Postglacial history and current population genetic diversity of a central-European forest plant *Hacquetia epipactis*

Postglaciální šíření a současná genetická diverzita populací středoevropské lesní byliny *Hacquetia epipactis*

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In the last decade, phylogeographical investigations have significantly contributed to our knowledge of the Quaternary history of several European species of trees in building forest ecosystems. In contrast, the phylogeography of mid- or low-altitude woodland understorey species that grow in moist and shaded forest habitats is still poorly understood. Here we focus on *Hacquetia epipactis*, a rare forest component of various types of deciduous forest communities, associated with *Fagus sylvatica*. We studied the genetic structure of populations of *H. epipactis* employing two molecular marker systems (AFLP fingerprinting and sequencing of several non-coding chloroplast DNA regions) to investigate the relationships among disjunctive groups of populations spanning its entire distribution in Europe (Dinaric Alps, Alps, Carpathians and adjacent Polish lowlands). The main goal of the present study was to explore the phylogeography and identify potential refugia and probable history of the development of the postglacial range of *H. epipactis*. We attempt to discuss this case study in the context of postglacial migration of forest forming species, especially beech, and postglacial assembly or co-migration of elements of forest communities. The non-coding chloroplast DNA showed a complete lack of genetic differentiation among populations, which may indicate a fast postglacial colonization from a single refugial area. AFLP data show no clear phylogeographical differentiation and indicate close relationships of the Dinaric and Carpathian/Moravian populations with a likely recent origin of the north-eastermost edge populations in Poland accompanied by a strong founder effect. Based on all the evidence, the most plausible scenario is a rapid, postglacial northward expansion from a Dinaric refugium, which concurs with the published postglacial scenario for beech. However, existence of a local refugium in the northern part of the present range is not excluded based on the distribution of genetic groups, which is also in congruence with the hypothetical last glacial history of beech. This suggests a possible shared migration history and role of *Fagus* expansion as the dominant species for the parallel establishment of Illyricoid species co-occurring in various beech-dominated communities. Limited gene flow among extant populations, due to disjunction and isolation at different spatial scales, is confirmed by significant correlation of genetic (pairwise Fₛ[sub]ₚₚₑₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖ¢

K e y w o r d s: AFLP, beech forest, cpDNA sequencing, disjunction, forest understorey, Holocene, illyricoid species, migration, molecular biogeography, woodland herb

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Introduction

The climatic fluctuations that characterized the Quaternary period (Pleistocene) had a substantial influence on the geographical distribution and evolutionary history of the European biota. These past processes and their role in shaping current biodiversity have been extensively studied and discussed, especially over the past 20 years (e.g. Hewitt 1996, Ehlers & Gibbard 2003). Recurrent series of glacial and interglacial periods, which occurred especially over the last million years, resulted in dramatic changes in species distributions due to shifts in the availability of ecological niches and concomitant latitudinal migrations and altitudinal range shifts. In the classical model for temperate biota, such changes involved range contractions and survival in refugia during glacials, and expansion and recolonization of more northerly areas by temperate biota from main refugia located in southern Europe after the cold periods (Taberlet et al. 1998). In a recent perspective, migration routes for more warm-demanding species were opened after the last glacial period the Vistulian (Würm, Weichselian) about 15,000 years ago (Hewitt 2004, Bhagwat & Willis 2008). The increase in warming and humidity resulted in the replacing the steppe-tundra by deciduous forests and establishment of edaphic and climatic conditions suitable for potential migrants and establishment of the temperate forest flora. It is expected that imprints of these historical processes will be found in the genetic structure of extant populations of species. Such data shed a new light on the geographic distribution, postglacial migration pathways, location of refugia and sometimes also ancestral areas of species (Taberlet et al. 1998, Kramp et al. 2009, Willner et al. 2009, Nieto 2011).

