cm capillaries and a POP-7 polymer (Applied Biosystems). In all samples, both strands were sequenced using the same primers as for the PCR.

The cpDNA and nrDNA sequences were aligned using Geneious 10.2.3 software (<https://www.geneious.com>). Additive nucleotide polymorphisms of ITS sequences were analyzed and coded using IUPAC nucleotide ambiguity codes.

Data analyses

Several basic statistics based on AFLP data were calculated for each population using the R-script AFLPdat (R

Development Core Team 2004; Ehrlich 2006): the total number of AFLP bands (P), percentage of polymorphic markers (% poly), Nei's gene diversity in populations (Div) (Nei 1987), and frequency-downweighted marker values (DW). Shannon index, the fixed numbers of bands, and the number of private markers restricted to defined geographical groups were assessed with FAMD software (Schlüter and Harris 2006). The Pearson correlation coefficient was used to check the possible correlation between the geographical location of a population and within-population genetic variation (Statistica 13.0 software).

General relationships of studied individuals were assessed by a principal coordinate analysis (PCoA) of the whole dataset based on Jaccard's similarity coefficient with FAMD software and by neighbor-joining (NJ) unrooted tree based on the population matrix of Nei and Li distances and bootstrapped using 1000 replicates with

FAMD software. The NJ analysis was also made on the population level (bootstrapped with 1000 replicates) for better visualization of the relationship between studied regions. Bayesian clustering of individuals was applied using STRUCTURE 2.2.3 (Falush et al. 2007), based on an admixture model with correlated allele frequencies. Numbers of K in the range 2–10 were tested with ten replicates per K ; 1×10^6 Markov chain Monte Carlo repetitions were applied with a burn-in period of 200 000. Outputs of all STRUCTURE runs were analyzed using the R-script Structure-sum (Ehrich 2006). Average similarity coefficients among runs were calculated for each K to verify the consistency of replicated runs. The following values were observed in order to assess the most appropriate number of clusters: (1) the $\ln P(D)$ values, estimates of the posterior probabilities provided in STRUCTURE outputs, examined as a function of increasing K ; (2) ΔK values, estimating the change in the likelihood function with respect to K , and estimated as an indicator of the most reliable clustering structure (Evanno et al. 2005).

The genetic structure of populations and variation levels were assessed by an analysis of molecular variance (AMOVA; Excoffier et al. 1992). Significance levels were determined using 1023 permutations. The analysis was conducted at three different hierarchical levels: within populations, among populations and among groups of populations (geographical groups and genetic groups detected by PCoA, neighbor-joining, and Bayesian analyses). AMOVAs and F_{ST} values were calculated using Arlequin 3.5.5 (Excoffier et al. 2007).

To test the correlation between the genetic and geographic distance of the studied populations, pairwise F_{st} values were calculated using Arlequin 3.5.5 software.

The alignment of ITS markers (Online Resource 2) and plastid markers (Online Resource 3) was analyzed using statistical parsimony as implemented in TCS Network (Clement et al. 2000) in PopART software (Leigh and Bryant 2015).

Results

AFLP data

AFLP analysis yielded 504 markers with 98% being polymorphic. The reliability of AFLP band was high, data quality test indicated repeatability of 98%. The number of bands recorded in populations varied from 232 (PR, Western Carpathians) to 321 (UZ, Eastern Carpathians), with a mean of 283.3 (SD=13.15). Polymorphic markers were detected in all studied populations in the range from 12.7% (PR, Western Carpathians) to 53.57% (RZ, Southern Carpathians), with a mean of 36.99% (SD=6.47). Generally,

the percentage of polymorphic markers was higher in the southern part of the sampled species range (Table 1), but this correlation was not statistically significant (Pearson correlation $p > 0.05$, $r^2 = 0.04$).

The Nei and Shannon coefficients for the populations ranged from 0.06 (PR, Western Carpathians) to 0.25 (AA, Alps) (mean 0.17, SD=0.03) and from 5.8 (PR, Western Carpathians) to 7.91 (AA, Alps) (mean 7.31, SD=0.26), respectively (Table 1). The frequency-downweighted values (DW) ranged from 6.28 (BZ, Balkan Peninsula) to 25.68 (BL, Balkan Peninsula) with a mean of 11.32 (SD=3.06). The private markers were present in few populations only throughout the species' range: B (Western Carpathians, 1 private marker), BW (Eastern Carpathians, 2), MJ (Balkan Peninsula, 1), PR (Western Carpathians, 1), and UY (Eastern Carpathians, 1). The Pearson correlation between geographical locations and genetic parameters was significant for Nei's index (for $N y = -0.05x + 0.45$, $r^2 = 0.2$, for $E y = 0.05 + 0.07$, $r^2 = 0.1$), DW (for $N y = -0.6x + 39.9$, $r^2 = 0.2$) and number of bands (for $E y = -1.6x + 221.1$, $r^2 = 0.1$).

All pairwise F_{st} values were significant ($P < 0.05$), and the mean value amounted to 0.34 (SD=0.10). The lowest values of pairwise F_{st} were found within regions and reached 0.02 between populations U and UZ (Eastern Carpathians). The highest value of 0.76 characterized geographically very distant populations PR (Western Carpathians) and BZ (Balkan Peninsula). Generally, F_{st} values between the PR population and other populations were relatively high, especially in relation to the populations originated from the Balkan Peninsula (0.64–0.76). For most populations, pairwise F_{st} ranged from 0.39 to 0.62.

