

Out of the Alps or Carpathians? Origin of Central European populations of *Rosa pendulina*

Z Alp nebo z Karpat? Původ středoevropských populací druhu *Rosa pendulina*

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The phylogeographical structure of the temperate shrub *Rosa pendulina* at 45 locations in Europe was studied using sequencing of a non-coding cpDNA region (*trnL-trnF*). Our study revealed a clear geographic structure of cpDNA haplotypes. Three main haplotypes were geographically widespread, but showed little overlap in their distributions, suggesting that postglacial expansion occurred from at least two distinct glacial refugia, probably located (1) at the edge of the Alps, N Apennines or Dinaric Alps, and (2) in the Balkan Peninsula or S Carpathians. All populations at locations in the Czech Republic and surrounding regions are of Carpathian origin. This finding disproved an Alpine origin of *R. pendulina* populations in the Šumava Mts (Czech Republic). A contact zone between Carpathian and Alpine migration routes of *R. pendulina* is probably located in the Danube valley.

Key words: cpDNA, glacial refugia, phylogeography, postglacial migration, *trnL-trnF* sequences

Introduction

Present distribution patterns of European plants are to large extent determined by the extensive climatic changes during the Quaternary, which caused repeated cycles of species migration throughout the continent (Hewitt 1999). Migration processes can be inferred only indirectly on the basis of palynological or phylogeographical evidence. The latter deals with the spatial distribution of gene lineages (Avice 2000).

Early phylogeographic studies on European taxa, dealing mainly with economically important tree species (e.g. *Alnus*, *Quercus*, *Fagus*; Demesure et al. 1996, Dumolin-Lapègue et al. 1997, King & Ferris 1998), focused on genetic patterns at the scale of the whole continent. Similar patterns of postglacial colonization routes were found for several taxa, and three main refugia in S Europe postulated (the Iberian and Italian Peninsulas and the Balkans; Taberlet et al. 1998). Putative refugial populations were found to harbour specific genetic lineages and sometimes also higher diversity than populations in recolonized areas (but see Widmer & Lexer 2001). The latter probably experienced bottlenecks during expansion after climate warming 13,000 years ago (Comes & Kadereit 1998).

Previous large-scale studies revealed several zones (Remington 1968, Swenson & Howard 2004) in Europe, where populations of the same or closely related taxa expanding

from different refugia came into contact (Hewitt 1988, 1999, Taberlet et al. 1998). Although these contact or suture (when hybridization is also involved) zones do not have identical locations for all taxa, four main areas can be identified: the Pyrenees, the Alps, central Europe north of the Alps and central parts of the Scandinavian Peninsula.

Based on palaeoecological evidence, Willis et al. (2000) and Willis & van Andel (2004) concluded that there was no forestless landscape in Central Europe during the last glacial as had been previously hypothesized. Thermophilous trees (*Carpinus betulus*, *Quercus*, *Corylus*, *Ulmus* and *Tilia*) must have survived in micro-environmentally favourable pockets in Central Europe. Recently published evidence on *Fagus sylvatica* (Magri et al. 2006) also suggests the presence of open forests in Central Europe. This evidence may change our view on glacial refugia of the Central European flora and the postglacial migration routes established by early phylogeographic studies.

The flora of the Czech Republic provides several examples of taxa with potentially different patterns of postglacial immigration. Sometimes it seems possible to unequivocally infer the origin of populations from their present distribution pattern. A few examples of taxa with an apparently Alpine origin are: *Soldanella montana* (distribution map in Slavík 1990), *Alnus viridis* (Kučera 1966, Plzáková 1973) and *Willemetia stipitata* (Kláštorský 1961, Kučera 1966), while *Cardamine glanduligera* (= *Dentaria glandulosa*; Slavík 1990, Lihová et al. 2007), *Cardamine amara* subsp. *opicii* (Hrouda & Marhold 1993), *Centaurea jacea* subsp. *oxylepis* (Hendrych 1985) and *Salix silesiaca* (Hendrych 1985) possibly migrated from the Carpathians to the area of the Czech Republic. There is, however, an array of species that could have come from both the Alps and the Carpathians (Hendrych 1985). This phenomenon potentially reflects the existence of a contact or suture zone in Central Europe, including the Czech Republic.

