

Phylogeography of *Lonicera nigra* in Central Europe inferred from molecular and pollen evidence

Fylogeografická studie druhu *Lonicera nigra* se zaměřením na střední Evropu – kombinace molekulárních a paleopalynologických dat

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The phylogeographic pattern of the temperate shrub *Lonicera nigra* (Caprifoliaceae) in Europe was inferred from molecular and fossil data. Population samples and pollen data from most of the contemporary natural distribution were analysed. While chloroplast DNA sequences revealed no intraspecific variation, AFLP data show a non-random geographic pattern. Two genetically different groups, distinguished by Bayesian clustering, divided the distribution area of *L. nigra* into south-western and north-eastern regions with a contact zone situated approximately in the upper part of the Danube Valley. Iberian populations constitute an additional distinct genetic group. Pollen evidence supports the genetic data, indicating that *L. nigra* might have survived in glacial refugia located in Central Europe. Nevertheless, this evidence should be considered only as indicative and supplementary, as an unambiguous determination of the species is not possible based on the information on pollen in the literature.

Keywords: AFLP, Central Europe, contact zone, cpDNA sequencing, pollen evidence, postglacial history

Introduction

The present distributions of plant and animal species are a result of historical processes. Species distribution patterns are modified by large-scale environmental changes and may vary among species depending on their ecology. In Europe, Quaternary climatic fluctuations strongly influenced the current composition of biota (Taberlet et al. 1998, Hewitt 1999, 2001). Palaeoecological and molecular methods can be used to investigate the sequence of events leading to contemporary species distributions. Palaeoecological methods (such as palaeopalynology, or analyses of charcoal or other macro-remains) can directly document the presence of a given species in a particular area in the past. However, such data are generally discontinuous in space and largely missing or methodologically unavailable for many species due to limited resolution at the species level. Fossil evidence can also provide information about the likely composition of ancient vegetation, but it can be difficult to establish whether the documented species were widespread in the study area or whether they occurred only in isolated patches (Willis & van Andel 2004, Jankovská & Pokorný 2008). Thus, when testing hypotheses it might be advantageous to combine palaeoecological evidence with other types of data.

Molecular methods are widely used in studies on the history of the distribution of plant and animal species. The following two types of molecular data are generally used in plant phylogeography: (i) sequences of chloroplast DNA, which are used to define haplotypes and (since they are non-recombinant and maternally inherited in most angiosperms) to infer the origin of populations (Taberlet et al. 1998, Hewitt 1999); (ii) amplified fragment length polymorphisms (AFLPs), which provide data on genome-wide genetic variation and are frequently used to reconstruct changes in the postglacial distribution of particular species (Schönswetter & Tribsch 2005, Ehrich et al. 2007). Moreover, population analysis of AFLP data can give additional information about population divergence, e.g. by calculating DWs (frequency-down-weighted marker values, Schönswetter & Tribsch 2005), which use the accumulation of rare markers to reflect long-term population isolation.

The most commonly inferred main southern glacial refugia (the Balkan, Iberian and Apennine Peninsulas), common postglacial colonization routes and contact zones among different genetic lineages (the Pyrenees, the Alps, Scandinavia and Central Europe) are postulated (Taberlet et al. 1998, Hewitt 1999). Glacial refugia represent areas of relative ecological stability that provided habitats for species survival more or less *in situ* during periods of climatic instability (Tribsch & Schönswetter 2003), which is reflected in the greater genetic diversity and unique genotypes recorded in these areas. In contrast, newly colonized regions are genetically depauperate. More recently, the possibility of full-glacial survival of temperate species at northern latitudes (in so-called northern or cryptic refugia; Stewart & Lister 2001) was assumed for some species based on fossil data (Willis & van Andel 2004). However, there is little molecular evidence for the existence of such northern refugia in Central Europe or the Western Carpathians.

The postglacial spread of populations from refugia led to the contact of previously isolated genetic lineages, which resulted in the formation of contact (suture) or hybrid (transitional) zones with secondarily increased genetic diversity (Taberlet et al. 1998, Hewitt 1999, 2004). Such contact zones are usually defined as areas in which different lineages meet, mix or hybridize (Remington 1968, Taberlet et al. 1998). Suture zones of several species tend to cluster in certain geographical areas; nevertheless, the exact location and other characteristics of such zones seem to be specific to each particular species (Hewitt 1999, Willis & van Andel 2004, Magri et al. 2006, Fér et al. 2007, Magri 2008, Dvořáková et al. 2010).

Contact zones in Central Europe for a diverse assortment of organisms, including plants, fish, amphibians, birds and mammals have been detected (Taberlet et al. 1998, Hewitt 1999, 2004, Gum et al. 2005 and references therein). Among plants, there is the Central European contact zone for the widespread grass *Festuca pratensis* (Fjellheim et al. 2006), the annual herb *Rhinanthus angustifolius* (Vrancken et al. 2009) and the temperate shrub *Rosa pendulina* (Fér et al. 2007).

Phylogenetic data supported by palaeoecological information is rare for many plant species because their pollen is either undetectable in fossil pollen profiles or often cannot be identified to the species level solely based on morphology (Beug 2004). Hence, only genera for which it is possible to identify the pollen to species or a narrow species-group level are suitable for studies combining fossil and molecular evidence on species survival during the late glacial maximum, e.g. *Picea* (Tollefsrud et al. 2008), *Fagus* (Magri et al. 2006), *Abies* (Liepelt et al. 2009) or *Cedrus* (Cheddadi et al. 2009).

The temperate shrub *Lonicera nigra* L. (*Caprifoliaceae*) is a suitable species for such a study because its pollen is detectable in pollen profiles, even though such evidence is rare. Although pollen grains of *L. nigra* can be distinguished from those of *L. xylosteum* and other *Lonicera* taxa at high magnifications (Punt et al. 1974), the data compiled refer either to the *L. xylosteum*-type [which can include *L. nigra* and *L. xylosteum* (Punt et al. 1974), sometimes also *L. alpigena* and *L. coerulea* (Moore et al. 1991), or even also *L. caprifolium* (Faegri & Iversen 1989)] or *Lonicera*-type (that can include any *Lonicera* species). Pollen production and dispersal in *Lonicera* is low, due to its mode of pollination [entomogamy (Chrtek 1997) – flowers with concealed anthers and large pollen size (Punt et al. 1974)]. Thus, even rare occurrences of single pollen grains are considered to be evidence of a local presence of the species in the past. However, the presence of *Lonicera* in a plant community does not guarantee its occurrence in the pollen assemblage (Pelánková & Chytrý 2009). Unfortunately, preservation of macro-remains of the genus *Lonicera* is extremely rare; its wood is too thin to be preserved as charcoal and there are no other palaeoecological data for this genus.

