Tree Genetics & Genomes (2017) 13: 47
DOI 10.1007/s11295-017-1137-9

ORIGINAL ARTICLE

# High genetic diversity and distinct origin of recently fragmented Scots pine (*Pinus sylvestris* L.) populations along the Carpathians and the Pannonian Basin

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Received: 24 May 2016 / Revised: 15 March 2017 / Accepted: 21 March 2017 / Published online: 27 March 2017


**Abstract** Historical evolutionary events highly affect the modern-day genetic structure of natural populations. Scots pine (*Pinus sylvestris* L.), as a dominant tree species of the Eurasian taiga communities following the glacial cycles of the Pleistocene, has survived in small, scattered populations at the range limits of its south-eastern European distribution. In this study, we examined genetic relationships, genetic divergence and demographic history of peripheral populations from central-eastern Europe, the Carpathian Mountains and the Pannonian Basin. Four hundred twenty-one individuals from 20 populations were sampled and characterized with both nuclear and chloroplast simple sequence repeat (SSR) markers. Standard population genetic indices, the degree of genetic differentiation and spatial genetic structure were analysed. Our results revealed that peripheral Scots pine populations retained high genetic diversity despite the recently ongoing fragmentation and isolation of the persisting relict populations. Analysis of molecular variance (AMOVA) showed 7% among-population genetic differentiation, and there was no isolation by distance among the island-like occurrences. Genetic discontinuities with strong barriers (99–100% bootstrap support) were identified in the Carpathians. Based on both marker types, populations of the Western Carpathians were delimited from those inhabiting the Eastern Carpathians, and two main genetic lineages were traced that most probably originate from two main refugia. One refugium presumably existed in the region of the Eastern Alps with the Hungarian Plain, while the other was probably found in the Eastern Carpathians. These findings are supported by recent palynological records. The strongest genetic structure was revealed within the Romanian Carpathians on the basis of both marker types. With only some exceptions, no signs of recent bottlenecks or inbreeding were detected. However, Carpathian natural populations of Scots pine are highly fragmented and have a small census size, though they have not yet been affected by genetic erosion induced by isolation.

**Keywords** Carpathians · Glacial refugia · Microsatellites · Phylogeography · *Pinus sylvestris*

Communicated by F. Gugerli

**Electronic supplementary material** The online version of this article (doi:10.1007/s11295-017-1137-9) contains supplementary material, which is available to authorized users.

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## Introduction

Among various factors, landscape-scale habitat loss, population fragmentation and isolation impose the highest risks for

species survival (Fahrig 2003; Ewers and Didham 2006). The long-lasting effects of fragmentation, which also trigger a loss of genetic diversity, may ultimately lead to population breakdown and extinction (Chapin et al. 2000). Natural (climate-driven) changes on a long-term historical timescale can leave imprints in spatial genetic patterns and the demography of a species' current spatial genetic structure (Bradshaw 2004). However, in the case of forest trees, genetic signals caused by fragmentation, such as inbreeding, genetic isolation and bottlenecks, appear in the population structure over a much longer period of time due to their long generation time and the effects of slow mutation, nucleotide substitution and speciation rates (Savolainen and Pyhäjärvi 2007; Kramer et al. 2008). This period can be as long as hundreds or thousands of years in conifer species (Petit and Hampe 2006). Peripheral populations living at the margin of a species' natural range are likely vulnerable to the genetic effects of population fragmentation (Lönn and Prentice 2002). Furthermore, isolation on the periphery potentially exposes populations to maladaptation, which can be caused by drift, inbreeding, selection, pleiotropy, linkage disequilibrium, homozygote disadvantage and gene flow (Crespi 2000; Kremer et al. 2012). On a spatial scale, these peripheral populations reflect the species' physiological boundaries, and they may also represent the climatic and adaptive limits of dispersal (Sykes et al. 1996). Therefore, peripheral populations are important for the future evolution and adaptation of a species, and they can serve as an initial gene pool for speciation (Lesica and Allendorf 1995; Chhatre and Rajora 2014). Thus, peripheral populations are considered important sources in phylogeographic, population genetic and demographic studies.

Scots pine (*Pinus sylvestris* L.) is a long-lived coniferous tree species of the Pinaceae family (Pravdin 1969), which occupies a continuous range as the dominant tree species of the Eurasian taiga communities. Furthermore, it is an essential species of various forest ecosystems (Matías and Jump 2012; Giertych and Mátyás 2013).

Peripheral populations of *P. sylvestris* have been widely studied across species' distribution range. Enzymatic polymorphism revealed overall low structuring of populations, but elevated differentiation was reported between populations that derived from different glacial refugia (Müller-Starck et al. 1992). Evaluations involving stands from the southern European provenances showed that they were distinct from the northern European populations (Mejnartowicz 1979; Kieliszewska-Rokicka 1981). Modern-day organelle and nuclear DNA marker studies highlighted that populations, especially on the southern margin of the Mediterranean region, present high genetic diversity and a complex geographic pattern (Robledo-Arnuncio et al. 2005). Similarly, genetic differentiation and high genetic diversity were identified in geographic regions of the Alps and the Apennines, at the species' Italian periphery (Labra et al. 2006; Scalfi et al. 2009; Belletti et al. 2012). The westernmost populations in the British Isles share a similar pattern, as the Scottish populations differ from the populations in continental Europe (Provan et al. 1998; Wachowiak et al. 2011). On a regional scale, populations from central-eastern Europe, and particularly the Carpathian Mountains and the Pannonian Basin, were recently studied by Bernhardsson et al. (2016). They revealed a low level of differentiation among the populations and the impact of Holocene population fragmentation.