One of the species for which both Alpine and Carpathian postglacial immigration into the area of the Czech Republic can be hypothesized is *Rosa pendulina* L. (*Rosaceae*). It occurs predominantly in mountains and hills in the Alps, Carpathians, Balkan Mountains, Apennines and Pyrenees (Meusel et al. 1965, Kurtto et al. 2004; Fig. 1a). In the Czech Republic, *Rosa pendulina* is common in mountainous and hilly landscapes, but it does not occur in lowlands (Slavík 1995: 38, Větvíčka 1995).

In this study, we analysed 45 samples of *R. pendulina* representing populations from the whole distribution area of the species. Using sequences of the non-coding *trnL-trnF* region of chloroplast DNA (Taberlet et al. 1991), allowed us to address the following questions: (1) Is there a phylogeographical structure in the distribution of cpDNA haplotypes? (2) Can we trace the glacial refugia of Central European populations? (3) Are there any contact zones in Central Europe? (4) Are the postglacial migration routes of *R. pendulina* comparable to those found in other plant species?

Methods

We analyzed individuals originating from 45 locations (one sample each) from the whole distribution area, including hypothetical refugia (Pyrenees, S Italy and Balkans; Table 1). Young leaves were collected in the field and dried in silica gel. Voucher specimens were deposited in the herbarium PRC.

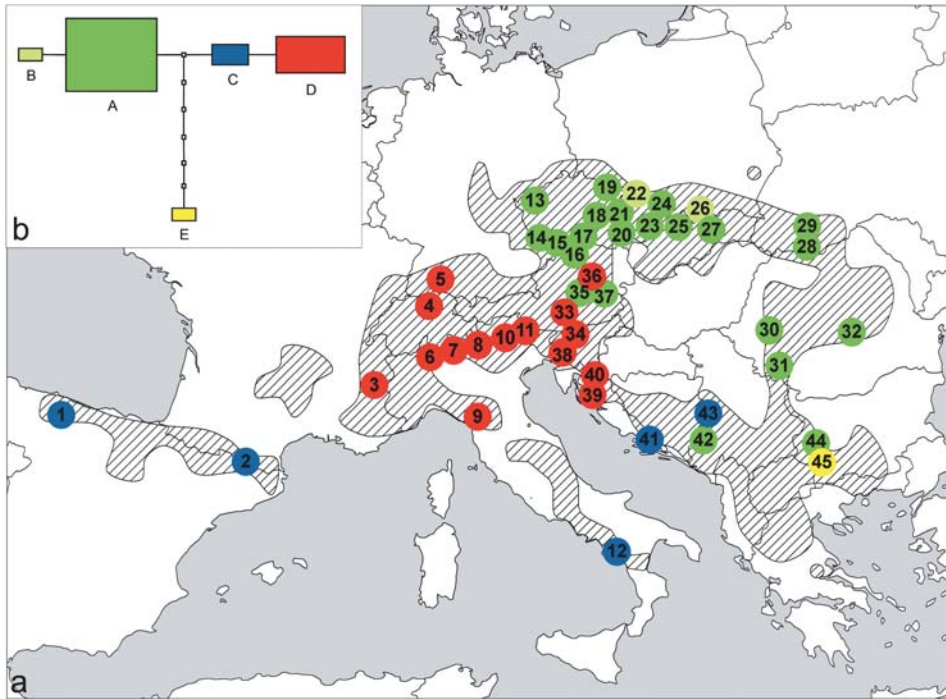


Fig. 1. – (a) Distribution area of *Rosa pendulina* in Europe (Kurtto et al. 2004) (shaded) and locations of the 45 samples (Table 1) used in *trnL-trnF* sequencing. Colour of dots refers to the five cpDNA haplotypes detected. (b) Parsimony network showing the relationships among the five cpDNA haplotypes detected. Box sizes are proportional to the number of samples with a particular haplotype. Small white squares represent missing haplotypes.

DNA extraction, PCR-RFLP and sequencing

Total DNA was extracted using the protocol of Doyle & Doyle (1987). DNA concentrations were measured photometrically and adjusted to 5 ng/μl.

We first searched for cpDNA variation in three non-coding regions (*psbC-trnS*, *trnK-trnK*, *trnC-trnD*; Demesure et al. 1995) using the polymerase chain reaction – restriction fragment length polymorphisms (PCR-RFLPs) with 11 restriction enzymes (*HinfI*, *Hin6I*, *MboI*, *Bsh1236RI*, *AluI*, *BsuRI*, *MspI*, *RsaI*, *PstI*, *BglII*, *HincII*, *TaqI*, *TaiI*, *TasI*, *TruII*). Restriction products were loaded onto an 1.8% agarose gel, electrophoresed and visualized under UV light after staining with ethidium bromide. Eleven samples, from Romania (no. 32; Table 1), Ukraine (29), Slovakia (27), Austria (33), France (3) and the Czech Republic (13, 16, 17, 18, 20, 22), were screened in order to detect variation in restriction profiles. We found no variation in any of the primer/restriction enzyme combinations.