Lonicera nigra is diploid ($2n = 18$; Browicz 1976, Chrtek 1997), self-incompatible, entomogamous and pollinated mainly by bumblebees (Willemstein 1987). Dark blue berries are dispersed by endozoochory, mainly by birds. In addition to sexual reproduction, clonal spread by root suckering or layering is recorded (Traiser et al. 1998). The distribution of *L. nigra* is restricted to Europe and extends from Central Europe to the Carpathians and Dinaric Alps in the south-east, to the Alps and Apennines in the south and to the Massif Central and Pyrenees to the south-west. This species reaches its northern limit of distribution in the Czech Republic. Being a submontane species, *L. nigra* is common in mountain regions and at low altitudes. It grows in forests and prefers forest edges and watersides (Browicz 1976, Chrtek 1997, 2002). Due to similar ecological requirements shared with other *Lonicera* species (*L. xylosteum* L. and *L. alpigena* L.), mixed populations may appear (Chrtek 1997, 2002). A hybrid between *L. nigra* and *L. xylosteum* (*L. xhelvetica* Brügger) is described, but there are no reliable data on its distribution (Browicz 1976, Bertová 1985, Chrtek 1997).

For the present study, cpDNA and AFLP genetic variation were analysed in populations from the entire range of *L. nigra* and palaeoecological data was compiled. The following questions were addressed by combining both types of data: (i) Does the molecular data indicate a phylogeographic pattern in the distribution of *L. nigra*? (ii) Is it possible to delimit contact zones between different genetic lineages? Where are these contact zones? (iii) Is it possible to delineate the probable glacial refugia of *L. nigra*? Is there any indication of full-glacial survival of this species in Central Europe? Is there a correlation between molecular and palaeoecological data? (iv) Is the phylogeographic pattern of *L. nigra* comparable with that of any other temperate, European plant species?

Materials and methods

Sampling

Leaf material of 150 individual plants from 31 populations (2–6 plants per population) of *L. nigra* was collected (Table 1). Sampling covered almost the entire contemporary natural range of this species. Field collections were conducted in 2006 and 2007. Material was

collected from shrubs separated by at least 10 m in order to prevent the collection of leaves from the same individual. Young, intact leaves were immediately dried in silica gel. Voucher specimens were deposited in the herbarium PRC.

DNA extraction

Total DNA was extracted from approximately three dried leaves per individual plant (about 15 mg of plant material), using the CTAB protocol (Doyle & Doyle 1987). DNA concentration was measured photometrically using BioPhotometer 6131 (Eppendorf).

Sequencing of cpDNA

Six non-coding chloroplast (cp) DNA regions (*psbA-trnH*, *rpoB-trnC*, *psbC-trnS*, *trnG-trnG2G*, *trnG2S-trnS* and *trnL-trnF*) were screened for possible variation. A test-sample set included individuals from six populations covering the whole distribution area [Pyrenees (population 1, Estany de Sant Maurici, Spain), Alps (7, Steiermark, Austria), Czech Republic (30, Jizerské hory Mts), Western Carpathians (14, Vihorlat Mts, Slovakia), Eastern Carpathians (13, Mt. Hoverla, Ukraine) and Balkans (10, Bjelasica Mts, Montenegro)]. Universal cpDNA primers (*trnL-trnF*, Taberlet et al. 1991; *psbC-trnS*, Demesure et al. 1995; *trnG-trnG2G*, Ohsako & Ohnishi 2000; *psbA-trnH*, Tate & Simpson 2003; *rpoB-trnC*, *trnG2S-trnS*, Shaw et al. 2005) were used for both PCR amplification and sequencing.

PCR amplifications were carried out in a total volume of 20 µl containing 5 ng of template DNA, 2 µl of 10× reaction buffer (Sigma-Aldrich), 0.2 mM of dNTP mix (Fermentas), 0.5 mM of MgCl₂ (Fermentas), 0.3 µM of each forward and reverse primers and 0.5 U of JumpStart RedTaq DNA polymerase (Sigma-Aldrich). Amplification was performed using an XP thermal cycler (Bioer Technology) with initial denaturation at 94°C for 60 s and 35 cycles of 94°C for 45 s, 60°C for 60 s and 72°C for 120 s. A final extension at 72°C for 10 min was performed. Amplification products were purified using the JetQuick PCR Product Purification Kit (Genomed).

Sequencing reactions were carried out using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions using the primers cited above. Purification of sequencing reactions was performed using an ethanol/sodium acetate precipitation provided with the sequencing kit. Purified reactions were run on an ABI 3130 Avant automated sequencer (Applied Biosystems).

AFLP

The double digestion was performed for 2.5 hours at 37°C. The reaction took place in a total volume of 5 µl containing 0.5 U of *EcoRI/MseI* enzyme mixture (AFLP Core Reagent Kit, Invitrogen), 250 ng of total DNA and 1 µl of 5× restriction buffer (AFLP Core Reagent Kit, Invitrogen). Restriction was immediately followed by ligation for 12 hours at 37°C. With the addition of 0.2 U of T4 DNA ligase (AFLP Core Reagent Kit, Invitrogen) and 4.8 µl of adaptor ligation solution (AFLP Core Reagent Kit, Invitrogen) directly to the restricted sample, the reaction volume was increased to 10 µl. Preselective amplification was carried out in a total volume of 5 µl containing 4.0 µl of pre-amplification mix (AFLP Pre-Amp Primer Mix I, Invitrogen), 0.5 µl of 10× polymerase buffer with

MgCl₂ (Sigma-Aldrich), 0.1 U of JumpStart RedTaq polymerase (Sigma-Aldrich) and 0.4 µl of restricted/ligated sample. The pre-amplification mix included *EcoRI*-primer (5′-GAC TGC GTA CCA ATT C - 3′) and *MseI*-primer (5′-GAT GAG TCC TGA GTA A - 3′). Pre-amplification proceeded under following conditions: 72°C for 120 s; 20 cycles: 94°C for 1 s, 56°C for 30 s, 72°C for 120 s and 60°C for 30 min.

Before selective PCR each pre-amplified sample was diluted 10×. The selective PCR reaction was conducted in a total volume of 10 µl containing 1.0 µl of 10× polymerase buffer with MgCl₂ (Sigma-Aldrich), 0.2 mM dNTP (Fermentas), 0.05 µM of fluorescence-labelled *EcoRI*-primer (Applied Biosystems), 0.25 µM of unlabelled *MseI* primer (Applied Biosystems), 0.2 U of JumpStart RedTaq polymerase (Sigma-Aldrich) and 2.3 µl of diluted pre-amplified sample. The selective PCR conditions were as follows: 94°C for 120 s, 65°C for 30 s and 72°C for 120 s. Eight cycles were performed under the following PCR conditions: 94°C for 1 s, 64–57°C for 30 s (in each subsequent cycle, the temperature was decreased by 1°C), 23 cycles: 94°C for 1 s, 56°C for 30 s, 72°C for 120 s and 60°C for 30 min. In total 63 selective primer combinations were tested and the following three combinations were selected as they gave the clearest and most reproducible signal (fluorescent dye in brackets): (FAM)-*EcoRI*-ACT + *MseI*-CAT, (NED)-*EcoRI*-AAC + *MseI*-CAG and (HEX)-*EcoRI*-ACG + *MseI*-CTC.