The PCoA scatter diagram of sampled individuals of *D. austriacum* predominantly displayed a division into three groups along the first two axes (Fig. 2). In general, these groups corresponded to geographical regions of origin. The most distinct was a group which contained plants from the mountain ranges of the Balkan Peninsula except the northernmost Dinaric population. The second group was composed of individuals from the South-Eastern Carpathians, Alps, Dinarides, and included a few individuals from the northern part of the range (populations PZ and G from the Western Carpathians and P from the Polish lowland). The third compact group included populations from the Western Carpathians, Sudetes, and the Polish lowlands. Subsequent axes in the PCoA did not display significant divisions in the data.

In the Bayesian analysis, the mean $L(K)$ and ΔK values indicated $K = 2-4$ as the most appropriate numbers of groups. These results are congruent with groups segregated by PCoA. At $K = 2$ the individuals showed a geography-related division between the Western Carpathians with Sudetes and Polish lowlands vs South-Eastern Carpathians, Alps, and

Balkan Peninsula. At $K=3$ the group of individuals from the Balkan Peninsula was distinguished as a separate group. At $K=4$, the group of individuals from the South-Eastern Carpathians and Alps were additionally split to the Eastern Carpathian group and the Southern Carpathian/Alpine group. However, individuals from the Alps showed some affinities to the Western Carpathians (Fig. 1), which was also congruent with the sample dispatch in the PCoA diagram (Fig. 2).

The neighbor-joining diagram based on population level of AFLP markers revealed the main split between the northern part of the sampled area (Western Carpathians, Sudetes, Polish lowland) and southern and western part (Alps, South-Eastern Carpathians, Balkan Peninsula—Fig. 3) which is congruent with the Bayesian analysis at $K=2$ (Fig. 6). The distinct position of the Balkan populations, except the Mt. Snežnik population, was highlighted with the relatively high bootstrap value (73). Mt. Snežnik population from the northern Dinarides showed affinity to the population from the Alps. Interestingly, individuals from lowland populations north of the Carpathians were partially separated from the Western Carpathians. As in other analyses, the neighbor-joining tree confirmed the genetic similarity of plants from the Sudetes and the Western Carpathians.

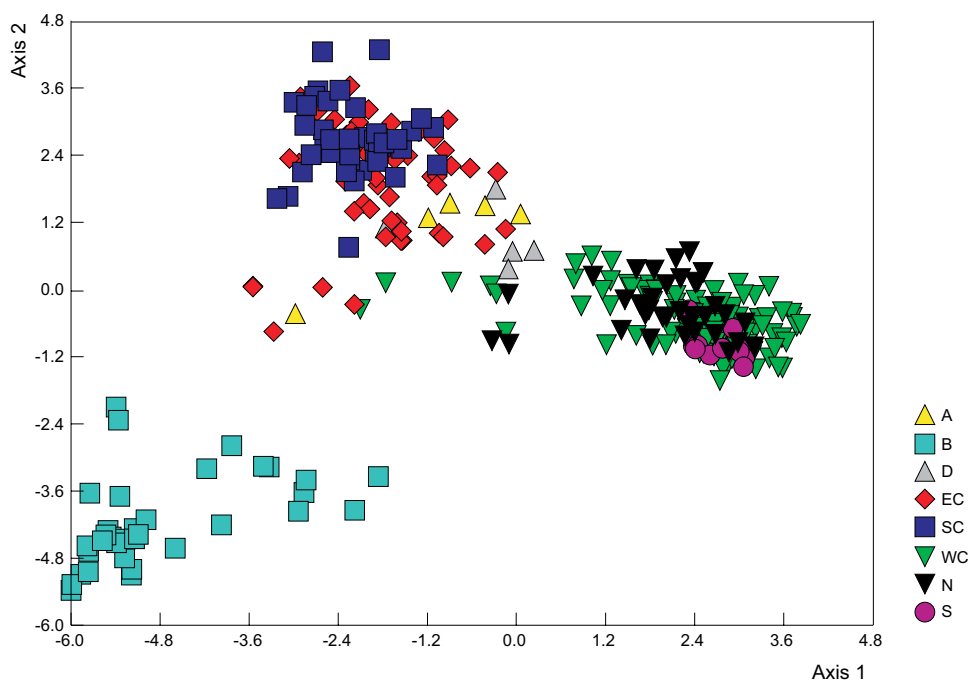
The results of AMOVA pointed, in general, at a higher proportion of variation within populations than between populations ($F_{st}=0.35$, Table 2). While testing the PCoA groups, the variation amounted to 22.8% among three groups, 18.6% among populations and 58.7% within populations ($F_{st}=0.41$). At the level of STRUCTURE groups, the variation among groups was 22.3%, 18% among

populations and 59.7% within populations ($F_{st}=0.40$). We also tested AMOVA when separating the Alps and part of South-Eastern Carpathians samples according to the STRUCTURE results yielding additional split at $K=4$ (see Fig. 1); it resulted in 17.1% of the variance between groups, 12.4% among populations, and 70.5% within populations ($F_{st}=0.29$). The percentage of variation between Western and Eastern Carpathians populations, where the main split was made for all data in the STRUCTURE analysis, was 18.1% with $F_{st}=0.37$.

nrDNA ITS variation

The ITS sequences displayed an intraspecific diversity and revealed eleven ribotypes in the data set (Table 1, Fig. 4). In populations where more than one individual was sequenced, a within-population variation of ribotypes was detected. Ribotype R4, distributed across the Carpathians, the Sudetes, and the Polish lowland populations, had the highest frequency in the whole data set. Populations from the Dinarides and the Northern Alps shared ribotype R2, which was also found in two individuals from the Polish lowlands population (P) and one individual from the Southern Carpathians (RA). Populations from the Balkan Peninsula bore two ribotypes not noted elsewhere: R6 dominating in all tested Balkan Peninsula populations and R1 detected in two individuals originated from the Rhodopes (BZ) and Vitosha (BV). Finally, the analysis detected also rare ribotypes, sometimes dispersed across geographically distant populations. Ribotype R5 was found in three populations,