Scots pine along the Carpathian Mountain range is distributed in island-like isolated populations (Fekete and Blattny 1913), but there are also scattered natural populations sustained in mixed forest stands, with broad-leaved species in the western Pannonian Basin at the foothills of the Alps (Pócs 1960; Fekete et al. 2014). The genetic structure of these peripheral populations of the Carpathian distribution was highly affected by the postglacial climate warming, forcing Scots pine to immigrate into edaphically specialized habitat types. Indeed, Scots pine natural populations are distributed in the Carpathians on a large elevation gradient, located in sites of divergent ecological conditions, including humid, cool peatbogs and sunny, dry, rocky outcrops. In addition, historical human-mediated activities further increased habitat fragmentation and considerably reduced population census sizes. In part as a consequence of this, only isolated and island-like populations have been sustained (Giertych and Mátyás 2013).

Drawing on macrofossil and pollen evidence, studies on the Quaternary vegetation history of *P. sylvestris* within this region conclude that Scots pine, along with other cold-tolerant and drought-tolerant conifer taxa, inhabited the Carpathians and the Pannonian Basin in the full glacial and later in the beginning of the postglacial period (Rudner et al. 1995; Rudner and Sümegi 2001; Jankovská and Pokorný 2008). In situ findings also suggest that conifers and in particular boreal and cool temperate tree taxa like Scots pine in central Europe and in the Carpathians survived the last glacial maximum (LGM 20,000–19,000 Before Present) in small, patchy and discontinuous glacial refugia (Willis et al. 1998; Magyari et al. 2014). Species would have had sustained populations in isolated, so-called cryptic refugia, with favourable conditions both for Scots pine and for other boreal and temperate species (Rull 2009, 2010; Sommer and Zachos 2009). Altogether, Scots pine had a complex spatio-temporal history in central-eastern Europe during the Holocene, influenced mainly by oscillations in the climate and also by anthropogenic impact, as a consequence of which overall reduction of population size was experienced (Feurdean et al. 2007). Pollen records also indicate that populations on the lowlands of the Pannonian Basin dramatically declined during the Holocene (Willis et al. 1995; Magyari 2011). Furthermore, on mid-altitudinal to high-altitudinal sites in the Carpathians, species abundance varied greatly by location (Willis 1994; Birks and Ammann 2000).

Despite combined pollen, macrofossils and organelle DNA analysis that could detect glacial refugia in the Carpathian Basin along the Danube, previous molecular studies performed in the region reported a lack of geographic structure both with mtDNA and cpDNA within the Carpathian Mountains (Cheddadi et al. 2006; Bernhardsson et al. 2016). Similarly, no variation and no phylogeographic structure in mitochondrial DNA were found in provenance trials conducted in the region by Čelepirović et al. (2009).

In this study, we combined nuclear and chloroplast microsatellite markers to discuss the results in the light of putative refugia occurring within the Carpathian region and to (1) highlight the current population structure of the selected peripheral populations from central-eastern Europe, most of which formerly were not included in molecular studies; (2) identify genetic relationships, degree of diversity and degree of divergence and infer gene flow between the stands; and (3) circumscribe putative refugia that existed in the time of the Pleistocene.

## Material and methods

### Plant material

Altogether, 20 natural and autochthonous populations were collected from the highly fragmented distribution of the species. In total, 421 individuals were analysed with nuclear and chloroplast SSRs (Table 1): five populations from the Pannonian Basin in western Hungary (HFE, HKO, HOR, HZA, HVA); four from the Western Carpathians (Tatra Mountains), i.e. the Low Tatras (STU, SLI) and the westernmost ridges of the High Tatras (SKV and SME); nine from Romania, four of which are found in the Transylvanian Central-Island Mountains (Apuseni) (RBI, RBE, RML, RMH); one in the western part of the Southern Carpathians, Latoriței (RPA); and four in the Eastern Carpathians (RFE, RCO, RMO, RPO). More distant populations from outside of the Carpathian region were also included as outgroup populations: one from the Bulgarian Rila Mountains (BYU) and one from the Central Estonian Plain (ESE). Our sampling plots are found in natural sites as part of nature reserves, and none of them are affected by forest activity. Accordingly, the Scots pine populations regenerate naturally. Most of the populations persist in ecologically extreme conditions, including dry, sunny outcrops with low soil availability, acidic peatbogs and dry sandy substrates with low nutrient content.

Needles were sampled from mature trees (8–30 from each population) with at least 30-m distance between individuals. However, in some of the populations, samples were taken from shorter inter-individual distances due to relatively small population size (from 0.02 to >5.00 km$^2$) and limited sample availability. Plant material was stored on silica gel and frozen at −80 °C until DNA extraction.

### Laboratory methods

Total DNA was extracted from 20 to 25 mg of plant material (1-year-old needles) by using DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). For the first attempt, ten nuclear microsatellites were used (SPAG 7.14, SPAC 11.4, SPAC 11.6 and SPAC 12.5 from Soranzo et al. (1998) and psyl16, psyl17, psyl19, psyl36, psyl42 and psyl57 from Sebastiani et al. (2012)). Later, two inconsistent loci (SPAC 11.6, SPAC 12.5) were excluded from further analysis. The remaining eight markers showed reliable banding patterns and were scored and used for further analysis. These markers proved to be highly polymorphic in our populations. Genotyping of individuals at chloroplast SSR loci was performed with four primer pairs, Pt-30204, Pt-15169, Pt-45002 and Pt-26081 originally developed for *Pinus leucodermis* (Vendramin et al. 1996). Forward nuclear primers were fluorescently labelled with 6-FAM (SPAG 7.14, SPAC 11.4, psyl17, psyl19, psyl42 and psyl57) and NED (psyl16, psyl36). Chloroplast primers were labelled with HEX (Pt-30204, Pt-26081) and 6-FAM (Pt-15169, Pt-45002). Polymerase chain reaction (PCR) details are provided in the Online Resource. Electrophoresis to detect PCR products were carried out on a 1% (*w*/*v*) ethidium bromide-stained agarose gel in 1× TBE buffer. After amplification, strong yields of PCR products were diluted 25 to 30 times for fragment sizing, which was performed on an automated sequencer ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA). Band scoring was analysed using PeakScanner 1.0 (Applied Biosystems 2006), and all size scores were visually checked.