Two precipitations produced the final purification. First, PCR products with 1 µl of sodium acetate and 25 µl of 96% ethanol were chilled for 20 min at -20°C. Precipitated products were spun at 4°C for 30 min at 12,500 rpm and the supernatant was discarded. Second, 100 µl of 70% ethanol was added and samples were spun at 4°C for 5 min at 12,500 rpm. Purified products were desiccated at 65°C for 10 min. Just before the products were run on the sequencer, 10 µl of the mixture HiDi formamide: GeneScan-500 Rox (20:1, Applied Biosystems) was added to each sample. Fragment analysis was performed on an ABI 3100 Avant automated sequencer (Applied Biosystems).

AFLP data analysis

Profile scoring was performed manually using the software GeneScan 3.7.1 (Applied Biosystems) and Genographer 1.6.0 (Benham 1999, Montana State University 1999 – <http://hordeum.oscs.montana.edu/genographer>). A presence/absence matrix of unambiguously scored AFLP bands was generated. In order to check reproducibility of the AFLP profiles, the whole AFLP procedure was repeated with 11 already analysed individuals and the error rate calculated as the ratio between the number of differences and the total number of compared fragments. This was done after suspicious and unreliable markers were deleted (Bonin et al. 2004). Afterwards, the presence/absence matrix of unambiguously scored AFLP bands was generated.

The isolation-by-distance pattern was investigated using Mantel tests (Legendre & Legendre 1998) implemented in ZT (Bonnet & Van de Peer 2002). The matrix of population pair wise F_{ST} -analogues (computed in Arlequin 3.01; Excoffier et al. 2005) was correlated with the matrix of inter-population geographic distances (computed using ArcGIS 9.0, ESRI). Significance was tested using 10,000 permutations.

The total number of AFLP fragments per population (FT, fragments total), the average number of fragments per individual (FA, fragments average), the number of unique fragments per population (EF, exclusive fragments) and the percentage of polymorphic fragments per

population (%PF, polymorphic fragments) were computed. Since these values depend on the number of individuals analysed per population, the values were calculated by re-sampling the whole dataset (special case of bootstrapping, see Kučera et al. 2008 for details) in order to achieve the same sample size for each population and prevent any loss of information. In this analysis, four individuals per population were selected at each step and a mean value from 1,000 replicates was calculated. The re-sampling procedure was executed using a script in Scilab (<http://www.scilab.org>). Similarly, using the re-sampling procedure and the Scilab script mentioned in Kučera et al. (2008), the rarity of AFLP markers was determined using the DW index (frequency-down-weighted marker values). The DW value was calculated for each population as the number of occurrences of each AFLP marker in a particular population divided by the number of occurrences of this marker in the total dataset. Finally, these values were summed (Schönswetter & Tribsch 2005). Higher DW values are expected in populations where infrequent markers have accumulated due to mutations during long-term isolation (Paun et al. 2008). Shannon's diversity index for each population was calculated in FAMD 1.108 (Schlüter & Harris 2006).

Population genetic structure was inferred using STRUCTURE 2.2.3 (Falush et al. 2007). This program applies a Bayesian model-based clustering method, which uses a Markov chain Monte Carlo (MCMC) algorithm to organize genetically similar individuals into clusters using multi-locus genotype data. The admixture model was used and independent allele frequencies were assumed. As AFLPs are dominant markers, a recessive allele model was used. The number of clusters (K) was limited to 1 to 10. For each K, ten runs were performed to test the stability of the results. The length of burn-in period was set to 100,000, and the MCMC chains after burn-in were run through an additional 1,000,000 replicates (Falush et al. 2007). All computations were done on the freely available Bioportal (<http://www.bioportal.uio.no>). The R-script Structure-sum-2009 (part of AFLPdat; Ehrich 2006) was used to summarize the output files and to calculate similarity coefficients between the replicate runs (Nordborg et al. 2005). The optimum number of populations/groups (K) was the one with consistent results over ten repeats and high similarity coefficient. The software CLUMPP 1.1.1 (Jakobsson & Rosenberg 2007) and Distruct (Rosenberg 2004) was used to create graphical outputs. Because an analysis of the entire dataset indicated that only those runs in which K = 2 converged to a consistent solution in ten repeats, a subsequent, separate analysis of each of these two partitions was conducted using the same parameters. Clustering results were plotted using ArcGIS 9.0 (ESRI).

Analyses of molecular variance (AMOVA; Excoffier et al. 1992) were computed to compare variability within and among populations. In addition to testing the STRUCTURE clustering results, AMOVA analyses were used to compare within- and among-cluster variation. All analyses were computed in Arlequin 3.01 (Excoffier et al. 2005) and the significance of the results was obtained from 1,000 permutations.

Palaeopalynological data

To search for pollen evidence describing the past distribution of *L. nigra*, pollen data was obtained from the Czech Quaternary Palynological Database covering the Czech Republic and Slovakia (PALYCZ; Kuneš et al. 2009) and the European Pollen Database (EPD; Fyfe et al. 2009) covering the area 39–52°N to 2°30'W–30°E, which includes Austria, Bulgaria, Germany, Spain, France, Greece, Hungary, Switzerland, Italy, Poland, Romania and Ukraine (see Electronic Appendix 1). Additionally, data from the same area, which are not

included in the PALYCZ or EPD databases, was also used (see Electronic Appendix 2). The area includes not only the entire contemporary range of *L. nigra* but also neighbouring areas. Chronologies proposed by both databases were used. For the sequences that lacked dates the periods proposed by their original authors were used. For many of the pollen profiles containing *Lonicera*, age was evaluated using a new depth-age model (linear interpolation between calibrated midpoints) constructed using Clam 1.0 (Blaauw 2010).

A map based on data from all the profiles examined was constructed, depicting the presence or absence of *Lonicera* or *Lonicera xylosteum*-type pollen grains. The records of *Lonicera* pollen were searched for the following periods: (i) Pleniglacial, 24–13 ky uncalibrated BP (–24 to –12.4 ky BC), (ii) Late-Glacial, 13–10 ky uncal. BP (–12.4 to –9.5 ky BC) and (iii) Early Holocene 10–7.5 ky uncal. BP (–9.5 to –6.4 ky BC) (Walker 1995, Brauer et al. 1999, Birks & Ammann 2000 and Tinner & Lotter 2001). The periods of time on the map show the oldest pollen record for each site and if there was at least one pollen grain recorded in an assemblage it is marked as a “presence”. Any site with no *Lonicera* pollen grain for all periods of time is marked as an “absence”.