Fig. 2 Principal coordinate analysis (PCoA) of the *Doronicum austriacum* individuals based on Jaccard's similarity coefficient of amplified fragment length polymorphism (AFLP) phenotypes (Axis 1 = 29.3%, Axis 2 = 16.1%). A Alps, B mountain ranges of Balkan Peninsula, D Dinarides, EC Eastern Carpathians, SC Southern Carpathians, WC Western Carpathians, N Polish lowlands, S Sudetes



one from the Southern Carpathians (RP), and two from the Polish lowlands (XM, XP). R3 was found in two isolated Western Carpathians populations (PM, PR) and R7 in two individuals: one from the Alps (AA) and one from the Western Carpathians (R). Ribotypes unique for populations were detected in different geographical regions (ribotypes R8-R11): Polish lowlands (XM), Eastern Carpathians (BW, U), Southern Carpathians (RP). The maximum parsimony network revealed the relation between detected ribotypes and pointed out three geographical regions important for the genetic variability: the Alps, the Carpathians, and mountain ranges of the Balkan Peninsula.

Plastid DNA variation

The studied plastid DNA (cpDNA) sequences had a similar level of variation as ITS. The analysis revealed twelve haplotypes but two of them were the most distinctive and frequent

(Table 1, Fig. 5). The most frequent was haplotype H4 distributed across the whole Carpathian range, the Alps and the Sudetes. The haplotype H2 occurred in the southern Balkan Peninsula populations. The haplotype H3 was found in two Polish lowland populations (XM, XK). The haplotype H1 occurred in two Western Carpathians populations (SS, SM). Remaining haplotypes were minor variants detected in single individuals across dataset: 3 haplotypes in the Balkan Peninsula populations (H10-H12), 2 haplotypes in the Southern Carpathians (H5, H8), 1 in the Western Carpathians (H6), 2 in the Polish lowlands (H7, H9). The populations from the Polish lowlands were the most diverse as four haplotypes were detected in six populations, out of which three were unique for the area.

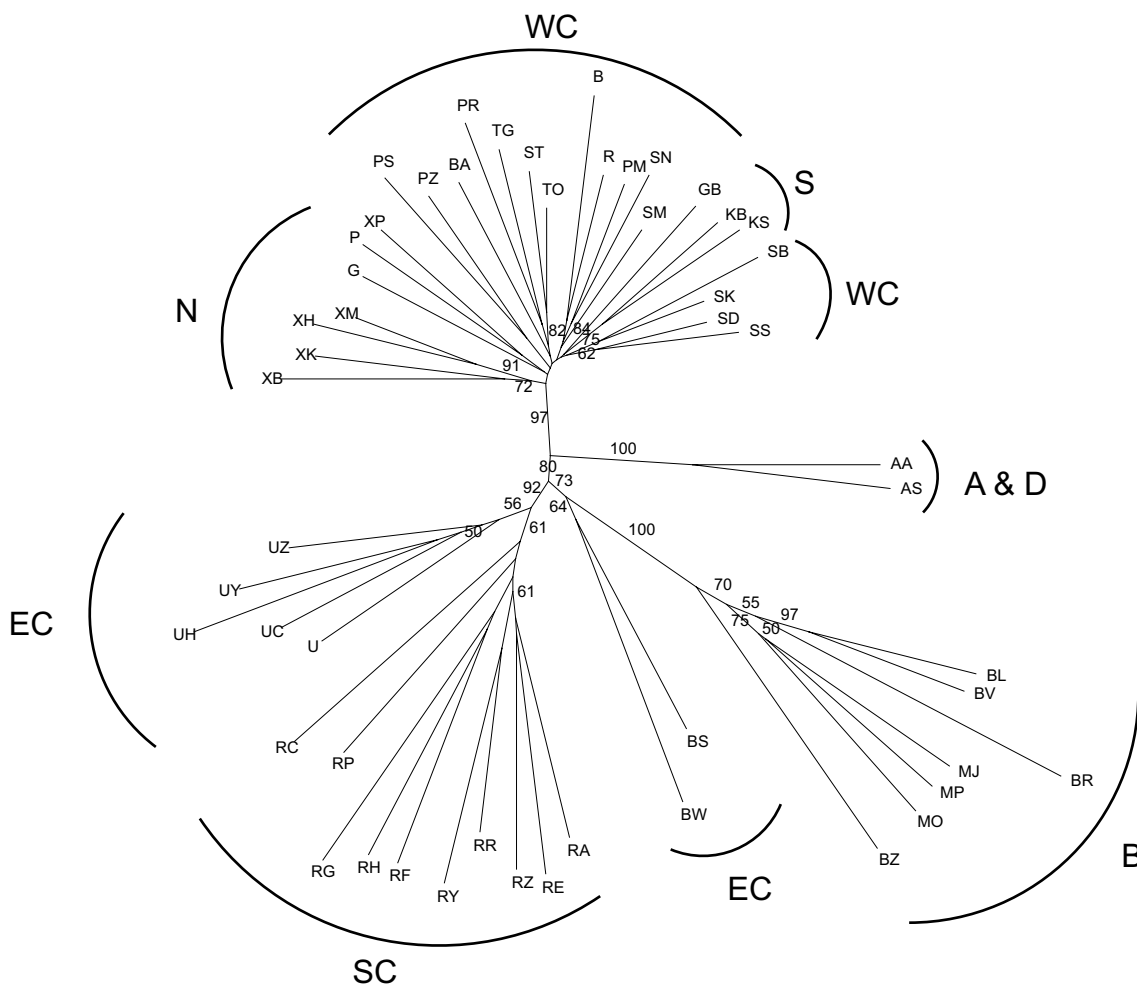


Fig. 3 Neighbour-Joining tree of the *Doronicum austriacum* populations based on AFLP markers (Nei and Li coefficient). Numbers indicate bootstrap values higher than 50% (1000 replicates) for main

splits. Bold names are for geographical regions as in the Fig. 2. Acronyms of populations correspond to Table 1