Therefore, sequencing of the *trnL-trnF* introns and spacer cpDNA was additionally used. PCR amplification with the universal primers c and f of Taberlet et al. (1991) was carried out in a volume of 19 μl reaction using 5 ng of template DNA, 2 μl of 10× reaction buffer (Sigma), 0.4 μl of dNTP mix (10 mM), 4 pmol of each primer and 0.5 U of REDTaq

Table 1. – List of 45 *Rosa pendulina* samples used for phylogeographical analysis with their code, locality, altitude, latitude, longitude, collectors, and *trmL-trmF* haplotype. AK – A. Kagalo (Lviv), AT – A. Tribsch (Salzburg), BF – B. Frajmann (Ljubljana), DR – D. Reich (Vienna), ER – E. Rejzková (Prague), HD – H. Dvořáková (Prague), JF – J. Fér (Kutná Hora), JK – J. Kučera (Rychnov nad Kněžnou), JL – J. Lihová (Bratislava), JV – J. Vojta (Prague), KB – K. Boublík (Prague), LF – L. Féroová (Prague), MaS – M. Stolarová (Prague), MK – M. Kolínk (Bratislava), MS – M. Slovák (Bratislava), MaS – M. Stolarová (Prague), PS – P. Schönswetter (Vienna), PST – P. Stanimirova (Sofia), PV – P. Vašák (Prague), TF – T. Fér (Prague), TW – T. Wohlgemuth (Birmensdorf), ZV – V. Zabloudil (Žďár nad Sázavou).

Code	Country	Locality description	Altitude	Latitude	Longitude	Collector	Haplotype
1	Spain	Cordillera Cantábrica Mts, La Vecilla de Curueño, 17 km N of the town, Puerto de Vegarada saddle.	1620	43.03	-5.49	TF, LF	C
2	Andorra	Soldeu, 4 km NNE of the town, Val d'Inclès.	2100	42.60	01.70	TF, LF	C
3	France	Provence-Alpes-Côte d'Azur, le Madeleine, 2.5 km of the town, mountain pass Col du Lautaret.	2067	45.03	06.40	MS	D
4	Switzerland	Zürich, 7 km S of the town, Girstel (Albis).	750	47.29	08.51	TW	D
5	Germany	Schwäbische Alb Mts, Beuron, 2.5 km SSE of the town, Knopfmacherfels.	750	48.03	08.95	JV	D
6	Italy	Lombardy, Varese, 6.5 km NNW of the town, Campo dei Fiori.	1100	45.87	08.78	TF, LF, JF, MaS	D
7	Italy	Lombardy, Sondrio, 32 km SWW of the town, Gerola Alta, Castello	1390	46.07	09.54	TF, LF, JF, MaS	D
8	Italy	Lombardy, Édolo, 15 km NE of the town, Vezza d'Óglio, Valle di Paghera	1500	46.21	10.41	TF, LF, JF, MaS	D
9	Italy	Toscana, Pievepelago, 10 km W of the town, Passo delle Radici.	1530	44.21	10.49	TF, LF, JF, MaS	D
10	Italy	Alto Adige, Bolzano, 23 km SEE of the town, Passo di Costalunga.	1740	46.40	11.62	TF, LF, JF, MaS	D
11	Italy	Veneto, Cortina d'Ampezzo, 10 km NW of the town, Misurina	1872	46.59	12.26	PV	D
12	Italy	Campania, Sala Consilina, 15 km NE of the town, Monte Cervati.	1860	40.29	15.48	TF, LF, JF, MaS	C
13	Czech Republic	Doupovské hory Mts, Karlovy Vary, 10 km E of the town, at the Pstružný potok brook.	510	50.24	13.06	JV	A
14	Czech Republic	Šumava Mts, Kašperské hory, 9 km SW of the town, in the valley of the Sekerský potok brook.	800	49.09	13.46	KB	A
15	Czech Republic	Šumava Mts, Prachatice, 4.5 km W of the town, the valley of the river Blanice.	612	49.01	13.94	PV	A
16	Czech Republic	Novohradské hory Mts, Kaplice, 15 km SE of the town, valley of the Pohořský potok brook.	780	48.65	14.65	KB	A
17	Czech Republic	Českomoravská vrchovina Mts, Jindřichův Hradec, 5 km E of the town, Jindřichské údolí valley.	480	49.14	15.07	KB	A
18	Czech Republic	Žďárské vrchy Mts, Přebyslav, 13 km NE of the town, the Štřítký důl nature reserve.	565	49.67	15.87	ZV	A
19	Czech Republic	Orlické hory Mts, Deštné v Orli. horách, 4 km N of the town, Bukáčka nature reserve.	1020	50.34	16.38	JK	A
20	Czech Republic	Drahanská vrchovina Mts (Moravian karst), Ostrov u Maceochy, 3.5 km SW of the town, Pustý žleb valley.	350	49.37	16.72	ER, TF, JV	A