Results

Molecular data

No variation was found among the 3,223 bp of six non-coding chloroplast DNA regions (379 bp in *psbA-trnH*, 426 bp in *rpoB-trnC*, 451 bp in *psbC-trnS*, 596 bp in *trnG-trnG2G*, 725 bp in *trnG2S-trnS* and 646 bp in *trnL-trnF*) in the sample of six populations covering the entire distribution range. A sequence of each region was submitted to the GenBank only once and the sequences are recorded there under the following accession numbers: GU076455 (*psbA-trnH*), GU076460 (*rpoB-trnC*), GU076465 (*psbC-trnS*), GU076470 (*trnG-trnG2G*), GU076475 (*trnG2S-trnS*) GU076480 (*trnL-trnF*). Thus, it is assumed that a single chloroplast haplotype occurs throughout the entire distribution area of *L. nigra*.

For the AFLP the three selective primer pairs that provided clearly readable profiles were selected (see Methods). In total, 205 unambiguously scored loci were selected from a range between 66 to 495 bp, of which 120 (59%) were polymorphic. The overall estimate of the error rate calculated from 11 repeated samples was 2.65%. The number of fragments per individual varied from 129 (population 28, Doupovské hory Mts, Czech Republic) to 154 (population 19, Českomoravské mezíhoří Hills, Czech Republic). After re-sampling, the average number of bands per individual was 142.14. The lowest values (134.94) were recorded in population 8 (Primorie-Gorski Kotar, Croatia) and the highest (150.78) for individuals from population 19 (Českomoravské mezíhoří Hills, Czech Republic). The percentage of polymorphic loci per population ranged from 5.60% (population 14, Mt. Vihorlat, Slovakia) to 11.44% (population 1, Catalan Pyrenees, Spain). These values correspond well with those of the Shannon index (Table 1).

There were no individuals with identical AFLP genotypes. Five populations contained one unique fragment: populations 11 (Kopaonik Mts, Serbia), 18 (Rychlebské hory Mts, Czech Republic), 19 (Českomoravské mezíhoří Hills, Czech Republic), 24 (Brdy Mts, Czech Republic) and 28 (Doupovské hory Mts, Czech Republic). In these populations, each individual harboured a specific fragment, which was not found in any of the other populations studied.

Table 1. – List of *Lonicera nigra* populations studied, details of their localities, distribution of AFLP fragments and genetic diversity measures. No. – locality number, which corresponds to those in Figs. 1, 2 and 3; N – number of analysed individuals; FT – total number of AFLP fragments per population; FA – average number of fragments per individual; EF – number of unique fragments per population; DW – frequency-down-weighted marker value of a population; %PF – percentage of polymorphic fragments per population; H_{Sh} – Shannon's diversity index. Repeated resamplings were made to achieve the same sample size in each population (for details, see "Materials and methods" and Kučera et al. 2008) and resulting mean values are presented for FT, FA, EF, %PF and DW. Collectors' names: AB – Anna Bucharová (Mariánské Lázně), HD – Hana Daneck (Prague), JK – Jan Košnar (České Budějovice), JV – Jaroslav Vojta (Prague), LD – Lucie Drhovská (Prague), LE – Libor Ekrt (České Budějovice), VZ – Vojtěch Zeisek (Prague).

No.	Country	Locality description	Altitude (m)	Latitude	Longitude	Collector	N	FT	FA	EF	%PF	DW	H_{Sh}
1	Spain	Pyrenees, Catalan Pyrenees, Estany de Sant Maurici, river valley near Sant Maurici lake.	1900	42.35 N	1.02 E	HD	5	143.66	135.14	0.83	11.44	7.19	5.25
2	Andorra	Pyrenees, Ransol, banks of the Vallira river.	1600	42.34 N	1.39 E	HD	4	147.24	142.78	0.35	6.40	7.63	4.35
3	Switzerland	Bern, Kaufdorf, 500 m NW of the village Hasli, Taanwald forest.	900	46.49 N	7.28 E	HD	5	142.45	137.18	0.01	7.35	5.33	4.62
4	Austria	Vorarlberg, Bludenz, near the tourist track, 1.5 km S of the village Raggal.	1600	47.11 N	9.50 E	HD	6	148.17	140.47	0.01	10.59	5.73	5.16
5	Germany	Bavaria, Tegernsee, 1 km W of the village Kreuth, tourist track to Mt. Leonhardstein.	870	47.38 N	11.44 E	HD	5	141.69	136.77	0.00	6.90	5.07	4.50
6	Austria	Lower Austria, Payerbach-Reichenau an der Rax, 3 km N of the town, near the tourist track.	900	47.43 N	15.51 E	HD	5	148.04	139.99	0.05	10.10	5.85	5.08
7	Austria	Steiermark, Graz, near the tourist track, 700 m E of the chalet Aiblwirt.	1100	46.57 N	15.07 E	HD	5	149.41	143.64	0.06	7.65	6.41	4.70
8	Croatia	Primorje-Gorski Kotar county, Delnice, close to road in direction of the National park Risnjak, 500 m from the town.	780	45.23 N	14.47 E	HD	6	142.36	134.94	0.04	10.44	5.82	5.11
9	Bosnia and Herzegovina	Bjelašnica Mts, 1.5 km W of the parking place at the ski centre.	1400	43.43 N	18.16 E	HD	6	143.79	136.18	0.05	10.45	5.68	5.14

No.	Country	Locality description	Altitude (m)	Latitude	Longitude	Collector	N	FT	FA	EF	%PF	DW	H_{sb}
10	Montenegro	Bjelasića Mts, Kolašin, banks of the brook near the chalet Jezertine.	1400	42.49 N	19.37 E	HD	5	147.15	141.54	0.03	7.46	5.77	4.67
11	Serbia	Kopaonik Mts, ski-run in direction Duboka, banks of the brook.	1740	43.16 N	20.50 E	HD	4	149.80	144.76	0.81	6.19	6.74	4.44
12	Romania	Caliman Mts, Rastolita, road through the valley of the Tihulețul brook.	1100	47.53 N	25.51 E	JK	5	148.85	142.12	0.02	8.63	6.27	4.95
13	Ukraine	Ukrainian Carpathian Mts, by the path from the village of Hoverla to Mt. Hoverla, banks of the brook.	1000	48.08 N	24.45 E	VZ	5	149.20	141.38	0.02	10.07	5.98	5.11
14	Slovak Republic	Vihorlat Mts, near the peak of the Mt. Vihorlat.	1060	48.53 N	22.06 E	HD	2	150.92	146.49	0.05	5.60	6.28	4.09
15	Slovak Republic	Spíš, Branisko pass, Příkro ridge, 3 km E of the village of Poľanovce.	820	49.01 N	20.51 E	HD	6	150.77	143.03	0.00	9.89	5.71	5.19
16	Slovak Republic	Velká Fatra, Lubochná, near the road, W edge of the town.	520	49.06 N	19.09 E	HD	5	147.77	140.33	0.22	9.91	5.61	5.05
17	Czech Republic	Moravskoslezské Beskydy Mts, Hlavatá, behind the village, along the river Bílá Ostravice.	650	49.24 N	18.23 E	HD	3	146.20	139.88	0.07	8.04	5.61	4.71
18	Czech Republic	Rychlebské hory Mts, Nýznerovské vodopády waterfalls, banks of the brook.	460	50.16 N	17.31 E	LD	5	153.41	148.98	0.97	5.92	7.47	4.48
19	Czech Republic	Českomoravské mezihoří Hills, Lanškroun, close to the town along the road in the direction of Jakubovice.	400	49.55 N	16.34 E	HD	5	158.31	150.78	1.42	9.44	8.83	5.07
20	Czech Republic	Železné hory Mts, Seč, 1.5 km S of the village, the castle hill of the ruin Oheb.	500	49.50 N	15.39 E	HD	5	154.19	147.61	0.01	8.35	6.50	4.91
21	Czech Republic	Českomoravská vrchovina Mts, Jihlava – Kosov, 700 m N of the village, Kosovská hůrka hill.	480	49.23 N	15.38 E	HD	5	154.21	148.39	0.07	7.35	6.68	4.69