Table 2 Analysis of molecular variance (AMOVA) of *Doronicum austriacum* including different hierarchical levels and geographical groups

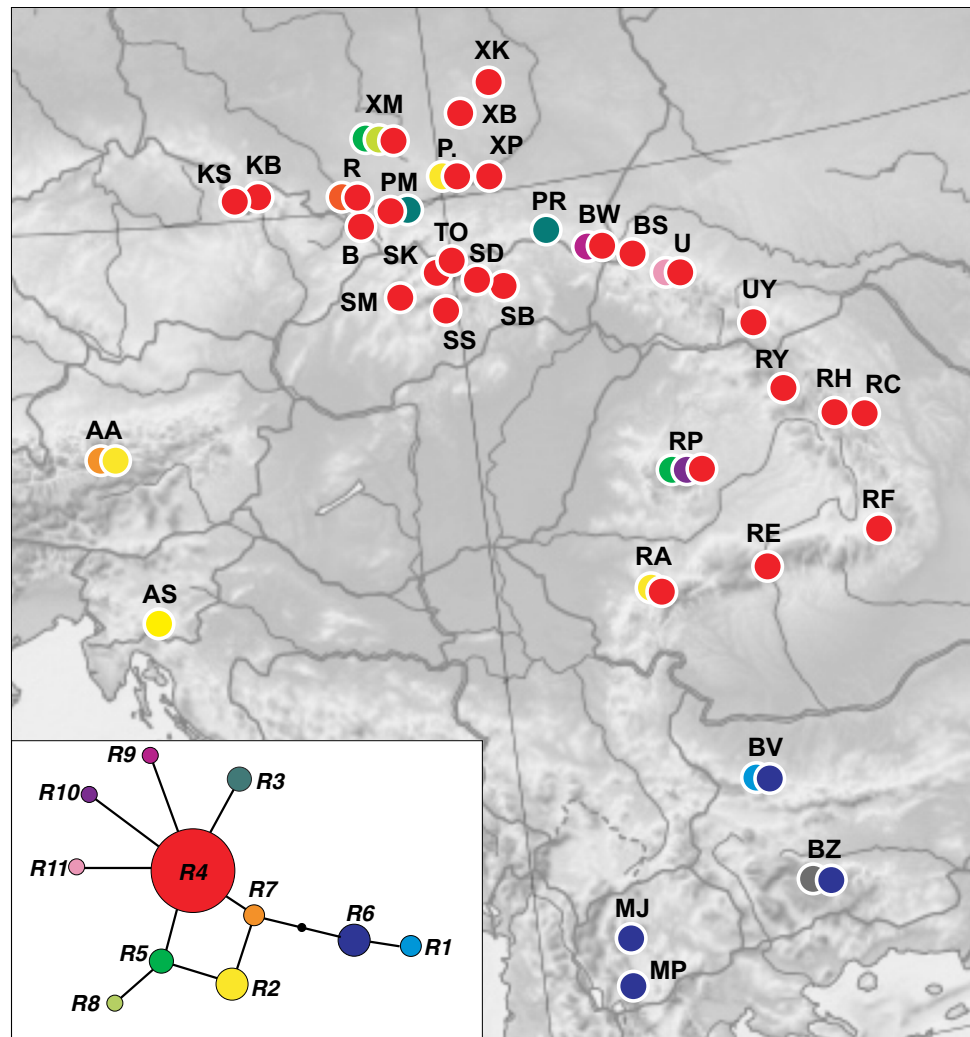
Source of variation	d.f.	Sum of squares	Variance components	% of variation	F_{ST}
1. Total: Among populations	51	9098.78	23.67	35.35	0.35
Within populations	245	10604.17	43.28	64.65	
2. Among geographical groups (Eastern Alps, Dinaric Alps, Eastern Carpathians, Southern Carpathians, Western Carpathians, Balkans, Sudetes, Polish lowlands)	7	4218.08	14.85	21.24	0.38
Among populations within groups	44	4880.69	11.81	16.88	
Within populations	245	10604.17	43.28	61.88	
3. Among Carpathian groups (Eastern, Southern and Western Carpathians)	2	1635.03	11.29	16.92	0.35
Among populations within groups	32	3608.19	11.82	17.70	
Within populations	170	7418.66	43.64	65.38	
4. Among Western Carpathians and Polish lowlands	1	222.69	2.16	4.06	0.26
Among populations within groups	22	2381.78	11.67	22.43	
Within populations	120	4591.09	38.26	73.51	
5. Among Eastern Carpathians, Southern Carpathians and Balkans	2	1432.49	13.89	18.25	0.35
Among populations within groups	21	2517.09	13.00	17.07	
Within populations	107	5270.68	49.26	64.68	
6. Among Southern Carpathians and Balkans	1	1154.79	24.56	28.94	0.42
Among populations within groups	15	1606.52	10.82	12.75	
Within populations	74	3661.54	49.48	58.31	
7. Among Eastern Carpathians and Southern Carpathians	1	204.74	1.69	2.63	0.23
Among populations within groups	15	1830.76	12.74	19.88	
Within populations	80	3972.23	49.65	77.49	
8. Among Eastern and Western Carpathians	1	1000.18	12.03	18.13	0.37
Among populations within groups	26	2957.84	12.24	18.46	
Within populations	136	5718.73	42.05	63.40	
9. Among Western Carpathians and Sudetes	1	158.03	2.53	4.94	0.26
Among populations within groups	18	1860.76	10.96	21.42	
Within populations	100	3766.23	37.66	73.64	
10. Among PCoA groups	2	3140.03	16.80	22.77	0.41
Among populations within groups	49	5958.75	13.71	18.58	
Within populations	245	10604.17	43.28	58.66	
11. Among STRUCTURE groups	3	3438.53	16.19	22.32	0.40
Among populations within groups	48	5660.25	13.07	18.01	
Within populations	245	10604.17	43.28	59.67	
12. Among Alps and Southern Carpathians	1	305.59	12.29	17.10	0.29
Among populations within groups	10	1007.59	8.92	12.43	
Within populations	55	2785.50	50.65	70.47	