21	Czech Republic	Podolícká pahorkatina Mts, Moravská Třebová, 5.5 km SE of the town, Radkov hill.	370	49.75	16.73	ER, TF, JV	A
22	Czech Republic	Jeseníky Mts, Karlov pod Prádkem, western border of the town, by the Kotelný potok brook.	760	50.03	17.26	ER, TF, JV	B
23	Czech Republic	Západní Beskydy Mts (Rožnovská brána), Rožnov pod Radhoštěm, bank of the Bečva river in the town.	360	49.46	18.16	ER, TF, JV	A
24	Czech Republic	Moravskoslezské Beskydy Mts, Ostravice, 2 km NE of the town, Lysá hora hill, at the Sopotný potok brook.	640	49.55	18.41	ER, TF, JV	A
25	Slovak Republic	Malá Fatra Mts, Martin, 19 km NNE of the town, above the Horný diery valley.	1060	49.24	19.09	TF	A
26	Slovak Republic	Belianské Tatry Mts, Poprad, 21 km N of the town, the Monkova dolina valley	800	49.26	20.23	TF	B
27	Slovak Republic	Slovenský raj Mts, Hrabušice, 4 km S of the town, the Suchá Belá valley, Glacká cesta.	850	48.94	20.39	MS, JL	A
28	Ukraine	Chornohora Mts, Yasynya, 14 km SE of the town, Mt. Petrosul.	1850	48.17	24.47	AK	A
29	Ukraine	Horhany Mts, Yaremcha, 10 km S of the town, nearby the village Mikulichin.	644	48.41	24.60	MK	A
30	Romania	Retezat Mts, Margina, 4.5 km N of the town, Cheile Scorota gorge.	1150	45.90	22.28	ER	A
31	Romania	Cernei Mts, Mehadia, 5 km E of the town, Domogled.	537	44.88	22.43	ER	A
32	Romania	Bucegi Mts, Braşov, 20 km S of the town, the Cheile Zaganului gorge.	1323	45.41	25.50	MK	A
33	Austria	Styria, Frauentalpe, Kaltwasser, 4 km E of the town.	2000	47.04	14.08	HD	D
34	Austria	Tirol, Karawanken Mts, Loibtal, 18 SEE of the town, Kleiner Grintoutz.	1600	46.41	14.48	PS, BF, DR	D
35	Austria	Styria, Hochschwab Mts, Seewiesen, W of the town, Gamssteig path.	1320	47.63	15.26	PS, AT	A
36	Austria	Lower Austria, between Kirchberg/Pielach and Lilientfeld.	690	48.00	15.32	HD	D
37	Austria	Styria, Schneecalpe Mts, Mürtzzuschlag, 5 km NE of the town, along the road to Schneecalpenhaus.	1000	47.67	15.60	HD	A
38	Slovenia	Primorska, Trnovski gozd Mts, Čaven.	1100	45.93	13.91	PS, BF	D
39	Croatia	Velebit Mts, Krasno Polje, 5 km SW of the town, Mali Rajinac hill.	1484	44.80	15.01	JV, PV	D
40	Croatia	Velika Kapela Mts, Zindovac, 2.5 km NE of the town, Klak hill.	795	45.25	15.15	JV, PV	D
41	Croatia	Biokovo Mts, Makarska, 3.5 km NE of the town, by the road to Mt. sv. Jure.	1392	43.32	17.05	JV, PV	C
42	Montenegro	National Park Durmitor, Žabljak, 1 km W of the town, Crno jezero lake.	1614	43.13	19.10	JV, PV	A
43	Serbia	Tara planina Mts, Mitrovac, nearby town.	743	43.86	19.41	JV, PV	C
44	Bulgaria	Vitosha Mts, Sofia, 6 km S of the town, between the peaks of Reznovete and Chernivrukh.	1906	42.57	23.30	PSt	A
45	Bulgaria	Rila Mts, Dupnica, 26 km SEE of the town, between Kirilova Polyana and Sukhoto ezero lake.	1850	42.16	23.41	PSt	E