### Data analysis

Micro-Checker (Van Oosterhout et al. 2004) was used to test all nSSR loci for null alleles and possible scoring errors derived from large allele dropout and the presence of microsatellite stutter bands. Then, standard population genetic diversity indices (number of alleles (*N*a), number of effective alleles (*N*e), number of private alleles (*N*p), expected heterozygosity ($H_e$), observed heterozygosity ($H_o$), inbreeding coefficient ($F_{IS}$)) were calculated for each population using GenAlEx v.6.5 (Peakall and Smouse 2006). For cpSSR loci, haploid genetic diversity (*h*) and *N*a, *N*e and *N*p were calculated with GenAlEx v.6.5. For estimates of haplotype diversity indices, haplotype analysis (Eliades and Eliades 2009) was used. Relying on the average squared sum of all allele size differences with the assumption of the stepwise mutation model (Morgante et al. 1998), we calculated mean genetic distance between individuals ($D_{sh}^2$; Goldstein et al. 1995). In addition, the number of haplotypes per population (*A*), the number of private haplotypes ($P_h$), the effective number of haplotypes ($N_e$) and haplotypic richness ($H_R$) were estimated using rarefaction (El Mousadik and Petit 1996).

**Table 1** List of the 20 island-like populations of *Pinus sylvestris* from the Central and Eastern European peripheral distribution of the species included in this study

| Code | Country | Region | Residential area | Latitude (°N) | Longitude (°E) | Altitude (m a.s.l.) | Est. area (km$^2$) | Samples | Comments |
|---|---|---|---|---|---|---|---|---|---|
| BYU | Bulgaria | RL | Yundola | 42.07 | 23.83 | 1561 | >5.00 | 29 | Montane forest |
| ESE | Estonia | CE | Selgise | 58.58 | 27.00 | 102 | >5.00 | 30 | Peatbog |
| HFE | Hungary | PB | Fenyőfő | 47.35 | 17.77 | 252 | 4.49 | 24 | Mixed forest |
| HVE | Hungary | PB | Pethőhenye | 46.87 | 16.92 | 306 | 0.04 | 19 | Mixed forest |
| HZA | Hungary | PB | Szalafő | 46.87 | 16.30 | 231 | 0.08 | 20 | Mixed forest |
| HOR | Hungary | PB | Csörötnek | 46.93 | 16.35 | 296 | 0.10 | 23 | Mixed forest |
| HKO | Hungary | PB | Kőszeg | 47.34 | 16.45 | 468 | 0.24 | 20 | Rock surface |
| SKV | Slovakia | WC | Kvacany | 49.18 | 19.54 | 799 | 0.48 | 20 | Rock surface |
| SME | Slovakia | WC | Zuberec | 49.27 | 19.63 | 815 | 0.07 | 20 | Peatbog |
| STU | Slovakia | WC | Svarin | 49.02 | 19.91 | 1107 | 0.70 | 20 | Rock surface |
| SLI | Slovakia | WC | Liptovszky Hrádok | 49.04 | 19.74 | 729 | 0.02 | 10 | Rock surface |
| RFE | Romania | EC | Fantana Brazilor | 46.50 | 25.26 | 953 | 0.32 | 26 | Peatbog |
| RPO | Romania | EC | Poiana Stampei | 47.30 | 25.12 | 878 | 1.43 | 20 | Peatbog |
| RMO | Romania | EC | Baile Tusnad | 46.13 | 25.91 | 1052 | 0.58 | 29 | Peatbog |
| RCO | Romania | EC | Lacu rosu, Bicaz | 46.80 | 25.79 | 981 | 0.04 | 19 | Rock surface |
| RPA | Romania | SC | Voineasa | 45.38 | 23.91 | 753 | 3.42 | 23 | Rock surface |
| RBI | Romania | CIM | Rosia | 46.84 | 22.37 | 393 | 0.13 | 8 | Rock surface |
| RBE | Romania | CIM | Posaga de sus | 46.49 | 23.36 | 524 | 0.84 | 20 | Rock surface |
| RML | Romania | CIM | Ponor | 46.33 | 23.34 | 925 | 0.10 | 10 | Peatbog |
| RMH | Romania | CIM | Calatele | 46.73 | 23.02 | 913 | 0.58 | 11 | Peatbog |

*RL* Rila Mountains, *CE* Central Estonian Plain, *PB* Pannonian Basin, *WC* Western Carpathians, *EC* Eastern Carpathians, *SC* Southern Carpathians, and *CIM* Central-Island Mountains (Apuseni)

Analysis of molecular variance (AMOVA) implemented in Arlequin v.3.5 software (Excoffier and Lischer 2010) was used for nSSR and cpSSR data to determine the partition of the genetic variation within and among populations. Significance tests were evaluated using a permutation approach with 999 replications. Similarly, Arlequin v.3.5 was used to detect deviation from expectations of Hardy-Weinberg equilibrium (HWE) at nuclear loci. The test of HWE employed a Markov chain approximation (Guo and Thompson 1992). The number of steps after the burn-in period was set to 1,000,000 with a 100,000 dememorization.

To test for correlation between geographical (kilometres) and genetic (Nei's unbiased genetic distance) distances between pairs of populations at both nSSR and cpSSR markers, dissimilarity matrices were generated and tested for isolation by distance (IBD; Wright 1943) with a Mantel test (Mantel 1967). The analysis was carried out in the online platform of Isolation-by-Distance Web Service (IBDWS) 3.16 (Jensen et al. 2005) and GenAlEx v.6.5, with 9999 and 1000 permutations, respectively.