Discussion

The flora and vegetation of the mountains in Central Europe have been strongly influenced by the Pleistocene climatic oscillations. Numerous studies using different aspects (climatic, paleobiological, palynological, and molecular) contribute to elucidating the changes in the past and their influence shaping contemporary species diversity. In this context, the Carpathian range is a good model because the impact of the last glaciation varied and was stronger in the northernmost than in the eastern and southern part (e.g., Pawłowski 1966;

Mojski 1993). This history is reflected in the genetic differentiation of the plant and animal species (reviewed recently by Mráz and Ronikier 2016). Here, we used different molecular markers to examine the genetic differentiation in *Doronicum austriacum* populations and discuss the historical factors that influenced the species' extant distribution. We also compare the present AFLP data with our previous analyses of two other typical tall-herb species to assess the general phylogeographical patterns at the scale of community.

Fig. 4 Location of nrDNA ribotypes of sampled *Doronicum austriacum*. Maximum parsimony networks analysis of ITS is showed in the left corner. The size of the circle corresponds to number of individuals for ribotype. Black circles indicate the missing ones. Acronyms of populations correspond to Table 1



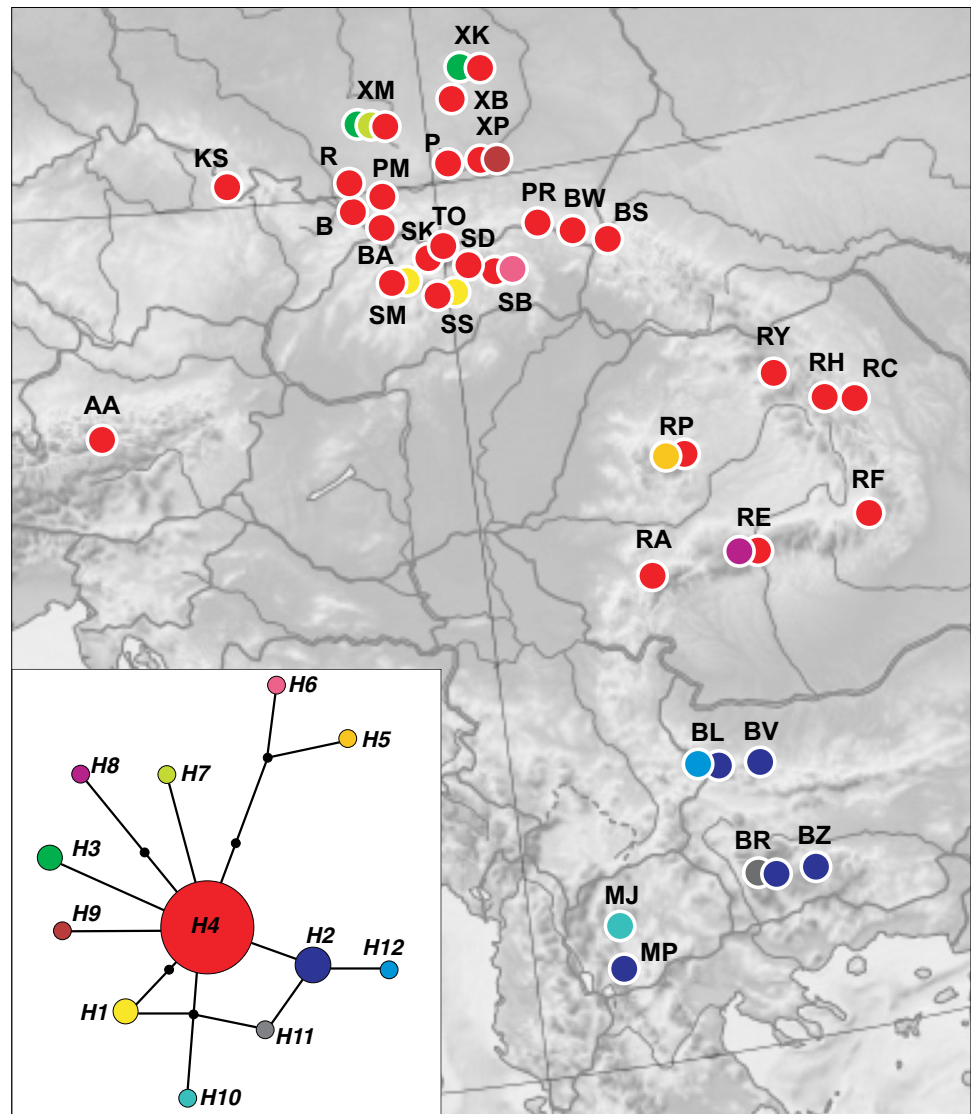
Genetic structure of *Doronicum austriacum* and comparative view on the phylogeography of tall-herbs in the Central European mountains