In studies on herbaceous plants, much attention has been focused on species inhabiting model disjunct habitats, especially alpine and arctic biomes because of their fascinating properties including high habitat heterogeneity and influence of both large-scale latitudinal climatic changes and local steep altitudinal gradients (for exemplary overviews see, e.g., Abbott & Brochmann 2003, Schönswetter et al. 2005, Alvarez et al. 2009, Ronikier 2011, Eidesen et al. 2013). Relatively little attention has been paid thus far to the phylogeography of herbaceous plant species in the forest understorey, which form an important component of forest ecosystems (Willner et al. 2009) and for which there are fewer comprehensive studies (Schiemann et al. 2001, Tyler 2002a, Rejzková et al. 2008, Svenning et al. 2008b, Kramp et al. 2009, Dvořáková et al. 2010, Slovák et al. 2012, Stachurska-Swakoń et al. 2012, Bartha et al. 2015). Phylogeographical patterns of such species may provide important insights into the postglacial processes of assembly of forest ecosystems. Studies based on macrofossil, palynological and genetic data provide evidence that temperate species could have survived the last glacial period not only in southern but also in western parts of Europe (Lascoux et al. 2004, Leipold et al. 2017). It is also well supported that some temperate forest species could also have survived the cold period in cryptic glacial refugia (Stewart & Lister 2001, Tyler 2002a, Willis & van Andell 2004, Magri et al. 2006, Bhagwat & Willis 2008, Stewart et al. 2010). They probably consisted of small forest patches in river valleys, which could contribute to warming-associated local expansions. In contrast, Leipold et al. (2017) show, that some species followed not only latitudinal but also longitudinal gradients during the last glacial maximum, that restricted species such as, for example, *Hippocrepis comosa* to several refugia.
Willner et al. (2009) and Brus (2010) also emphasize the importance of phytogeographical analyses for a better understanding of the processes of migration, regarding colonization or persistence of forest plants in cryptic refugia. Several forest herbaceous species and shrubs growing in beech forests were certainly associated with beech communities before the last glaciation and their common Holocene presence might reflect the shared history of postglacial recolonization from refugia. It seems that beech spread first, forming a forest ecosystem that was gradually inhabited by herbaceous species (Oberdorfer & Mueller 1984, Willner et al. 2009).

*Hacquetia epipactis* (Scop.) DC. (*Apiaceae*) is an early flowering herbaceous species occurring in deciduous forests in central and southern Europe, with a disjunctive distribution and narrow ecological amplitude. It is a stenotopic plant, i.e. highly sensitive to habitat factors such as shade and moisture. This understorey species is closely associated with the occurrence of *Fagus sylvatica* (Ellenberg 1996). Inherent contemporary association of *H. epipactis* with forests dominated by *F. sylvatica* indicates a shared glacial and postglacial history of these two species (Hendrych & Hendrychová 1985, Brus 2010). On the other hand, Willner et al. (2009) suggest these species may have survived in the forest glacial period within the Carpathians (explaining the isolated occurrence of *H. epipactis* in this area), and later colonized the area north to the Carpathians.

The main goal of the present study was to unravel the phylogeographical pattern of *H. epipactis*, as a stenotopic forest herbaceous plant, and determine the genetic diversity in its current populations. Accordingly, the aims were: (i) to identify the location of potential glacial refugia of *H. epipactis* and explain the origin of the central-European populations with particular emphasis on peripheral populations at the northern edge of species’ distribution in Poland, (ii) to assess the level of genetic diversity and differentiation of *H. epipactis* populations, and (iii) to discuss the phylogeography of *H. epipactis* in the context of the postglacial history of beech as the founding species of related communities.

**Material and methods**

**Study species**

*Hacquetia epipactis* (Scop.) DC. is the only species in the monotypic genus *Hacquetia*. It belongs to the subfamily *Saniculoideae* and it is suggested that it is phylogenetically closely related to the genus *Sanicula* (Calviño & Downie 2007, Kadereit et al. 2008). It is a rhizomatous, clump-forming, perennial species (about 5–10 cm in height) with glossy pale green leaves that only fully develop after flowering. It grows predominantly as a clonal plant, but both vegetative and generative reproduction occur (Duda et al. 2001, Guzik et al. 2008). The tiny yellow flowers appear in early spring and are carried in dense spherical umbels. Seeds are dispersed in July. It is a diploid species with chromosome number 2n = 16 (Májovský et al. 1974, Dobea & Hahn 1997). Biogeographically, it is an Illyrian species, i.e. belongs to the group of species with a distribution centre in the north-western Balkan Peninsula. It has a disjunctive distribution covering north-eastern Italy (Friuli, Veneto), south-eastern Austria, Slovenia, Serbia, Croatia (Dinaric Alps), as well as the Czech Republic, Slovakia and Poland (Meusel et al. 1965, Hendrych & Hendrychová 1985). The main area of this species’ distribution is located in southern Europe,
mainly in Slovenia and Croatia where *H. epipactis* is locally common. In central Europe, the species is restricted to isolated sites in the Czech Republic (Moravia), Slovakia and southern Poland (Beskidy Mts, Western Carpathians). There are also a few additional localities in the eastern Polish uplands (Lubelska Upland), where *H. epipactis* reaches its north-eastern distribution limit (Tumidajowicz 1964, Hendrych & Hendrychová 1985). Altitudinal range of *H. epipactis* covers areas from lowlands (e.g. in the Czech Republic, Hodonín, 170 m a.s.l.) to low- to mid-altitudes in the mountains (Slovakia, Malá Fatra Mts, 720 m a.s.l.). *Hacquetia epipactis* grows on fertile soils that are highly rich in organic matter, with a high humus content, from slightly acidic to slightly alkaline, formed on various types of bedrock, including limestone, sandstone, shale and loess. The inherent soil pH is from slightly acid to slightly alkaline, with high humus content. This species grows in deciduous forests that belong to the order *Fagetalia sylvaticae*. In southern Europe, it is an important characteristic and diagnostic species of the *Aremonio-Fagion* alliance (Horvat et al. 1974, Surina 2002, Trinajstić & Pavletić 2004, Brus 2010).