Different approaches were employed to investigate the spatial genetic structure of the populations. A Bayesian clustering approach implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000) was used to infer groups or subpopulations in the nSSR dataset. We performed the analysis with an admixture model with correlated allele frequencies and a LOCPRIOR setup (according to preliminary test runs). The method uses sampling locations as prior information in the case of a relatively weak signal of structures (Hubisz et al. 2009). *K* value was set to 1–10 with a burn-in period of $10^5$ steps followed by $10^6$ repetitions of Markov chain Monte Carlo (MCMC). Fifteen repetitions were set for each run. The web-based STRUCTURE HARVESTER (Earl and von Holdt 2012) was used to apply the Evanno method (Evanno et al. 2005) to detect the value of *K* (the number of genetic groups) that best fit the data. The 15 simulations were averaged using CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007) and represented in the form of bar graphs using POPHELPER (Francis 2016). We used BAPS 6.0 (Corander and Marttinen 2006; Corander et al. 2008a, 2008b) to conduct hierarchical clustering analyses of the chloroplast microsatellite dataset. BAPS was run with the maximal number of groups (*K*) set to 2–20 (equal or larger than the population number), and each run was replicated five times.

Genetic discontinuities corresponding to the change in genetic variation among populations were identified with BARRIER 2.2 (Manni et al. 2004) on both nuclear and chloroplast SSR datasets. Spatial geographic coordinates were connected with Delaunay triangulation, and the corresponding Voronoi tessellations were projected. To identify the genetic barriers, Monmonier's maximum difference algorithm (1973)

was applied, which traces a barrier along the Voronoi tessellation, starting from the edge for which the distance value is maximal. The boundary proceeds across adjacent edges until the forming boundary reaches the limit of the Delaunay triangulation or closes itself by forming a loop around a population. Within the analysis, barriers were set from 1 to 9. To test the significance of the detected barriers, 1000 resampled bootstrapped (population pairwise) $D_A$ genetic distance matrices (Nei's chord distance; Nei 1978) were calculated in MICROSATELLITE ANALYSER (MSA) software (Dieringer and Schlötterer 2003).

To detect any recent severe reduction in effective population size or possible expansion events in Scots pine populations, BOTTLENECK 1.2.02 was used on the nSSR dataset (Cornuet and Luikart 1996). Bottlenecks cause low-frequency alleles to become transitorily less abundant (<0.1), while more intermediate-frequency alleles increase (Luikart et al. 1998). BOTTLENECK correlates expected heterozygosity ($H_e$) with observed heterozygosity ($H_o$) at mutation-drift equilibrium. The two-phased model (TPM) of mutation was applied as the most appropriate for microsatellite data (Di Rienzo et al. 1994; Piry et al. 1999). We used 5% of multistep changes and a variance among multiple (12) steps (Piry et al. 1999). For each population, 2000 simulations were performed. Significance was assessed using the implemented Wilcoxon sign-rank test, which determines whether or not the average of standardized differences between $H_o$ and $H_e$ is significantly different from zero (Cornuet and Luikart 1996). Significant heterozygote excess relative to the number of alleles indicates a recent population bottleneck. Additionally, the 'mode shift' qualitative descriptor of allele frequency distribution was applied to discriminate bottlenecked populations (Luikart and Cornuet 1998).

## Results

### Chloroplast microsatellites

All four chloroplast microsatellite markers amplified successfully, and polymorphism was found at all loci (Online Resource Table S1). Haplotype analysis of the SSRs revealed 4 to 13 size variants per locus. A total of 36 size variants at the four loci were identified. These size variants combined into 141 haplotypes, 87 of which were private, having frequencies of <1.0%. The number of haplotypes detected in each population was 3 to 23. Haploid diversity ($h$) was balanced along the populations (without any outlier value) and ranged from 0.349 in RFE to 0.703 in RBI. Similarly, the number of alleles (*Na* 2.5–5.0) and the number of effective alleles (*Ne* 1.706–3.558) were balanced without any outstanding differences. Mean number of private alleles was the highest in SME (1). The overall mean of genetic distance between individuals ($D_{sh}^2$) was 5.792, but in SME (17.070), RBI (20.893) and RCO (11.986), this value was substantially higher than the average (Online Resource Table S1).

The geographic distribution of the 20 highest frequency haplotypes is reported in Fig. 1. There were differences in haplotype frequencies between the Eastern Carpathian populations on one hand and the Transylvanian Central-Island Mountains (Apuseni) and Southern Carpathian populations on the other. Moreover, Western Carpathian populations showed differences in haplotype proportions and occurrences in haplotypes when the four populations were separated into two groups: High Tatra (SME, SKV) and Lower Tatra (SLI, STU). It is also noticeable that three of the Hungarian populations (HZA, HVE, HFE) harboured only 7–8 of the 20 most common haplotypes. The average occurrence along the range of the 20 common haplotypes was 4–5, with some exceptions.

Nei's genetic distance between populations varied from 0.063 to 0.664, with a mean of 0.181. AMOVA analysis including all populations resulted in $\Phi_{PT} = 0.074$ ($p < 0.001$), meaning that 7% of genetic variance resides among and 93% within populations. Within the Carpathians (excluding ESE and BYU), AMOVA presented $\Phi_{PT} = 0.081$ ($p < 0.001$). The average gene flow (Nm; calculation based on $F_{ST}$) was estimated at 6.272 migrants per generation. The Mantel test revealed significant negative correlation between geographic and genetic distances among populations ($r_{xy}$ −0.176, $p < 0.045$). The Bayesian approach of population structuring estimated with BAPS identified three main clusters ($K = 3$; Fig. 2), which formed geographically distinct groups. Two major groups were detected: Populations from western Hungary clustered together with those of the Eastern Carpathians and the Bulgarian population (1), while populations of the Western Carpathians (Tatras) and the Transylvanian Central-Island Mountains (Apuseni) grouped together with the Southern Carpathian and Estonian populations (2). Population SME from the Tatras was separated as a distinct group.