No.	Country	Locality description	Altitude (m)	Latitude	Longitude	Collector	N	FT	FA	EF	%PF	DW	H_{sb}
22	Czech Republic	Českomoravská vrchovina Mts, Jindřichův Hradec, 1 km E of the railway station Jindřiš, Jindřišské údolí valley.	500	49.08 N	15.03 E	HD	5	152.92	146.52	0.24	8.55	7.22	4.92
23	Czech Republic	Šumava Mts, České Žleby, 1.1 km E of the town, Spálenišťské hill.	955	48.52 N	13.47 E	LE	5	150.26	143.73	0.01	8.95	6.25	5.01
24	Czech Republic	Brdy Mts, Slavětín u Březnice, 1.5 km NW of the railway station, Špalková hora hill.	450	49.31 N	13.52 E	HD	5	149.33	142.12	0.35	9.86	6.16	5.04
25	Czech Republic	Slavkovský les Mts, Mariánské Lázně, Žizkův vrch hill, nearby the town.	720	49.58 N	12.47 E	AB	5	146.99	140.30	0.01	9.05	5.76	4.91
26	Germany	Thuringia, Thüringer Wald, Gehlberg, banks of the brook near the railway station Gehlberg.	600	50.41 N	10.45 E	HD	5	147.21	141.79	0.07	7.21	6.40	4.60
27	Germany	Thuringia, Schleiß, 700 m E of the castle Burgk, tourist track along the river Saale.	420	50.32 N	11.43 E	HD	4	142.08	137.01	0.22	7.39	5.45	4.49
28	Czech Republic	Doupovské hory Mts, Ostrov, 1 km SW of the town, Ostrovské rybníky nature reserve.	420	50.29 N	12.92 E	JV	5	149.72	141.19	1.07	11.01	7.16	5.25
29	Czech Republic	Lužické hory Mts, Česká Kamenice, 3 km E of the town, Pustý zámek natural monument.	400	50.48 N	14.27 E	HD	5	146.22	142.02	0.24	5.65	6.17	4.28
30	Czech Republic	Jizerské hory Mts, Oldřichov v Hájích, Viničná cesta road, along the watercourse.	500	50.51 N	15.08 E	HD	5	147.34	141.43	0.34	7.76	5.95	4.68
31	Czech Republic	Krkonoše Mts, Špindlerův Mlýn, Labský důl valley, along the tourist track near to the town.	850	50.44 N	15.36 E	HD	4	152.64	147.77	0.19	6.12	6.25	4.43

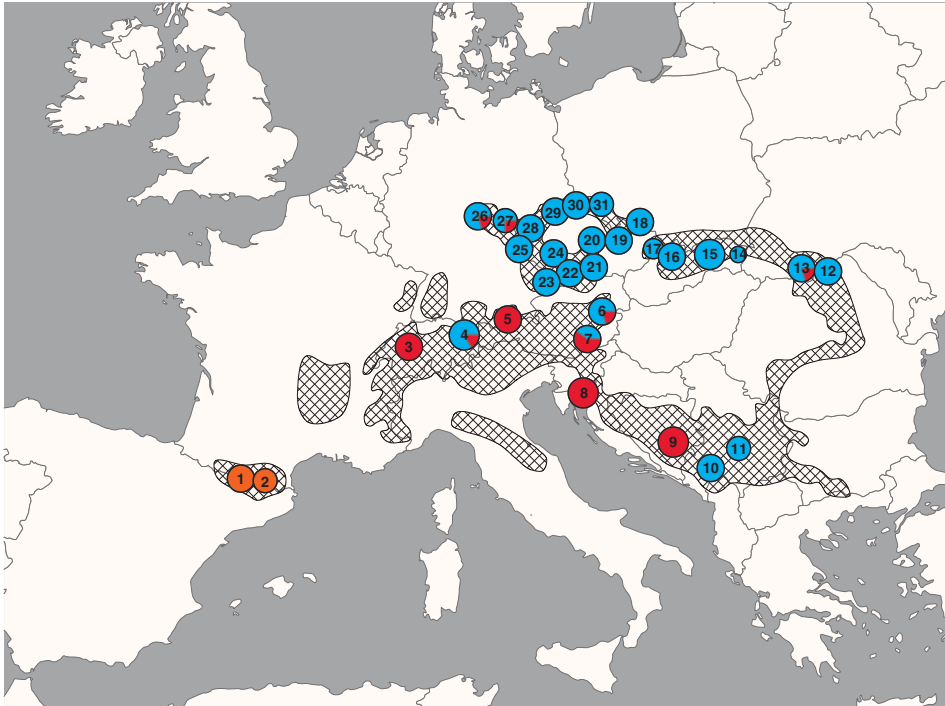


Fig. 1. – Distribution (crosshatched) of *Lonicera nigra* in Europe (taken from Meusel & Jäger 1992) and location of the 31 populations (Table 1) used for the molecular analyses. The colours of the dots refer to the three AFLP genotype groups (A1, A2, B) resolved using Bayesian analyses (Fig. 2).

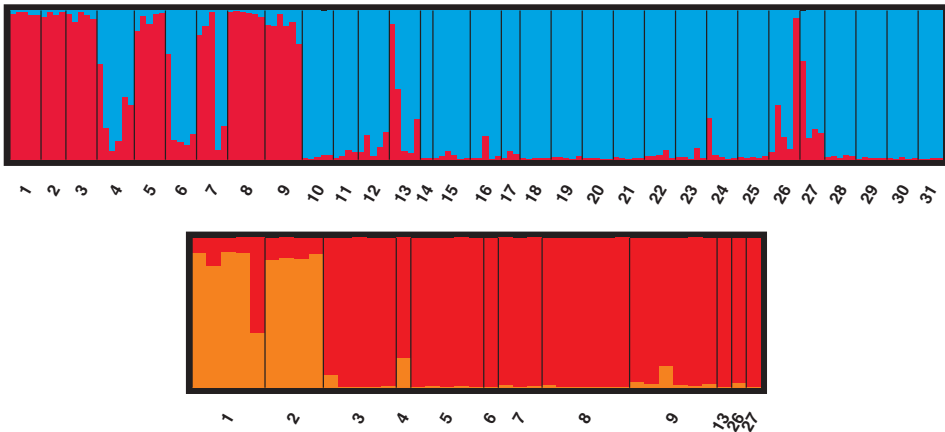


Fig. 2. – Graphical output of the Bayesian analyses showing the probabilities of (a) classifying all individuals in groups A and B; (b) classifying group A individuals in subgroups A1 and A2. Group colours are the same as in Fig. 1.