**Study area and sampling of populations**

Samples for the present study were collected from 15 populations of *H. epipactis* across the species’ geographical range in Europe (Table 1, Fig. 1). Individuals within populations were sampled randomly at distances that generally allowed covering the spatial extent of the populations’ genetic diversity. Fresh leaves were sampled from each population and quickly transported to the laboratory in bags with water. DNA isolation was carried out immediately after coming back from the field. In total, 133 individuals of *H. epipactis* were included in the analysis (with 8 to 10 samples per population).

**DNA isolation and genetic analysis of the species**

Total genomic DNA was isolated from fresh tissue using freeze-dried and powdered material using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Tissue was disrupted using a mortar and liquid nitrogen. Quality and quantity of the isolated DNA was estimated using a fluorometer (Eppendorf, Hamburg, Germany) and its integrity estimated against λ-DNA on 1% agarose gel stained with ethidium bromide. Individuals of *H. epipactis* from the most distant populations (pop. 1 – Królewski Las, pop. 11 – Lucanska, pop. 12 – Loibl and pop. 15 – Plitvice) were used for screening the chloroplast DNA (cpDNA) sequence variation. The following cpDNA regions were tested: *trn*S(UGA) – *trn*M(CAU) with *trn*SUGA and *trn*MCAU primers (Demesure et al. 1995), *trn*L(UAA) – *trn*F(GAA) with “c” and “f” primers (Taberlet et al. 1991), 5’*rps*12 – *rpl*20 with 59*rpl*S12 and *rpl*L20 primers, *trn*H(GUG) – *psb*A with *trn*H(GUG) and *psb*A primers (Shaw et al. 2005), 3’*rps*16 – 5’*trn*Q region with *rps*16x2F and *trn*K(UUU)x1 primers, *rpl*32 – *trn*L with *trn*L(UAG) and *rpl*32-F primers (Shaw et al. 2007).

PCR reaction mix for cpDNA amplification included (in the total volume of 20 μl): 1U Taq recombinant polymerase (ThermoFisher Scientific), 10X Taq Buffer supplied with the enzyme, 1 mM MgCl₂, 0.4 μl of each primer (at 10 mM), 0.4 mM dNTP, 1% of BSA (Thermo Scientific), and 1 μl of DNA template. PCR cycle was performed using a PS2 or Veriti Thermal Cycler (ThermoFisher Scientific) with the following parameters: 10 min at 95°C, followed by 35–38 cycles of 30 s at 95°C, 1 min at appropriate annealing temper-
Table 1. – Geographical origin and genetic characteristics of the sampled populations of *Hacquetia epipactis* analysed using AFLP markers.