The main barriers to gene flow as detected with BARRIER on the cpSSR dataset delimited the Eastern Carpathian populations with highest bootstrap support of 99.5% (Online Resource Fig. S1). Between the Eastern and the Southern Carpathian populations, the barrier was only weak (34.3%). Another strong barrier (with up to 86.1% support) was drawn around the RML population in the Transylvanian Central-Island Mountains (Apuseni). In the High Tatra, SME population also showed genetic discontinuity with a 58% supported barrier.

### Nuclear microsatellites

All loci largely conformed to the HWE and showed no significant deviations, although a few populations partially showed minor deviations, which cannot be associated with null alleles or non-neutral behaviour. Micro-Checker test confirmed the lack of null alleles.

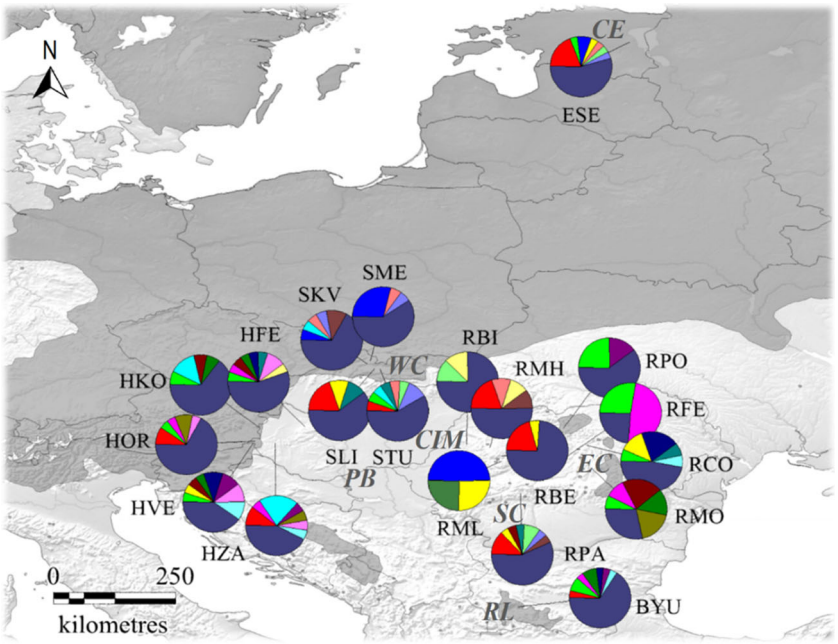


**Fig. 1** Geographic location and distribution of the cpSSR haplotypes with the frequency of the 20 most common haplotypes among the populations sampled. The slate grey portion of the pie charts represents the remaining, less frequent haplotypes. The acronyms stand for the population code in Table 1. The natural distribution of Scots pine is marked in *grey* according to the EUFORGEN database, with modifications by the authors. *RL* Rila Mountains, *CE* Central Estonian Plain, *PB* Pannonian Basin, *WC* Western Carpathians, *EC* Eastern Carpathians, *SC* Southern Carpathians and *CIM* Central-Island Mountains (Apuseni)

A moderate level of intra-population variability was found. The mean of $H_e$ was 0.586, which ranged from 0.493 in RMO to 0.648 in HKO. $H_o$ was 0.589, ranging from 0.488 in RMO to 0.652 in HKO. The latter HKO population showed the highest value, both in the number of alleles (*Na* 8.250) and in the number of effective alleles (*Ne* 4.862). The mean number of private alleles was remarkably high in RPO (1.750), while in the rest of the populations, the overall mean was