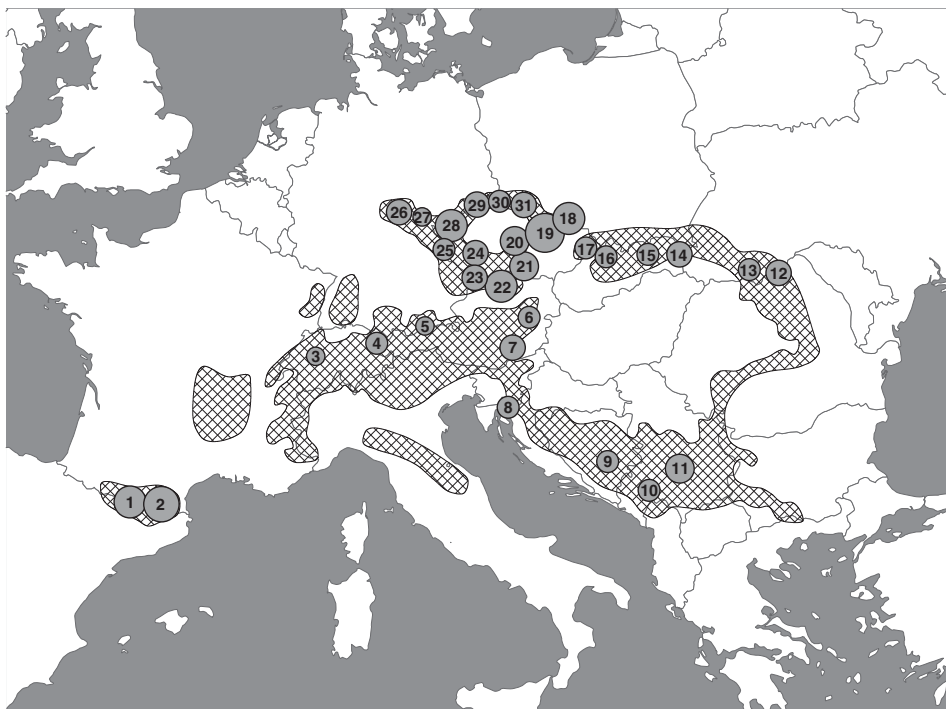


Fig. 3. – Frequency-down-weighted marker values for the 31 populations of *Lonicera nigra* studied. Dot sizes are proportional to the values (see Table 1 for exact values). The distribution of *L. nigra* in Europe is crosshatched.

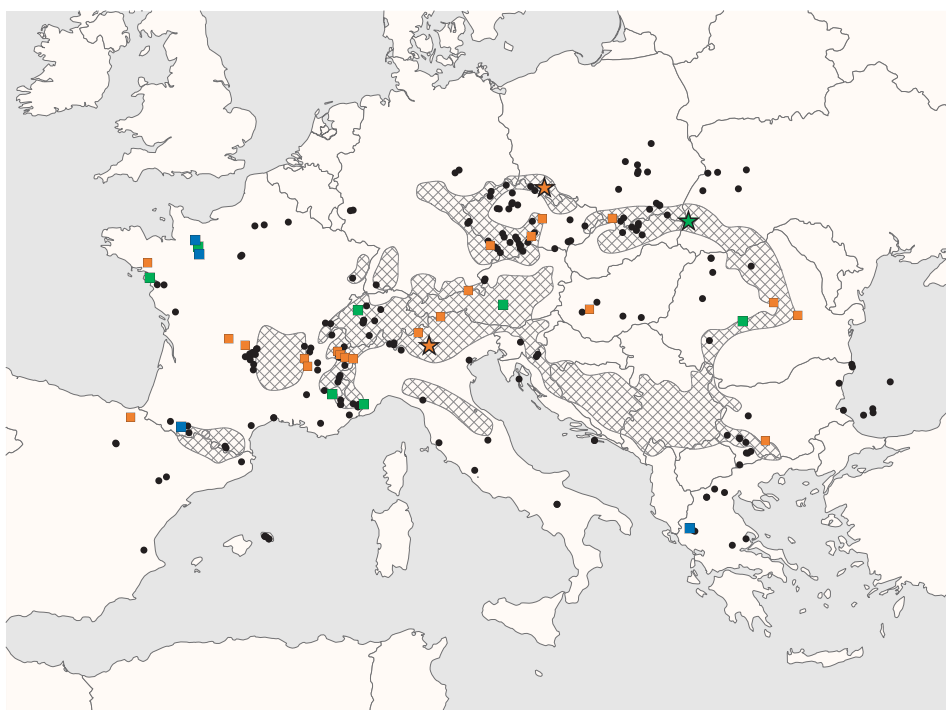


Fig. 4. – Locations in Europe from which the pollen data records studied came (absence of any *Lonicera* pollen – black dots, presence of *Lonicera sp.* pollen – squares, presence of *L. xylosteum*-type pollen – stars, Periodization: Pleniglacial – blue, Late-Glacial – green, Early Holocene – brown).

The re-sampled DW-index values ranged from 5.07 (population 5, Bavaria, Germany) to 8.83 (19, Českomoravské mezihoří Hills, Czech Republic), with an average of 6.29. The highest DW values were recorded in the Pyrenees (population 1, Catalan Pyrenees; 2, Andorra) and the Czech Republic (population 28, Doupovské hory Mts; 22, Českomoravská vrchovina Mts; 18, Rychlebské hory Mts). Populations with the lowest DW-values occurred primarily in the Alps (population 5, Bavaria, Germany; 3, Bern, Switzerland; 4, Vorarlberg, Austria), but also in the Carpathians (population 17, Moravskoslezské Beskydy Mts, Czech Republic; 16, Veľká Fatra Mts, Slovakia; 15, Spiš, Slovakia) and, surprisingly, on the Balkan Peninsula (population 9, Bjelašnica Mts, Bosnia and Herzegovina; 10, Bjelasica Mts, Montenegro). Two other populations with low DW-values were recorded on the western edge of the distribution range (population 27, Thuringia, Schleiz, Germany; 25, Slavkovský les Mts, Czech Republic) (Table 1, Fig. 3).