<table>
<thead>
<tr>
<th>No</th>
<th>Locality/Code</th>
<th>Country/Region</th>
<th>Altitude (m a.s.l.)</th>
<th>N</th>
<th>No. of total bands</th>
<th>Percentage of polymorph. bands</th>
<th>Nei’s diversity index</th>
<th>Shanon diversity index</th>
<th>DW index</th>
<th>No. of private bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Królewski Las</td>
<td>Poland/Lubelska Upland</td>
<td>240</td>
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<td>143</td>
<td>19</td>
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<td>0.099</td>
<td>3.01</td>
<td>0</td>
</tr>
<tr>
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<td>Poland/Lubelska Upland</td>
<td>280</td>
<td>8</td>
<td>146</td>
<td>35</td>
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<td>199</td>
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<td>0.268</td>
<td>4.59</td>
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<td>Poland/Wielickie Foothills</td>
<td>360</td>
<td>8</td>
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<td>7</td>
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<td>8</td>
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<tr>
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<td>Lucanska</td>
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<tr>
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<td>Slovenia/Halsean Hills</td>
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<td>8</td>
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<tr>
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<td>Plitvice</td>
<td>Croatia/Dinaric Alps</td>
<td>710</td>
<td>10</td>
<td>265</td>
<td>77</td>
<td>0.280</td>
<td>0.213</td>
<td>10.24</td>
<td>4</td>
</tr>
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</table>
ature tested for each region using a gradient block and 1 min at 72°C, followed by a final extension step of 10 min at 72°C. Prior to sequencing, PCR products were purified using GeneMATRIX PCR/DNA Clean Up Purification Kit (Eurx, Gdańsk, Poland). Sequencing of amplicons was carried out in two directions, using amplification primers and the BigDye 3.1 chemistry diluted with 5x sequencing buffer (ThermoFisher Scientific) following the manufacturer’s guidelines. Post-reaction purification was done using an EDTA/ethanol precipitation protocol and sequence analysis was performed on an ABI 3130 automated DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) at the Institute of Botany, Polish Academy of Sciences.
The AFLP procedure was performed following Vos et al. (1995) with slight modifications. 100-150 ng template DNA was digested with 2 U of *Mse*I and 5 U of *Eco*RI restriction enzymes (New England Biolabs, Ipswich, USA) in a 20 μl mixture of 1× NEBuffer-2 (containing 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂ and 1 mM Dithiothreitol, at pH 7.9) and Bovine Serum Albumin (New England Biolabs). Digestion was performed in a Veriti Thermocycler (Applied Biosystems, Carlsbad, USA) for 3 hours at 37°C. Subsequently, 20 μl of ligation mixture containing *Mse*I and *Eco*RI adapters (Sigma-Aldrich, St. Louis, USA), 1U T4 Ligase and 2 μl of slightly acidic 1× T4 DNA Ligase Reaction Buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, and 10 mM Dithiothreitol, pH 7.5) (New England Biolabs) was added. Ligation was performed for 3 hours at 30°C. The resulting primary template was diluted 10 times with H₂O and stored at -20 °C until use. AFLP fingerprints were obtained using a two-step polymerase chain reaction (PCR) amplification. The first step (preamplification) was performed on a primary template using a primer pair based on the sequences of the *Mse*I and *Eco*RI adapters, with one additional selective nucleotide at the 3’ end: ‘A’ for *Eco*RI and ‘C’ for *Mse*I primer. Preamplification reactions (25 μl) contained 5 μl of diluted template, both primers, 0.5 U Taq DNA polymerase with 750 mM Tris-HCl buffer (pH 8.8), 200 mM (NH₄)₂SO₄, 0.1% (v/v) Tween 20 (Fermentas, Glen Burnie, USA), 10 mM of each dNTP, and 1.5 mM MgCl₂. The amplification profile was 2 min at 94°C, followed by 25 cycles of 30 s at 94°C, 30 s at 56°C and 2 min at 72°C, and finally an extension step of 10 min at 72°C. The quality and quantity of the preamplification product was checked on 1% agarose gel. The remaining product was diluted 20× and used for selective amplification with three selective nucleotides. Twenty-eight combinations of the selective *Eco*RI and *Mse*I primers were screened out of which we chose six primer pairs for the selective amplification: ACC/CAT, ACA/CGA, ACA/CAC, ATG/CAT, ACC/CAC. Selective *Eco*RI primers were labelled with a 5-FAM or JOE fluorochrome. The selective amplification reaction mix (20 μl) contained 0.5 μM of each *Eco*RI and *Mse*I primer, 0.5 mM of each dNTP, 1.5 mM MgCl₂, and 0.5 U Taq DNA polymerase, with 750 mM Tris-HCl buffer (pH 8.8) and 5 μl of diluted pre-selective products. The selective amplification profile was as follows: 4 min at 94°C, followed by a touchdown cycler protocol of 35 cycles of 30 s at 94°C, 64°C for 1 min with the annealing temperature decreased by 1 °C each cycle for the first 10 cycles, 1 min at 72 °C and finally an extension step of 10 min at 72 °C. After selective amplification, the fluorescent-labelled selective amplification products were diluted 10× in Sample Loading Solution (SLS) with the addition of DNA size standards (DNA Size Standard Kit 400), and separated on a separation gel (GenomeLab™ Linear Polyacrylamide) in an automated sequencer (GenomeLab™ GeXP Genetic Analysis System, Beckman Coulter, Brea, USA). Electrophoresis was performed for 60 min at 6000 V. Analysis of some individuals was repeated in order to test the reproducibility of the analysis. As suggested by Bonin et al. (2004), to estimate the reproducibility of the AFLP results, DNA from 15 randomly chosen samples, collected as duplicates (11% of the whole sample set) was extracted twice during the DNA isolation procedure. The test samples were analyzed independently throughout the whole procedure. The genotyping error rate was calculated as the ratio of mismatches (1 vs. 0) over matches (1 vs. 1) of the replicated samples (Bonin et al. 2004). Raw data were collected and aligned with the internal size standard using Beckman Coulter Fragment Analysis Software. To create a binary matrix, amplified fragments in the range of 100–450 bp were scored as present (1) or absent (0) across AFLP profiles.
**cpDNA and AFLP data analyses**

The cpDNA sequences were manually aligned using BioEdit v. 7.1.11 (Hall 1999) or DNA Baser Sequence Assembler v4 (Heracle BioSoft 2014) and checked for nucleotide variation.