A strong differentiation between populations from the Western Carpathians and South-Eastern Carpathians was the most apparent feature of the phylogeographical pattern in *D. austriacum* based on AFLP data. The border between these two main groups (STRUCTURE at $K=2$, Figs. 1, 6b, NJ tree, Fig. 3) was congruent with that detected for two other tall-herb species, namely *Cicerbita alpina* and *Ranunculus platanifolius* (Fig. 6a, c; Stachurska-Swakoń et al. 2012, 2013a). Genetic indices and differentiation of AFLP markers for the northernmost Carpathian populations indicate that the species survived during at least the last cold period not only in the southern part of the Carpathians but also in the valleys of the Western Carpathians. The possibility of the presence of refugia in this part of the Carpathians was also pointed for other tall-herb species based on genetic studies (Stachurska-Swakoń et al. 2011, 2012, 2013a). Local

refugia are also supported by palynological and palaeobotanical studies (e.g., Mamakowa and Środoń 1977; Jankovská and Pokorný 2008). Although not statistically significant, a northward trend of decreasing genetic diversity may reflect more restricted and nowadays isolated populations in the northern part of the range.

The genetic border area between the Western and South-Eastern Carpathians for *Doronicum austriacum* detected in AFLP data is located in the Beskid Niski Mts., a low-elevation massif forming a disjunction for high-mountain species. The populations from the Bieszczady Mts.—the northernmost part of the Eastern Carpathians, are characterized by high rarity markers (DW and private markers). We could postulate the origin of extant populations there from local refugia. Similarly, high values of DW index were detected for *R. platanifolius* in the Bieszczady Mts. and for *C. alpina* sampled in Chornohora, both in the northern part of the Eastern Carpathians (Stachurska-Swakoń et al. 2012, 2013a). The Bieszczady Mts. were recognized as an important area for differentiation of the genus *Aconitum* (Mitka

Fig. 5 Location of cpDNA haplotypes of sampled *Doronicum austriacum*. Maximum parsimony networks analysis of cpDNA is showed in the left corner. The size of the circle corresponds to number of individuals for haplotype. Black circles indicate the missing ones. Acronyms of populations correspond to Table 1



2003, Boroń et al. 2011) and were postulated as a refugial area for two forest herbaceous plants *Galium schultesii* and *Stellaria holostea* (Mitka et al. 2014, 2015b). On the other side of the border area, the easternmost studied Western Carpathian populations of *D. austriacum* (PR, PS, Beskid Sądecki Mts) have today isolated locations and they are depauperated but genetically belong to the Western Carpathian lineage.

While the main intra-Carpathian phylogeographical split is a common feature of all tall-herb taxa compared and also corroborates a more generally detected break in mountain biota (Mráz and Ronikier 2016), the spatial genetic pattern of *Doronicum austriacum* also displays important differences within the group of tall-herbs. It concerns especially the southernmost part of the geographical range. The most diverging feature is a significant genetic differentiation of southern populations from the Balkan Peninsula, which form a separate AFLP lineage, distributed across the mountain

ranges from the east (Rila, Rhodopes, Vitosha) to the west (Baba, Jakupica, Osogovska Planina) (emerging at a $K=3$ in the STRUCTURE; Fig. 6), additionally supported by unique haplotype and ribotype variants. This is in line with studies documenting diversification of taxa evolving in spatiotemporal isolation in the Balkan Peninsula (e.g., Stefanović et al. 2008; Bardy et al. 2010; Surina et al. 2011; Olšovská et al. 2016; Glasnović et al. 2018). However, in the comparative phylogeographical context of tall-herb communities, in both *Ranunculus platanifolius* and *Cicerbita alpina* the populations from the Balkan Peninsula formed a genetically coherent group with the Southern Carpathian populations suggesting the common history despite a significant extant disjunction (Fig. 6; Stachurska-Swakoń et al. 2012, 2013a). Distribution of lineages in *D. austriacum* points to a different history despite the same habitat preferences, extant distribution, and phytosociological affinities. Instead, the South-Eastern Carpathian plants appear more closely related