The Bayesian analysis of the complete dataset using STRUCTURE produced consistent results for only those runs in which $K = 2$ (similarity coefficient = 0.99, among 10 repeats; Fig. 2a). Individuals from the same population clustered together in the same group; only six populations [three populations from the Austrian Alps (population 4, Vorarlberg; 6, Lower Austria; 7, Steiermark), two from Germany (population 26, Gehlberg; 27, Schleiz) and one from the Eastern Carpathians (13, Hoverla Mt., Ukraine)] comprised a mixture of individuals that appeared in both groups (Fig. 2a). The first group (A; 'Pyrenean-Alpine group') includes the populations from the Pyrenees, the Alps, the western Balkan Peninsula and Thuringia (Germany). The easternmost individual of the group A was identified in the only mixed population in the Carpathians (population 13, Mt. Hoverla, Ukraine). The second group (B; 'Balkan-Carpathian group') includes all the populations located in the Czech Republic, the Carpathians (except one individual from Mt. Hoverla, see above) and the central part of the Balkan Peninsula. Some individuals were assigned by STRUCTURE to both groups A and B, but with different probabilities, suggesting they are of hybrid origin. These suspected hybrids were detected in mixed populations from Thuringia (population 26, Gehlberg; 27, Schleiz, Germany), the Alps (population 4, Vorarlberg, Austria) and the Eastern Carpathians (population 13, Mt. Hoverla, Ukraine). Each of these populations (except the population from Mt. Hoverla, Ukraine) is situated close to the border between the two clusters in Central Europe.

Bayesian analysis was repeated for each group separately. Analysis of group A produced consistent results for $K = 2$ (Fig. 2b) and separated populations from the Pyrenees (subgroup A1; 'Pyrenean group') from the rest of the group (subgroup A2; 'Alpine group'). All models with higher K values produced inconsistent results with low similarity coefficients for the 10 runs. Analysis of group B revealed no structure (models for $K \geq 2$ produced results with very low similarity coefficients among runs).

The Mantel test resulted in a highly significant positive correlation between genetic and geographic distance ($r = 0.276$, $P = 0.019$), indicating an isolation-by-distance pattern. The AMOVA analysis revealed 36.78% variation among populations, whereas 63.22% was due to within-population variation (Table 2). The AMOVA analysis of groups based on the first STRUCTURE clustering results (Pyrenean-Alpine group vs. Balkan-Carpathian group) detected 13.43% variation between the two groups. Variation between the Pyrenean and Alpine groups was 24.27%, indicating a clear separation between these two regions.

Table 2. – Results of AMOVA (1,000 permutations) for (A) 150 individuals from 31 populations, (B) 150 individuals forming two clusters based on the first analysis using the program STRUCTURE (Pyrenean-Alpine vs. Balkan-Carpathian group), and (C) 38 individuals forming two clusters, based on the second analysis using STRUCTURE (Pyrenean vs. Alpine group). All values are highly significant ($P < 0.001$).

	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation index F_{st}
A	Among populations	30	925.20	4.71	36.78	
	Within populations	119	962.72	8.09	63.22	
	Total	149	1887.92	12.80	100.00	0.37
B	Among groups	1	117.08	1.86	13.43	
	Within groups	148	1759.47	11.97	86.57	
	Total	149	1876.55	13.83	100.00	0.13
C	Among groups	1	54.39	3.23	24.27	
	Within groups	36	362.38	10.07	75.73	
	Total	37	416.77	13.30	100.00	0.24

Palaeopalynological data

The palaeopalynological data (Fig. 4) confirmed the Pleniglacial presence of *Lonicera* in the north-western Pyrenees (Mardones & Jalut 1983), north-western France and Greece. Only the site in the Pyrenees is within the contemporary range of *L. nigra*. The presence of *Lonicera* pollen is documented at this site until the end of the Late-Glacial period. The other two Pleniglacial *Lonicera* pollen records are doubtfully for *L. nigra*.

During the Late-Glacial period, *Lonicera xylosteum*-type pollen (in the sense of Faegri & Iversen 1989) is documented only in the Eastern Carpathians (Poland, Tarnawa Wyżna; Ralska-Jasiewiczowa 1989), while *Lonicera*-type pollen grains are recorded for sites in the northern foothills of the Southern Carpathians (Romania, Avrig; Tantau et al. 2006) and at four sites along the entire length of the ridge of the Alps (Italy, Selle di Carnino and south-east France, Lac Saint Léger, Beaulieu, unpubl. data; Switzerland, Lobingensee, Ammann 1985; Austria, Dürrenecksee-Moor, Krisai et al. 1989). With the exception of the site at Avrig (where 14 Late-Glacial samples containing *Lonicera* pollen grains document the continuous presence of the genus at this locality), *Lonicera* pollen was confirmed just once at each site during the Late-Glacial period and just once during the Holocene.

Records of *Lonicera*-type pollen from the Early Holocene document its presence in the Bulgarian Rhodopes (Huttunen et al. 1992), the Carpathians (Rybníček & Rybníčková 2002, Tantau et al. 2003, 2009), the Bohemian Massif (Rybníčková 1974, Rybníčková & Rybníček 1988, Svobodová et al. 2002), the Alps (Rybníček & Rybníčková 1977, Oegg 1988, Zoller et al. 1998, Clerc, unpubl. data) and the Massif Central (Guenet 1993). The *Lonicera xylosteum*-type from the Early Holocene in the sense of Moore et al. (1991) is recorded for the sandstone landscape of Broumovsko (Czech Republic; Pokorný & Kuneš 2005) and in the sense of Punt et al. (1974) in the Central Alps (Italy; Pini 2002).

Discussion

No plastid DNA variation detected in Lonicera nigra

Based on the molecular data there is little genetic variation in *L. nigra*. No variability was recorded in chloroplast DNA, although in many other species these regions are very variable (e.g. Shaw et al. 2005). Nevertheless, complete uniformity or very low variation of chloroplast non-coding regions at the continental scale is probably not exceptional as it has been recorded for *Carex pilosa* (Rejzková et al. 2008), *Corylus avellana* (Palmé & Vendramin 2002), *Fraxinus excelsior* (Heuertz et al. 2004), *Carex atrofusca* (Schönswetter et al. 2006) and *Carex curvula* (Pusçaş et al. 2008). The absence of genetic variation in *L. nigra* at northern latitudes (e.g. the Bohemian Massif) might be due to a rapid postglacial colonization resulting in homogeneity of cpDNA (cf. Hewitt 2004, Maliouchenko et al. 2007, Rejzková et al. 2008). However, in the case of this species no variation in chloroplast DNA has been recorded even in southern populations. Moreover, the extensive dispersal of the fleshy fruits of *L. nigra* could support the case of a rapid range expansion and lack of variation among populations, as is reported for several other taxa with fleshy fruits dispersed by birds or mammals (Mohanty et al. 2001, 2002, Hampe et al. 2003).