In the case of the AFLP analysis, basic diversity statistics such as total number of bands, percentage of polymorphic markers, Shannon’s diversity index and number of private bands were calculated using FAMD v. 1.25 (Schlüter & Harris 2006). Nei’s gene diversity values were calculated using POPGENE v. 1.32 (Yeh et al. 1999). An additional measure of divergence, the DW index (Schönswetter et al. 2005) was calculated using the R-script AFLPdat (Ehrich 2006). Analysis of molecular variance (AMOVA) within and between populations or between groups (\(F_{st}\)) was performed using ARLEQUIN 3.5.1.3 (Excoffier & Lischer 2010) with 1000 permutations to test the partitioning of genetic variation within and among populations, as well as the importance of the main geographically defined groups of populations: pop. 1–2 (Lubelska Upland), pop. 3 (Śląska Upland), pop. 4 (Wielickie Foothills, Western Carpathians), southern Poland (pop. 5 – 7) and a larger group encompassing the three previous areas: pop. 1–8 (Beskid Śląski Mts, Western Carpathians), pop. 11 (Malá Fatra Mts, Western Carpathians) and pop. 12–15 (Alps and Dinaric Alps). ARLEQUIN software was also used for calculating the correlation between the genetic (\(F_{st}\)) and geographic population pairwise distance matrices using the Mantel test with 1000 permutations; scatter plot was constructed in STATISTICA 12.0 (StatSoft 2014). Bayesian non-hierarchical clustering of individuals was applied using STRUCTURE 2.3.4 (Pritchard et al. 2000, Falush et al. 2007), based on an admixture model with correlated allele frequencies. The numbers of defined groups (\(K\)) from 2 to 10 were tested with 10 replicates per K. 1,000,000 Markov Chain Monte Carlo repetitions were applied with a burn in period of 200,000. The most likely number of groups was assessed using several indicators such as the “LnP(K)” distribution, repeatability among runs for each \(K\), the modal value of the “\(\Delta K\)” or “mean(L'(K))/sd(L(K))” (Evanno et al. 2005, Hou & Lou 2011). Output data from all runs were analyzed using STRUCTURE HARVESTER (Earl & von Holdt 2012) with implemented CLUMPP (Jakobsson & Rosenberg 2007). DISTRACT (Rosenberg 2004) and GSVIEW v 4.8 (Ghostugum Software 2006) were used for producing graphical displays of STRUCTURE results. Additionally, we applied the NeighborNet method to the distance matrices, bootstrapped using 1000 replicates, with SPLITSTREE v.4 (Huson & Bryant 2006).

**Results**

*Genetic diversity within and among populations of Hacquetia epipactis*

In the case of cpDNA sequences, our analysis showed no variation among analysed individuals and populations of of *H. epipactis* in any of the tested non-coding cpDNA regions.

The obtained AFLP data had a high reproducibility. The genotypic error rate of the AFLP fragments detection was estimated to < 3%. The individual number of markers ranged from 103 (pop. 2, Żółkiewka/ Lubelska Upland) to 334 (pop. 15, Plitvice/Dinaric...
Alps) and the average number of markers per population varied from 143 (pop. 1, Królewski Las/Lubelska Upland) to 265 (pop. 15, Plitvice/Dinaric Alps) with a mean of 191 (SD = 42). The percentage of polymorphic markers varied from 18% (pop. 13, Stari Grad/Haloze Hills) to 77% (pop. 15, Plitvice/Dinaric Alps) with a mean of 42% (SD = 9.6) (Table 1). Nei’s diversity index varied from 0.065 (pop. 1, Królewski Las/Lublin Upland) to 0.280 (pop. 15, Plitvice/Dinaric Alps) with a mean of 0.145 (SD = 0.058). Private bands occurred in five populations from different geographical regions: the highest number (4) was found in pop. 15 (Plitvice/Dinaric Alps). Single private bands were also found in pop. 5 (Rozumice/Głubczyce Plateau), pop. 8 (Třinec/Beskid Śląski Mts) and pop. 9 (Vsetín/Vsetínské vrchy Hills) (Table 1). Nei’s Genetic Identity Index (Nei 1972) that describe the genetic similarity between regions varied from 0.79 between pop. 1–2 (Lubelska Upland) and pop. 12 (Alps), to 0.94 between pop. 4–7, located in southern Poland and pop. 3 (Śląska Upland). The rarity index (DW) varied considerably among populations, ranging from 2.94 in pop. 13 (Slovenia/Haloze Hills) up to 10.24 in pop. 15 (Plitvice/Dinaric Alps) (Table 1).

AMOVA attributed 38.8% of the genetic variability between populations and 61.2% within populations (Fst = 0.39, P < 0.001, Table 2). When a population group level was introduced, 6.3% of the variability was recorded between the groups and 32.9% between populations within the groups. Differences among selected geographical groups based on the F statistics values indicated that the highest differentiation was in the pop. 12–15 (Alps) vs pop. 1–2 (Lubelska Upland), Fst = 0.51, and the Alps (pop. 12–15) vs the rest of populations (Fst = 0.40) (Electronic Appendix 1). Lower Fst values between groups of populations were recorded for groups defined in the northern part of the range: pop. 8–10 (northern Czech Republic) and pop. 11 (Lucanska/Malá Fatra Mts), Fst = 0.31; Lubelska Upland (pop. 1–2) vs pop. 5–7 from southern Poland localities (Fst = 0.37) and localities in southern Poland (pop. 5–7) vs northern Czech Republic (pop. 8–10), Fst = 0.38.