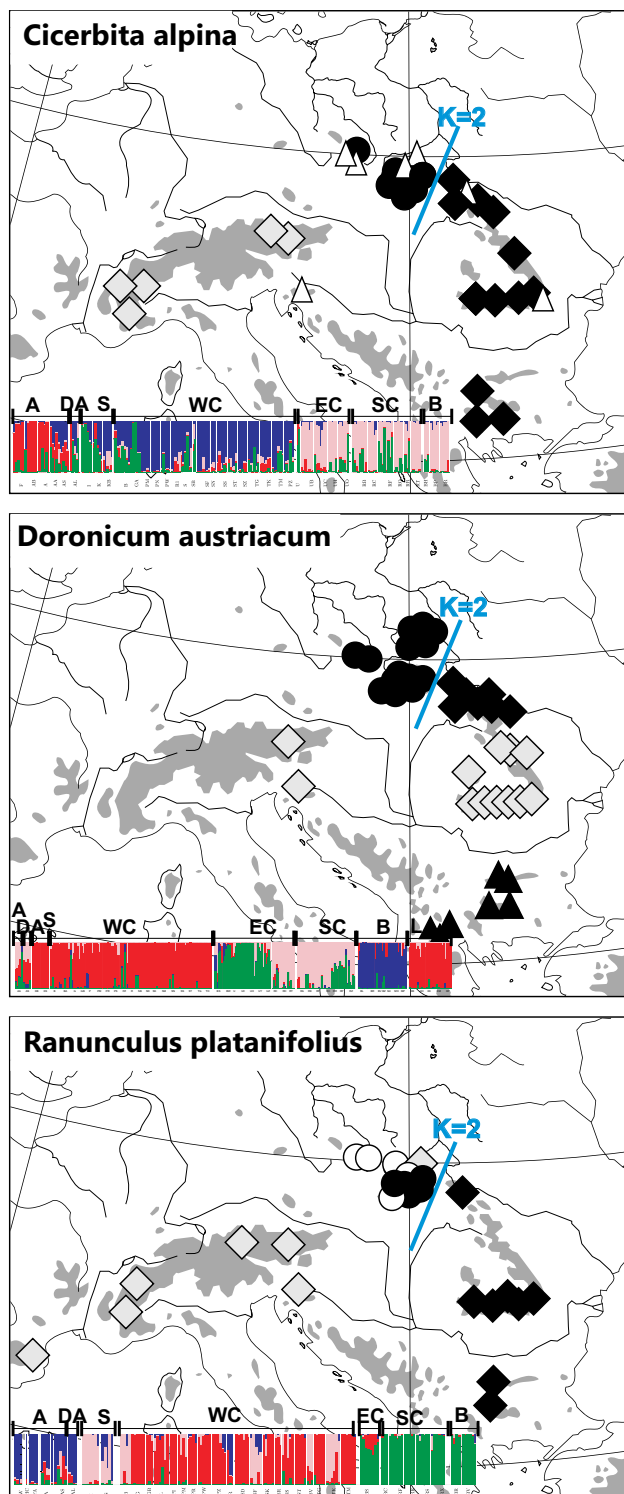


Fig. 6 The simplified genetic pattern of three tall-herb species according to AFLP results (Bayesian analysis, STRUCTURE software, $K = 4$). *Cicerbita alpina*—42 populations, 226 individuals, (Stachurska-Swakoń et al. 2012); *Doronicum austriacum*—52 populations, 297 individuals, *Ranunculus platanifolius*—38 populations, 183 individuals (Stachurska-Swakoń et al. 2013a). Detailed STRUCTURE clustering in geographical alignment of sampled populations inserted in the left corner (abbreviations of geographical regions as in Fig. 2, abbreviations of names as in Table 1)

to those in the Alps. This additionally alters the phylogeographical pattern of tall-herbs where other species rather displayed a northwest-/southeast-oriented differentiation. The genetic divergence pattern in *D. austriacum* detected in all our data sets may likely corroborate a taxonomic distinction of the Balkan populations. As recently discussed by Skof et al. (2019) in their phylogenetic study, *D. austriacum* contains two lineages which may represent two different evolutionary units corresponding to Central European *D. austriacum* s.str. and the once described but neglected South-Eastern European *D. orphanidis* Boiss (*D. austriacum* Jacq. subsp. *giganteum* (Griseb.) Stoj. and Stef.). As *D. austriacum* demonstrates variability in the morphological features, many Flora accounts (e.g., Tutin et al. 1976; Assyov and Petrova 2012; Dimopoulos et al. 2013, 2016) do not list a separate Balkan taxon. The Balkan Peninsula is a major biodiversity center which provided a long-term stability of habitats for mountain species. Recent molecular analyses repeatedly provided support for delimitation of allopatric, endemic taxa described in the past (e.g., Lakušić et al. 2006; Kučera et al. 2010; Ronikier and Zalewska-Gałosz 2014) or newly delimited (e.g., Aleksić et al. 2018). Here, taking into account the morphological trait variability (Álvarez Fernández 2003) and limited phylogenetic resolution available (Skof et al. 2019), a comprehensive morphological and molecular analysis including variable markers and an exhaustive sampling from the Balkan Peninsula is necessary to resolve the problem of whether the Balkan range constitutes a distinct taxon and how it is related to *D. austriacum* populations from more northerly latitudes.

At the scale of among-massif population relationship, a clear congruence across all three tall-herb species studied is in a close genetic relationship between the Western Carpathian populations and those from the Sudetes, part of the Central European Hercynian mountains. Their northerly location caused the most significant influence of the last glaciation due to the close proximity of the continental ice sheet and the presence of mountain glaciers are documented (Starkel 1991; Badura and Przybylski 1998). Several case studies have documented the affinity of species populations from the Sudetes to the Western Carpathians—interpreted as trace of postglacial recolonization from the latter area or intermediate lower stands—not only in tall-herb species but also other mountainous elements, such as *Aconitum* ssp. (Mitka et al. 2007), *Arabidopsis halleri* (Wasowicz et al. 2016) *Hieracium silesiacum* (Ronikier and Szeląg 2008), *Melampyrum sylvaticum* (Tešitel et al. 2009), *Swertia perennis* (Urbaniak et al. 2018).

In the geographically largest group, some further differentiation can be inferred from the AFLP phylogeography of *Doronicum austriacum*, consistently with geographical areas of population groups, such as the Southern Carpathians and Eastern Carpathians. Several studies documented a possible

different history of species populations located in different mountain massifs in these two large regions (e.g., Hurdu et al. 2016; Kolář et al. 2016; Lendvay et al. 2016; Wasowicz et al. 2016). Also, the Alps together with the northernmost Dinarides populations formed a separate group at this scale but this area is represented by a much smaller sampling in our study, which limits possible discussion.