DNA Polymerase (Sigma) on a Mastercycler ep gradient S thermal cycler (Eppendorf) with denaturation at 94 °C for 60 s, 35 cycles of 45 s denaturation at 94 °C, 45 s annealing at 52.2 °C and 2 min extension at 68 °C, followed by 10 min final extension at 70 °C. Amplification products were subsequently purified using the JetQuick PCR Purification Kit (Genomed). Sequencing reactions were performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions using the primers cited above. Purification of sequencing reactions was carried out using an ethanol/sodium acetate precipitation provided with the sequencing kit. Products were run on an ABI 3100 Avant automated sequencer (Applied Biosystems).

Data analysis

Sequences from both strands were manually assembled and aligned using CLUSTALX v 1.83 (Thompson et al. 1997). The alignment was improved manually. Informative insertions/deletions were coded using simple indel coding (Simmons & Ochoterena 2000) implemented in SEQSTATE (Müller 2005).

A parsimony network of haplotypes (Templeton et al. 1992) was made with TCS 1.21 (Clement et al. 2000), which constructs a haplotype network with a minimum number of required mutational steps among haplotypes. The position of haplotypes in the network gives information regarding their age since older haplotypes are located internally in the network (Posada & Crandall 2001). The input file includes the alignment and insertions/deletions were coded as two additional characters using C for the presence of insertion and A for its absence. The gaps were treated as missing data, but with additional indel coding (see above).

Results

The total length of the aligned *trnL-trnF* region was 836 bp. Eleven positions were variable: nine point mutations and two indels (one and four bp in length, respectively). Based on this variation, five different haplotypes were determined (A–E; Table 2). GenBank database accession numbers are: haplotype A – EF591622, B – EF591623, C – EF591624, D – EF591625, E – EF591626. Three haplotypes (A, C, and D) were widely distributed, haplotypes B and E only occurred locally (Fig. 1a). Haplotype A occurred in the Balkans, Carpathians and in all samples from Central Europe north of the Alps except sample 5 (Schwäbische Alb, Germany) and 36 (Lower Austria). Haplotype B was only found in the Jeseníky Mountains (22; Czech Republic) and in one sample from the Slovak Republic (26). The distribution of haplotype C included the Pyrenees, S Italy and the W Balkans. All populations of *R. pendulina* in the Alps and surrounding areas have haplotype D. Haplotype E was only found at one Bulgarian locality, namely in the Rila Mts (45).

Haplotypes A and B found in the Carpathians and in Central Europe north of the Alps differed by only one point mutation (Fig. 1b). Similarly, the S European haplotype C and the Alpine haplotype D also differed by one point mutation. These two groups of haplotypes differed by two point mutations. The haplotype E, found in the Balkans, was more distinct from all the other haplotypes, differing by five point mutations and two indels from haplotypes A and C.

Table 2. – Variable positions in the *trnL-trnF* cpDNA region defining five haplotypes (A-E) in *Rosa pendulina*. The number of sites at which a particular haplotype was found is indicated.

Haplo- type	Number of sites	Variable positions (bp)										
		45	131	266	291	548	554	625–628	645	756	769	
A	22	G	A	A	G	T	T	–	G	C	C	
B	1	G	A	A	G	T	C	–	G	C	C	
C	5	G	A	A	G	T	T	–	T	C	G	
D	12	G	A	A	G	G	T	–	T	C	G	
E	1	–	G	C	T	T	T	TTCA	G	T	G	

Discussion

This study suggests a geographic organization of the *trnL-trnF* cpDNA variation with three geographically widespread common haplotypes that showed little overlap in their distributions. The present day distribution of cpDNA haplotypes of *R. pendulina* suggests that postglacial expansion originated from two major refugia. One was located in the Balkan Peninsula or Carpathians. From here, migration to the whole E and Central Europe occurred. This is one of the most common scenarios for the colonization of Central Europe as demonstrated for *Fagus sylvatica* (Magri et al. 2006) and *Carpinus betulus* (Grivet & Petit 2003). The other refugium was located somewhere at the edge of the Alps, the N Apennines or Dinaric Alps. From here the species spread throughout the Alps and surrounding regions. A similar refugial area has also been postulated for *Alnus glutinosa* (King & Ferris 1998), *Fraxinus excelsior* (Heuertz et al. 2004) and *Abies alba* (based on isozymes; Konnert & Bergmann 1995).