Table 2. – Analysis of molecular variance (AMOVA) of the Hacquetia epipactis populations studied.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>% of variation</th>
<th>Fst</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among populations</td>
<td>14</td>
<td>4151.9</td>
<td>33.1</td>
<td>38.8</td>
<td>0.39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Within populations</td>
<td>139</td>
<td>5005.2</td>
<td>52.1</td>
<td>61.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The Mantel test revealed a statistically significant positive relationship between geographical and genetic distances (r = 0.418, P < 0.05) across all of the regions sampled (Fig. 2), indicating significant progressive isolation by distance in H. epipactis populations. In turn, correlation between geographic distances and the Nei’s Genetic Identity (Pearson correlation) was not statistically significant (r = −0.4081, P = 0.363).

Inference of genetic groups and relationships among populations

Bayesian analysis of the population genetic structure using STRUCTURE showed stable results with a high similarity coefficient for 10 replicated runs (> 0.96) only for K = 3...
Fig. 3A. The \( \Delta K \) also showed one distinct peak at \( K = 3 \) and this value was regarded as relevant and it is interpreted here. Three STRUCTURE clusters are distributed across the whole species’ range and thus indicate in all disjunct areas a generally high level of admixture in populations. Accordingly, no clear geographically driven segregation of genetic groups could be observed, only some weak trends can be defined. One group dominates in the north-eastern part of the area with the easternmost Polish populations from the edge of the general distribution range (pop. 1–2, Królewski Las and Żółkiewka) being almost homogeneously assigned to this group. Remaining two groups are more widespread in the south and centre of the distribution area. In general, in most populations there was contribution from all three phylogroups indicated by the STRUCTURE analysis (Fig. 3A).

The Neighbor-Net analyses indicated a more pronounced structure in the data set, however, with moderate bootstrap support for distinguished groups (Electronic Appendix 2). Individuals formed several clusters and in some populations individuals coherently clustered together but several clusters were heterogeneous in terms of populations and geographical groups; for instance, individuals from pop. 12 (Loibl/Karawanken Alps) were grouped in one cluster with individuals from pop. 15 (Plitvice/Dinaric Alps), southern Poland (pop. 3, 5–6) and northern Czech Republic (pop. 8–9). In general, two tendencies could be detected in the Neighbor-Net analysis: some of the populations and individuals were grouped in geographically widespread clusters and some formed more homogeneous, regional groups, especially supporting some segregation of populations from the north of the Carpathians.
Fig. 3. – Results of the Bayesian admixture analysis of AFLP data for populations of *Hacquetia epipactis* using STRUCTURE software, analysis for K = 3 (see text for details). (A) Partitions of genetic groups (different colours) detected in the analyses across populations shown in the synthetic diagram and (B) in the geographical context with pie chart graphs indicating the share of genetic groups in populations. (C) Tentative location of refuge areas for *Fagus sylvatica* in central Europe during the last glacial maximum and main colonization routes during the postglacial period, based on Magri et al. (2006).
Discussion

The flora and vegetation of deciduous forests in Europe has been strongly influenced by the Pleistocene climatic oscillation. Numerous palynological and molecular studies contributed to understanding these past changes in the distributions of trees and shrubs (Huntley & Birks 1983, Konnert & Bergmann 1995, Demesure et al. 1996, Dumolin-Lapègue et al. 1997, Ferris et al. 1997, King & Ferris 1998, Hewitt 1999, Palmé & Vendramin 2002, Petit et al. 2002, Grivet & Petit 2003, Heuertz et al. 2004, Magri 2008, Svenning et al. 2008a, Daneck et al. 2011). Much less is known about the genetic diversity patterns and history of forest herbaceous species, also important for the postglacial development of forest communities, e.g. with Fagus sylvatica (Schiemann et al. 2001, Tyler 2002a, Rejzková et al. 2008, Svenning et al. 2008b, Kramp et al. 2009, Brus 2010, Dvořáková et al. 2010, Slovák et al. 2012). An important question for the knowledge on extant assemblages is to what extent their components shared a common history and whether they may have coexisted in past assemblages and comigrated from the same glacial refugia. This may be expected in forest understorey herbaceous plants such as H. epipactis, often characterized by narrow and specific ecological demands.