In *Doronicum austriacum* we coupled the AFLP analysis with an overview of nrDNA and cpDNA sequences. More conservative plastid DNA showed twelve haplotypes, but only two of them had a high frequency and were distributed across larger areas. One of them was exclusive for the Balkan Peninsula populations (H2), while the second common haplotype dominated in the Carpathians, Alps and Sudetes (H4). In general, although all haplotypes were weakly diversified, all Balkan populations were strictly segregated from all other populations. Accordingly, plastid DNA variation, as well as ITS variation (also showing dominance of a single ribotype across the Carpathians) did not corroborate the within-Carpathian split displayed by AFLP data. The divergence between northern- and southernmost populations of *D. austriacum* was apparent in the phylogeny of the genus *Doronicum* (Álvarez Fernández et al. 2001), where two samples of *D. austriacum* were studied from the northern (Bieszczady Mts., Eastern Carpathians, Poland) and southern (Nomos Florinis, West Macedonia, Greece) parts of the distribution range. These two sequences differed by 11 nucleotide substitutions. The variation of plastid markers is assumed to reflect a more ancient differentiation (e.g., Eidesen et al. 2007), and it may reflect a taxonomically distinctive divergence (see also above).

The remaining haplotypes and ribotypes, closely related to the most common ones, were rare in our samples and dispersed in different regions (Figs. 4, 5). Interestingly, several such rare variants were identified in the relict low-elevation populations north of the Carpathians, which may indicate their age longer than postglacial (see below).

Diversity and origin of the subalpine plant populations to the north of the Carpathians

At the northern edge of the species' geographical distribution several isolated populations of *Doronicum austriacum* occur at low elevations either within lower Carpathian ranges or to the north of the Western Carpathians (Bróz and Przemyski 1992; Zajac and Zajac 2001; Stachurska-Swakoń and Kuź 2011) of which six populations representing four main localities were represented in this study (Table 1). The Polish lowland localities of *D. austriacum* are considered to be of the relict origin (Szafer 1930). They are all distinct, i.e., small isolated populations, located in the alder-ash forest along streams, and *D. austriacum* is usually accompanied there by other mountain species (A. Stachurska-Swakoń,

unpublished observations). Based on our AFLP results, we can postulate that these populations originated from local peripheral refugia in the northwestern part of the Carpathians. Indeed, the lowest pairwise *Fst* values for these populations were connected with the Beskid Żywiecki population (PZ) in the Western Carpathians. This population, located in the deep forested valley of the Potok Cebulowy creek, is characterized by high genetic diversity indices (after populations from Munții Retezat in the Southern Carpathians and from North Limestone Alps; Table 1). Although it is an isolated population in a low-elevation, forested massif, it may have persisted in this site for a long time. Interestingly, this site harbors a rich mountain flora including other tall-herbs and even alpine species such as *Viola biflora*, with locations much below their altitudinal amplitude (Bartoszek et al. 2015).

On the other hand, the species could theoretically have persisted in the areas north of the Carpathians throughout the last glaciation or even longer because after the San II glaciation no continental ice sheet exceeded the Świętokrzyskie Mts to the South (Lindner 2005). In such a case, we could expect microevolutionary processes which took place in geographically isolated glacial refugia at low altitudes (Kadereit et al. 2004; Mitka et al. 2013, 2015a; Pachschtöll et al. 2015). While our AFLP data do not indicate any differentiation of these populations, the sequence data reveal some distinct haplotypes in this area. For instance, a specific plastid haplotype, absent from the Western Carpathians (H3), were detected in population XM and the northernmost locality XK (Świętokrzyskie Mts.) (Fig. 5). Two specific ribotypes were detected in populations P (R2) and XM, XP (R5; Fig. 4). The R2 ribotype was shared with the Alpine and Dinaric populations. Such rare haplotypes may indicate a longer isolation but a denser sampling of sequences in populations would be needed to further explore this possibility. A phenotypic study was conducted for one of the lowland populations (Stachurska-Swakoń and Kuź 2011) discovered by Piech (1924). However, the morphological observation pointed at the phenotypic response to diverse lowland condition rather than evolutionary adaptation, as also noticed for lowland populations of another mountain species, *Veratrum lobelianum* (Stachurska-Swakoń et al. 2018).

Conclusions

Our results support the existence of different local refugia in the main regions where *Doronicum austriacum* occurs. Hence, we can assume that the species has survived at least most recent climatic oscillations of the Quaternary within the respective regions and exhibited local vertical shifts rather than long-distance migrations across regions. This general situation is similar to those previously found in

two other tall-herb species: *Cicerbita alpina* and *Ranunculus platanifolius* (Stachurska-Swakoń et al. 2012, 2013a). However, location of specific genetic breaks was only partly congruent across these species (Fig. 6) with the major difference in the southernmost part of the range where the southernmost Balkan populations form a strongly divergent group in contrast to other species studied (this differentiation being also the main pattern supported by plastid haplotype and nuclear ribotype distributions). In contrast to *C. alpina* and *R. platanifolius*, in the case of *D. austriacum* this may reflect a deeper evolutionary differentiation at the taxonomic level.

Our detailed analysis of the northern part of the range in the Western Carpathians and to the north of them, most affected by the past glaciations, indicates that numerous isolated populations may have persisted not only in major mountain ranges but also at lower elevations, likely in deep valleys where they occur today. The history of detached, low-elevation populations to the north of the mountains remains unclear although AFLP data suggest their close affinity to the Western Carpathians.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Information on Electronic Supplementary Material

Online Resource 1. GenBank accession numbers of plastid *trnL*-F region and nuclear ribosomal ITS of sampled individuals of *Doronicum austriacum*.

Online Resource 2. The alignment of ITS1 + ITS2 markers of *Doronicum austriacum* individuals used for analyses.

Online Resource 3. The alignment of cpDNA (*trnL*-F region) of *Doronicum austriacum* individuals used for analyses.

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