We found a single common haplotype of *R. pendulina* in all three hypothetical southern refugia (Iberia, Italy and the Balkans; Taberlet et al. 1998). This haplotype (C) probably did not contribute to the present-day genetic pool of the Central European populations. The distribution of haplotype C on both the western and eastern shores of the Adriatic Sea suggests a connection between refugia in the Italian and Balkan Peninsulas as previously observed in common ash (Heuertz et al. 2004), oaks (Fineschi et al. 2002, Petit et al. 2002) and ivy (Grivet & Petit 2002). Indeed, during the last glacial maximum, the sea level of the Adriatic Sea was considerable lower, and there was possible contact between the Italian and the Balkan floras, especially in the northern part of the Adriatic region (Frenzel et al. 1992, Adams 1997). The most genetically distinct haplotype E was found at a single location in Bulgaria (45). A geographically more detailed study will be necessary to evaluate the haplotype diversity in the Balkan Peninsula.

The hypothesis that *R. pendulina* survived in Central Europe during the last glaciation (see also Willis et al. 2000, Willis & van Andel 2004) cannot be completely rejected on the basis of our results. A more variable molecular marker (e.g. AFLPs; Vos et al. 1995) should be used in addition to our cpDNA data, since it may provide some indication of bottlenecks or founder effects (Tribisch et al. 2002).

A contact zone between the Carpathian and Alpine migration pathways of *R. pendulina* probably is located in the Danube valley. This is indicated by the present day distribution gap between the continuous Alpine area and Danube river in Germany and Austria (Haeupler & Schönfelder 1989, H. Niklfeld et al., University of Vienna, unpublished data)

at low elevations. An alternative explanation is that the migration from the Alpine refugium was slower than the expansion from the Carpathian source. Despite the gap between the Alpine and Carpathian parts of *R. pendulina* distribution, some migration across the Danube valley was detected. The Carpathian haplotype A was detected at Hochschwab (sample no. 35, Table 1) and Schneetalpe (37) in central E Austria.

Based on the geographical distribution of cpDNA haplotypes, all individuals in the Czech Republic and surrounding regions appeared to have a Carpathian origin and probably originated from Carpathian or Balkan refugia. Therefore, an Alpine origin of the *R. pendulina* in the Šumava Mts (Hendrych 1985) seems unlikely. The low altitude landscape along the Danube has probably constituted an ecological barrier, which has prevented the Alpine populations of *R. pendulina* from dispersing further north (see above).

Haplotype B was found in the Jeseníky Mountains (22; Czech Republic) and the Belianské Tatry Mts (26; Slovak Republic). It is related to the common Carpathian haplotype A from which it differs by a single point mutation. This haplotype probably evolved recently or during the postglacial recolonization of Central Europe as indicated by the terminal position of the haplotype in the network (Posada & Crandall 2001).

It is possible to conclude from our study that sequences of the non-coding cpDNA region (*trnL-trnF*) of *Rosa pendulina* can be used to reveal geographical patterns in genetic variation of this species. Postglacial history of *R. pendulina* and the origin of the Central European populations can be traced using this molecular marker. It is apparent that the current Central European populations originated from at least two glacial refugia. The populations occurring north of the Alps, including those in the Šumava Mts appear to have a Carpathian origin.

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Souhrn

Použitím PCR-RFLP a sekvenování nekódujících úseků cpDNA byla studována fylogeografická struktura mezi 45 populacemi růže převislé (*Rosa pendulina*) v Evropě. Naše studie ukázala jednoznačné geografické uspořádání diversity chloroplastové DNA. Tři hlavní haplotypy jsou geograficky široce rozšířené a vykazují jen velmi malé přesahy v distribuci. Tato situace svědčí o tom, že postglaciální kolonizace probíhala nejméně ze dvou odlišných glaciálních refugií, která byla lokalizována (1) na okraji Alp, severních Apenin anebo Dinárských Alp a (2) na Balkánském poloostrově nebo v Karpatech. Všechny populace v České republice a okolí jsou karpatského původu. Toto zjištění vyvrací spekulace o možném alpském původu šumavských populací *R. pendulina*. Kontaktní zóna mezi alpským a karpatským migračním proudem je pravděpodobně údolí Dunaje.

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