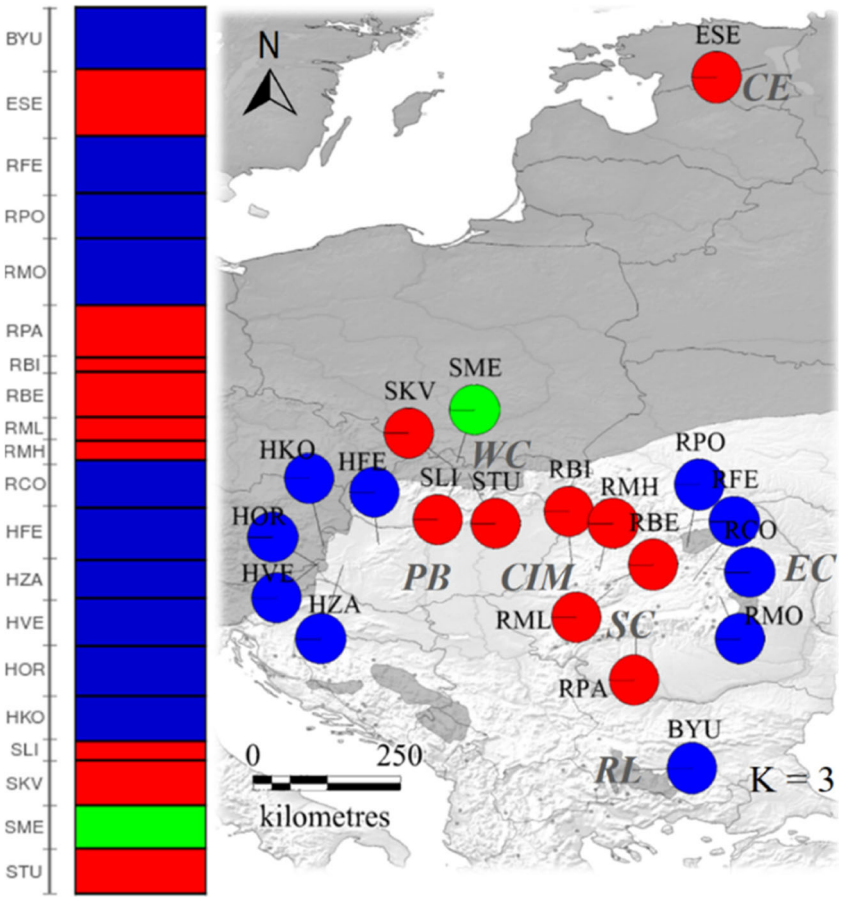


**Fig. 2** Estimated population structure for $K = 3$ according to BAPS analysis (Corander and Marttinen 2006; Corander et al. 2008a, 2008b). The acronyms stand for the population code in Table 1. The natural distribution of Scots pine is marked in *grey* according to the EUFORGEN database, with modifications by the authors. *RL* Rila Mountains, *CE* Central Estonian Plain, *PB* Pannonian Basin, *WC* Western Carpathians, *EC* Eastern Carpathians, *SC* Southern Carpathians and *CIM* Central-Island Mountains (Apuseni)

0.268. Inbreeding coefficient ($F_{IS}$) varied greatly, ranging from −0.2411 in RFE to 0.2830 in RPO, with a mean of −0.0329 (Online Resource Table S1).

AMOVA showed high molecular variance within individuals (90%) and relatively low molecular variance among individuals (3%), while among populations, only a 7% variation was observed. Overall, $F_{ST}$ was 0.071 ($p < 0.001$), while within the Carpathians, $F_{ST}$ was 0.075 ($p < 0.001$). The number of migrants per generation (Nm) was estimated at 3.247. The Mantel test of IBD yielded no significance value ($r_{xy}$ 0.085, $p < 0.233$), and the distribution of Nei's genetic distances over the geographic region did not show limited or restricted gene flow (Online Resource Table S1).

By evaluating nuclear SSR variation with STRUCTURE when $K = 2$, the two groups were as follows (Fig. 3): (1) Eastern Carpathian populations with the Bulgarian population (BYU) and the northernmost population from Estonia (ESE) and (2) western Hungarian, Western Carpathian populations and the Southern Carpathian population. Populations from the Transylvanian Central-Island Mountains (Apuseni Mts.) were distributed in both clusters: RMH, RML and RBE in cluster 1 and RBI in cluster 2. At $K = 3$, two peat bog populations (RMO, RPO) from the Eastern Carpathians were differentiated forming cluster 1. Although populations from Bulgaria and Estonia showed grouping with the Eastern Carpathian populations at $K = 2$ and $K = 3$, these populations were highly admixed (Fig. 3).

To identify underlying subclusters, the two clusters (namely cluster 1 and cluster 2) were reanalysed separately (data not shown). Within cluster 1, RFE was separated, while in cluster 2, high admixture was detected without any clear substructuring among the Hungarian, Slovakian and Romanian populations.

BARRIER analysis identified major genetic discontinuities with high bootstrap support (from 70.9 to 100%) around the Eastern Carpathians, separating these populations from the rest (Online Resource Fig. S1). Additionally, the single RCO population from rocky substrate was separated within the Eastern Carpathians with a 99.5% highest support. As in the case of the cpSSR dataset, around the RML population, a strong barrier (up to 92.2% support) was detected around the RML population. All the other barriers between the populations were weak and indicated a non-significant separation with <45% bootstrap support.

The BOTTLENECK analysis showed no evidence of significant excess or deficit of heterozygosity in most populations (19) under the TPM model. As an exception, a recent decline (heterozygote excess) was detected in the RML population from the Central-Island Mountains (Apuseni). This