AFLP based phylogeographical pattern, delimitation of a contact zone

Although no variation in cpDNA was detected, AFLP analysis revealed a clear geographic structure in the variation, as three clusters of AFLP genotypes were identified using Bayesian clustering. The separation of the populations from the Pyrenees (confirmed by AMOVA analysis) is in accordance with their geographic isolation and presence of pollen of a *Lonicera* species in this area during the Pleniglacial and Late-Glacial periods. It is likely that plants from this refugium did not contribute to postglacial expansion into other parts of Europe. Similarly, a distinct genetic group of *Polygonatum verticillatum* is present in the Cantabrian Mountains (Kramp et al. 2009) and of *Alnus glutinosa*, *Fraxinus excelsior*, *Meum athamanticum* and *Rhinanthus angustifolius* in the Pyrenees (King & Ferris 1998, Hewitt 1999, Heuertz et al. 2004, Huck et al. 2009, Vrancken et al. 2009). While there is only a slightly different unique chloroplast haplotype of *Rhinanthus angustifolius* in the Pyrenean population; an analysis of AFLP data separates this population as a clearly differentiated group (Vrancken et al. 2009). On the Iberian Peninsula, there are also genetically different populations of several alpine plants, such as *Pritzelago alpina* (Kropf et al. 2003), *Cardamine alpina* (Lihová et al. 2009) and *Androsace vitaliana* (Dixon et al. 2009).

The remaining area of distribution of *L. nigra* is occupied by two genetically and geographically defined groups (Alpine and Balkan-Carpathian) separated by a contact zone in which there are mixed populations and individuals with intermediate genotypes (Fig. 1). This contact zone is located approximately in the upper part of the Danube Valley and reaches the north-western boundary of the distribution range of *L. nigra*. Towards the eastern part of Central Europe, the two above-mentioned genetic groups are divided by the Pannonian basin, which forms a natural barrier uninhabited by *L. nigra* due to inhospitable environmental conditions. This contact zone of *L. nigra* is present in a similar area to that of other plant species such as *Festuca pratensis* (Fjellheim et al. 2006) and *Rhinanthus angustifolius* (Vrancken et al. 2009). The contact zone between two main haplotype

lineages of *Rosa pendulina* is also suggested to lie in the Danube Valley (Fér et al. 2007); however, subsequent analyses of the AFLP pattern in this species showed that the contact zone is wider, reaching the southern boundary of Austria (Daneck et al., in preparation).

Because admixed populations of *L. nigra* occur only south of the Danube and at the north-western edge of its range, it is hypothesized that the postglacial expansion from the contemporary northern or north-western part to the southern area of the range (Balkan-Carpathian lineage) reached the Danube Valley more quickly than the Alpine genetic lineage, which migrated from the south or southwest. Thus, it seems that only individuals from the Balkan-Carpathian genetic lineage crossed the Danube Valley, which probably constitutes a barrier to range expansion. In addition, it is also likely that the contemporary contact zone was established by the leading-edge colonization phenomenon (Hewitt 1993, 2004), suggesting that the northward migration of the Alpine lineage was prevented by the colonization of suitable habitat by the Balkan-Carpathian lineage.

Location of glacial refugia

Frequency-down-weighted marker values, which indicate long-standing isolation and consequent accumulation of rare markers, were used to detect divergent populations (Schönswetter & Tribsch 2005, Paun et al. 2008). This isolation may also indicate in situ glacial survival rather than a postglacial population origin. High DW values for some Central European populations (19, Českomoravské mezihorí Hills; 22, Českomoravská vrchovina Mts; 18, Rychlebské hory Mts) may thus indicate glacial survival of *L. nigra* in this area. Indeed, the presence of glacial refugia in Central Europe is suggested (based on molecular data) for several plant and animal species, e.g. *Saxifraga paniculata* (Reisch et al. 2003), *Cochlearia bavarica* (Koch 2002), *Carex pilosa* (Rejzková et al. 2008), *Clethrionomys glareolus* (Deffontaine et al. 2005) and *Ursus arctos* (Sommer & Benecke 2005).

Fossil pollen indicates that species of *Lonicera* occurred in Central Europe during the Late-Glacial period and Early Holocene. Nevertheless, this evidence should be considered only as indicative and supplementary, as this pollen data does not allow unambiguous determination of the species. The majority of the evidence refers only to the genus (*Lonicera*-type), but in three cases [the sandstone landscape of Broumovsko, Czech Republic (Pokorný & Kuneš 2005), the Eastern Carpathians (Ralska-Jaszewiczowa 1989), and Italian Central Alps (Pini 2002)] it is specific to *L. xylosteum*-type. The data for the *Lonicera xylosteum*-type from the Late-Glacial (Fig. 4) indicate possible glacial refugia for species of *Lonicera* in the Eastern Carpathians. On the other hand, the Early Holocene occurrences suggest either a very rapid postglacial spread or the glacial survival of *Lonicera* in the Bohemian Massif, and in the Alps and Carpathians, with subsequent population growth at the start of the warm period.

In addition, the genus level pollen evidence supports the hypothesis that some species of *Lonicera* survived in glacial refugia situated along the edge of the Alps. This accords with the existence of Central European glacial refugia for montane forest species and even some temperate broadleaf species (Willis et al. 2000, Jankovská & Pokorný 2008). In landscapes, where the topography is rugged, it is likely that most glacial refugia were located in mountain valleys or deep gorges, which offer protection against cold winds and where there is a high mesoclimatic humidity (Jankovská & Pokorný 2008).

In conclusion, molecular and palaeopalynological data indicate that *Lonicera nigra* may have survived in Central European glacial refugia located in the Carpathians and/or in the eastern perialpine region. Additional refugia north or south of the Alps or on the Balkan Peninsula cannot be excluded, but it was not possible to address this hypothesis using the data set presented. This data also indicates that despite low overall differentiation, the postglacial spread occurred along at least two migratory routes, which resulted in the contact zone in Central Europe.

See <http://www.preslia.cz> for Electronic Appendix 1, 2.

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Souhrn

Fylogeografická studie temperátního druhu *Lonicera nigra* L. (*Caprifoliaceae*) byla provedena s využitím molekulárních a pyloanalytických údajů, jejichž zastoupení v datových souborech pokrývá celý současný areál tohoto taxonu se zaměřením na oblast střední Evropy. Ačkoli sekvenování chloroplastové DNA odhalilo pouze jediný haplotyp v rámci druhu *L. nigra*, AFLP data vykazují genetickou variabilitu a její nenáhodné geografické uspořádání. Pro analýzu molekulárních dat byla použita Bayesova shluková analýza, jejíž výsledky ukazují rozdělení současného areálu druhu na dvě hlavní části (A; Pyrenejsko-Alpská a B; Balkánsko-Karpatská skupina) s kontaktní zónou probíhající zhruba v údolí horní části toku Dunaje. Doplnující analýza dále vyčlenila populace z Iberského poloostrova jako samostatnou geneticky odlišnou skupinu. Přestože pyloanalytická data nebylo možno určit na úroveň druhu, poukazují ve shodě s výsledky molekulárních analýz na možnost, že některé populace *L. nigra* mohly přežít klimaticky méně příznivé období čtvrtohor v glaciálních refugiích ve střední Evropě. Důkaz na základě pylu je ovšem nutno pokládat za pouze doplňkový, vzhledem k nemožnosti přesného taxonomického rozlišení pylových zrn v rámci rodu *Lonicera*.

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