It has been demonstrated that a combined analysis of chloroplast DNA and fingerprinting (AFLP) markers has a high discriminatory potential and can be efficiently used for inference of phylogeographical history of populations, gene flow and postglacial plant migrations (e.g. Schönswetter et al. 2006a, Ronikier et al. 2008). The results indicate a lack of genetic variation in the cpDNA regions in H. epipactis studied. Undiversified cpDNA at the intraspecific level has been previously described in various species, for instance in Carex atrofuscus despite screening 1.5–2.0% of plastid genome (Schönswetter et al. 2006b) or in Nardus stricta using 21 cpDNA primers (Zoric 2013). Palmé et al. (2003) screened several plastid regions of Salix caprea and document the absence of a clear geographic structuring of the haplotypes. Lack of cpDNA variability could be expected also for endemic and relict species of plants, such as H. epipactis. This lack of differentiation could reflect a single origin of postglacial H. epipactis populations based on a single refuge area, although its location remains elusive with respect to the unresolved genetic data. Populations may be also connected with the northern part of the Balkan Peninsula (Dinaric Alps), which is a diversity hotspot of beech-associated species (Willner et al. 2009) and documented as an important glacial refugium of Fagus sylvatica (Magri et al. 2006, Brus et al. 2010). The homogenous genetic structure of cpDNA is rather characteristic of populations from northern Europe and, in the case of H. epipactis, it could indicate a rapid postglacial migration. This would be in accordance with the analysis of the migration of F. sylvatica based on pollen records that indicate an early and vigorous expansion from the northern Balkan refugia into the present area in the Czech Republic (Moravia) and from here further northwards, possibly forming plant communities with Illyricoid species (Magri et al. 2006, Magri 2008).

The observed high levels of genetic admixture revealed by AFLP data can be a direct effect of fast postglacial expansion of H. epipactis. This is similar to results previously reported for Sanguisorba minor (Tausch et al. 2017) and Hippocrepis comosa (Leipold et al. 2017) that clearly indicates a northward migration from southern refugia (e.g. Iberian Peninsula). In addition, the detected lack of a phylogeographical structure is congruent with the homogeneous cpDNA data and together indicate a rapid postglacial spread of
this species along with the expansion of beech, which shows the same clear genetic similarities between Dinaric and Czech/Moravian populations (Magri et al. 2006). Within such a scenario, this species could have reached the Carpathians in the Holocene, Atlantic period, 8.5–6.5 kyr ago (Hendrych & Hendrychová 1985) or later as suggested for beech (Magri et al. 2006), and subsequently populations could expand into eastern Poland (Fig. 3C). It is difficult to assess what the distribution range of *H. epipactis* looked like in older periods of time but it is probable that it coexisted with *F. sylvatica* communities or grew in other types of deciduous forests growing under similar environmental conditions. According to Hendrych & Hendrychová (1985), it is impossible that this species was present in the Pleistocene in the Carpathians and southern Moravia. The current distribution range of *H. epipactis* is fragmented, and the Danube valley and western dry areas of Hungary form a barrier that divide both parts of the distribution range (Fér et al. 2007).

Hendrych & Hendrychová (1985) propose that a fast post-Pleistocene migration of *H. epipactis* occurred across the present area of Hungary in the Atlantic period, but probably this part of the range disappeared due the unfavourable climatic conditions following the Subboreal period (3.7–0.6 kyr ago). While no clear pattern of differentiation was detected, the high $F_{st}$ value from the AMOVA analysis of the entire data set ($F_{st} = 0.39$) and correlation of pairwise $F_{st}$ with distance (Fig. 2) may reflect isolation of *H. epipactis* populations due to disjunct distribution and habitat fragmentation (at local and larger scales) and to limited recent gene flow. In all areas populations are isolated from each other (Table 2, Fig. 1) and this is a general characteristic of the range (Tumidajowicz 1964, Hendrych 1981). The recent gene flow between populations seems indeed to be limited only to the nearest populations. The results can be compared with e.g. Reisch & Bernhardt-Römermann (2014) who found, using a much larger AFLP data set, much higher genetic variation among populations of rare species of plants, than widely dispersed species. This is mostly likely an effect of more scattered gene flow among isolated plant populations. Also Premoli et al. (2001) mention, that the geographic distance indicates a high probability of genetic variation within, as well as among populations.

However, it has to be noted that clonal growth might also influence the genetic structure and diversity of *H. epipactis*, especially in populations with low numbers of polymorphic markers, which often coincides with genetic homogeneity and little admixture.

The DW values (Table 1) reveal a higher number of rare markers in the Dinaric Alps and South-Eastern Alps (pop. 15, Plitvice/Dinaric Alps – 10.24; pop. 12, Loibl/Karawanken Alps – 7.39) and the Western Carpathians (pop. 11, Lucanska/Malá Fatra Mts – 7.63; pop. 8, Třinec/Beszki Śląski Mts – 6.22) (Fig. 1). While a hypothesis of a fast postglacial migration from a single refugium in the south seems the most plausible, the distribution of DW values may also indicate a trace of long-term isolation in a local refugium not only in the southern part of the range but also within the disjunct Carpathian area. This species is locally common in Moravia (Czech Republic) and the Slovak Republic and from this area it could have expanded northwards (Beskidy Mts in Poland) and eastwards (Lubelska Upland) (Fig. 1, 3B). A more complex formation of the disjunct range with a rapid postglacial migration from the Dinaric Alps in parallel with a local earlier lineage within the Carpathians may be supported to some extent by such aspects as the Neighbor-Net clusters showing widespread lineages on the one hand and several more localized northern lineages on the other (Electronic Appendix 2) and gradual changes in admixture patterns with latitude (Fig. 3A). In fact, close genetic relationships between the two areas
inferred for populations of beech concur with the postulated local, northern *Fagus* refugium in Moravia indicated by palaeobotanical evidence (Magri et al. 2006; Fig. 3C) and this could be mirrored by *H. epipactis*. Further insights into this herbaceous plant based on other marker systems would be necessary to effectively verify this hypothesis. Also, although we included several populations from every part of the distribution of this species in this study, a denser population coverage in the main areas of distribution (especially the Dinaric Alps and the Carpathians) may improve the data resolution and reduce the uncertainty of the phylogeographical inference.