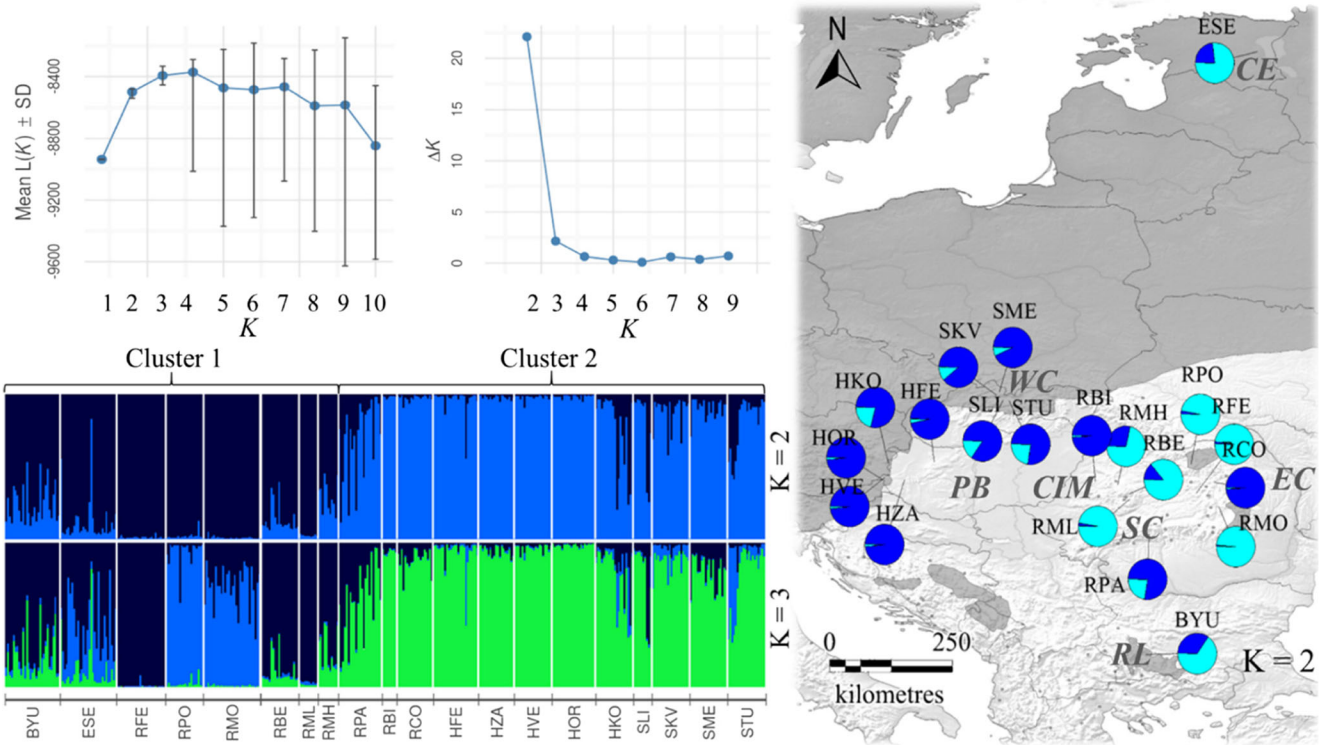


**Fig. 3** Estimated population structure for $K = 2$ and $K = 3$ of assignment analysis performed in STRUCTURE (Pritchard et al. 2000). $K = 2$ clusters of individuals detected by Evanno et al. (2005) implemented in STRUCTURE HARVESTER (Earl and von Holdt 2012) plotted on a geographic map. The most likely membership to one of the $K$ clusters is presented by the colour of the individual's thin line. The acronyms stand for the population code in Table 1. The natural distribution of Scots pine is marked in *grey* according to the EUFORGEN database, with modifications by the authors. *RL* Rila Mountains, *CE* Central Estonian Plain, *PB* Pannonian Basin, *WC* Western Carpathians, *EC* Eastern Carpathians, *SC* Southern Carpathians and *CIM* Central-Island Mountains (Apuseni)

was also supported by the Wilcoxon sign-rank test. Additionally, a shift in the distribution of allele frequencies was detected in the RML population. In the case of non-bottlenecked Scots pine populations, the mode shift indicator test identified a normal L-shaped form of allele frequency distribution as expected in populations that are near to mutation-drift equilibrium. On the basis of the distribution of allele frequencies, we can assume that populations are randomly mating.

## Discussion

This study aims to assess the genetic diversity and differentiation, based on nuclear and chloroplast microsatellite marker analysis, of *P. sylvestris* populations considered to be natural and inhabiting the species distribution range limits along the Carpathians and the Pannonian Basin. Both marker types showed overall high genetic variation along the range studied, suggesting that population fragmentation events might have taken place relatively recently.

Although the two marker types exhibit particular genetic patterns, there were also some congruencies in the detected spatial genetic structures of populations. BAPS and STRUCTURE analysis delimited Western Carpathian populations that proved to be different from those inhabiting the eastern range of the Carpathians (Figs. 2 and 3). Furthermore, Eastern Carpathian populations were differentiated by cpSSR markers from the populations of the Transylvanian Central-Island Mountains (Apuseni) (Figs. 1 and 2). The differentiation in the genetic pattern along the Carpathians has been recognized in earlier studies of other conifer species. In the case of *Picea abies* or *Abies alba*, mitochondrial minisatellite regions and nSSRs delimited lineages of the Western and Eastern Carpathians, and accordingly, these patterns suggest different origins of populations from distinct glacial refugia (Tollefsrud et al. 2008; Liepelt et al. 2009; Gömöry et al. 2012). *Salix herbacea*, an arctic-alpine species from the Carpathian region, showed identical population structure highlighted by BAPS and STRUCTURE and confirmed the distinct origin of the Carpathian populations in question (Alsos et al. 2009). Moreover, our revealed haplotype pattern in Scots pine is highly congruent with that observed by Höhn et al. (2009) for *Pinus cembra*, where the populations of the Western and the Eastern Carpathians were spatially separated on the basis of chloroplast SSR variation. However, this separation was not significant in the study by Lendvay et al. (2014), which was done using nuclear SSR markers. Based on a cluster analysis of cpSSR haplotype frequencies in *P. abies*, Bucci and Vendramin (2000) revealed genetic differentiation between Western and South-Eastern Carpathian populations. These findings correspond with the main geobotanical regions described earlier for the Carpathians (Georgescu and Donita 1965; Zemanek 1991; Ronikier 2011).

The Western Carpathian population Medzi bormi (SME) from Slovakia was the most outstanding, showing the overall highest number of private alleles ($Np$ = 1.000) and forming a distinct cluster in the BAPS analysis. Moreover, BARRIER analysis also separated this population from those inhabiting the same range with a moderate bootstrap support (58%). This might be explained by hybrid individuals of *Pinus rhaetica* (*P. sylvestris* x *Pinus mugo*) reported earlier from this peat bog (Staszkiewicz 1994; Kormut'ák et al. 2013). Bottleneck and restricted gene flow was detected by BARRIER in the Mluha population (MLA) from the Transylvanian Central-Island Mountains, which might be a consequence of the decreased population census size where closely related individuals are mating within an isolated stand. On the basis of a very early first description of the Mluha peatbog in which Scots pine is not mentioned (Csató 1885), one plausible explanation would be the recent colonization of the peatbog by this species. Later, a low population size was mentioned by Pacurar et al. (2010). Additionally, the Fantana Brazilor (RFE) population from the Eastern Carpathians appeared as a conspicuously different group in the substructure analysis and also showed a distinct haplotype proportion in the region. It is possible that this stand originates from a distinct refugium or might bear signs of historical human influence.