The northernmost populations, in particular those at the north-eastern edge of the distribution range in the Lubelska Upland, had the lowest DW values and lowest genetic diversity measures (Table 1). The admixture STRUCTURE analysis indicated that latter were also the most uniform and included them in one of the widespread genetic groups (Fig. 3A). Here, a scenario of a relatively recent dispersal and foundation of these remote populations accompanied by a strong founder effect, seems more plausible than a long-term persistence in this area. It is also supported by a common cluster of the edge populations with some genotypes from the Carpathian foothills in the Neighbor-Net (Electronic Appendix 2). This recent colonization could also concur with postglacial northward expansion of beech. It should be noted, however, that low diversity and homogeneity of these populations may also be influenced by the potential role of clonality (Paul et al. 2016).

In conclusion, the detected phylogeography of *H. epipactis*, characterized by close relationship of southern (Dinaric) and northern (Carpathian) populations with a founder effect at the north-eastern edge seems to show clear analogies with the view on the persistence of beech and its expansion in the area studied. The analogous pattern inferred for *H. epipactis* supports its close association with beech forests. Hence, the reconstruction of the glacial and post-glacial history of *H. epipactis* should indeed be connected with its phytosociological context (Willner et al. 2009, Brus 2010). Many other Illyricoid species are closely associated with *F. sylvatica*, including diagnostic species of associations (e.g. *Anemono trifoliae-Fagetum, Hacquetio-Fagetum, Vicio oroboidi-Fagetum, Lamio orvalae-Fagetum, Omphalodo-Fagetum*) from the Illyrian beech forests, classified into the alliance *Aremoinio-Fagion* (Török et al. 1989, Brus 2010). Besides *H. epipactis*, it also includes the following species: *Aremonia agrimonyoides, Lamium orvala, Epimedium alpinum, Vicia oroboides, Anemone trifolia, Euphorbia curniolica, Omphalodes verna* and *Scopolia curniolica*. Further case studies involving other representatives of these beech-associated communities will allow the testing of the extent to which the parallel migration history may represent a community-scale pattern.

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

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Souhrn

Fylogeografický výzkum v posledním desetiletí výrazně přispěl k poznání role několika evropských dřevin při formování lesních ekosystémů v průběhu čtvrtohor. Naproti tomu historie rozšíření vlhkomilných a stínomilných druhů podrostu lesů nížin a středních poloh je dosud známa jen velmi málo. Zaměřili jsme se proto na druh Hacquetia epipactis, coby vzácného průvodce bučin a dalších typů opadavých lesů se zastoupením buku. Ke studiu genetické struktury jeho populací a odhalení vztahů mezi jednotlivými areály disjunktního areálu druhu (Dinářské hory, Alpy, Karpaty a přílehlé polské nížiny) jsme využili dva molekulární markery (AFLP a sekvenování několika nekódujících úseků chloroplastové DNA). Hlavním cílem práce bylo identifikovat potenciální refugia a možnou historii vývoje areálu druhu během postglaciálu. Výsledky jsme porovnali s poznatky o postglaciálním šíření buku, zejména buku, a dalších druhů lesního podrostu. Nekódující úseky chloroplastové DNA neodhalily žádnou diferencii mezi populacemi, což může poukazovat na rychlou postglaciální kolonizaci území z jediného původního refugia. Výsledky AFLP také neukázaly jakoukoliv zřetelnou fylogeografickou diferencii, pouze naznačují bližší vztahy dinárských, karpatských a moravských populací s pravděpodobně později vzniklými populacemi na severovýchodním okraji areálu a projevy tzv. efektu zakládacího. Na základě všech dostupných dat se jako nejpravděpodobnější scénář postglaciální historie H. epipactis jeví jeho rychlé rozšíření z dinárského refugia severním směrem, které se shoduje s dříve popsanou postglaciální migrací buku. Existence místního refugia v severní části současného areálu ale není vyloučena, což je rovněž v souladu s hypotetickou historií buku. Tyto výsledky ukazují na možnou migrační historii buku coby dominanty a illyrských druhů na bučiny vázaných. Signifikantní korelace mezi genetickou a geografickou vzdáleností potvrzuje omezený genový tok mezi současnými vzájemně izolovanými populacemi.

References


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