On the basis of both chloroplast and nuclear microsatellite markers that presented congruent structure with the previously mentioned conifer species, it is likely that the Carpathian populations of Scots pine harbour genetic material originating from at least two separate refugia, dating back to the Pleistocene. One refugium might have been situated around the Eastern Alps and the Hungarian Plain with the Danube region (Cheddadi et al. 2006; Tribsch and Schönswetter 2003), and the other might have existed in the Eastern Carpathians, where a high abundance of fossil pollen remains of diploxyon Pinus species was reported (Feurdean et al. 2011). These two possible refugia were also reported for subalpine and alpine perennial plant species, such as *Hipochoeris uniflora* (Mráz et al. 2007) and *Campanula alpina* (Ronikier et al. 2008; Ronikier and Zalewska-Gałosz 2014), which present similar delimitations in population structure and support the North-Eastern Alpine and East Carpathian refugia. Eastern Carpathian populations might have also served as source populations in later Holocene colonization towards northern latitudes as described for other coniferous species (Latałowa and van der Knaap 2006; Feurdean et al. 2007; Tollefsrud et al. 2008). This is probably the case, since the Estonian population and the more southern Bulgarian population clustered together.

Levels of genetic diversity revealed in our study with nSSR markers (mean expected heterozygosity ($H_e$: 0.586) showed similar values as those found in Bulgarian populations (Naydenov

et al. 2011), but the values are much lower than those in the more southern peripheral populations from the Apennines and the Southern Alps studied by Scalfi et al. (2009). Likewise, we have found overall higher values compared to those reported by Bernhardsson et al. (2016) from the Romanian-Hungarian region. This might be attributed to the larger sampling area of our study, which included not just the Romanian Carpathians but also the Western Carpathians (the Tatras), and involved a higher number of Hungarian populations. However, it should be noted that this difference can be also the consequence of different marker set, type and number, respectively.

Chloroplast haploid diversity values (*h*: 0.546) were also lower than the diversity detected by similar markers (Vendramin et al. 1996; Soranzo et al. 1998) at the edge of the range populations in Italy by Scalfi et al. (2009) and in Spain by Robledo-Arnuncio et al. (2005). The lower chloroplast population diversity indices might show signs of isolation and fragmentation of the studied populations as the consequence of restricted gene flow on a regional scale or might be due to the genetic drift, which affects more strongly the populations with decreased census size. As the uniparentaly inherited chloroplast genome is more susceptible to genetic drift compared to the nuclear genome involved in the recombination of the meiosis (Birky et al. 1989; Petit et al. 1993), the low values in cpSSR diversity might show early signs of population fragmentation. Genetic discontinuity was also detected in both datasets with BARRIER analysis.

As expected, a high number of cpSSR haplotypes was detected (36 size variants combined into 141 haplotypes) all over the range studied in the Carpathian populations. A high number of haplotypes was also reported in earlier studies by Naydenov et al. (2005), Robledo-Arnuncio et al. (2005) and Cheddadi et al. (2006), since these microsatellite regions have very high mutation rates (Vendramin and Ziegenhagen 1997; Vendramin et al. 1998; Provan et al. 1998).

In most of the populations studied, we did not find signs of inbreeding, as in our study, $F_{IS}$ values were overall negative (−0.0329), except in the cases of HVE, SME and RPO, for which $F_{IS}$ values were found to be positive (0.184, 0.197 and 0.283). Bernhardsson et al. (2016) reported an overall positive $F_{IS}$ value, potentially as a consequence of artificially maintained and human-restored populations. Alternatively, we consider that in HVE, SME and RPO, the small population distribution area (0.02–4.49 $km^2$) and highly isolated habitat have increased the rate of selfing and might exhibit higher viability of selfed offspring (Savolainen et al. 1992), resulting in a slightly increased positive $F_{IS}$ value. Although small and isolated populations are more vulnerable to inbreeding (Ellstrand and Elam 1993), our overall results regarding most of the population studied are in accordance with earlier statements according to which inbreeding takes generations to develop, and/or even with a restricted gene flow, populations still maintain gene exchange. By estimating gene flow between populations, we detected a relatively high number of possible migrants per generation. Between populations, the value of the number of migrants per generation was fairly large (Nm = 6.272) for chloroplast and moderate (Nm = 3.247) for nSSRs.

In accordance with the lack of signs of inbreeding, BOTTLENECK analysis provided evidence that the Carpathian populations studied are not influenced by a recent genetic bottleneck. Long-lasting signs of bottlenecks require multiple generations to appear. Furthermore, the effects can vary not only based on the reduction size but also depending on the duration period (Busch et al. 2007; Peery et al. 2012). It is most plausible that populations that today are isolated have undergone a recent fragmentation and isolation event. Macrofossil and pollen records indicate that conifer species like *P. sylvestris* with diploxylon pollen type have survived the LGM in the Carpathians and the Pannonian Basin (Rudner et al. 1995; Rudner and Sümegi 2001; Magyari 2011), and a strong withdrawal and population decline began only between 8000 and 10,000 years BP (or even later, depending on geographic location and elevation) in the Late Glacial/Holocene transition period to mid-Holocene (Tantau et al. 2003, 2006; Feurdean and Bennike 2004; Feurdean et al. 2007, 2012). Transition from coniferous stands to mixed forests has been detected by Mihai et al. (2007), and recently, within the last decades, increasing clear-cutting of the coniferous forests for pasturing has been reported by Motta et al. (2006).

Both marker types in our study presented a relatively high among-population differentiation, as in the cases of other peripheral study sites in the Italian Alps and the Apennines (Scalfi et al. 2009; Belletti et al. 2012). In our results, population differentiation was $\Phi_{PT} = 0.071$ in the case of nuclear and $\Phi_{PT} = 0.074$ for chloroplast SSRs within the range studied. This relatively high differentiation detected might be due to historical demographical events and presumably are not the consequence of pollen exchange restriction and/or effect of geographic isolation caused by the complex architecture of the Carpathians. The barriers identified among regions are not considered impervious, because neither the high level of gene flow nor the low inbreeding values support this. Our estimated degree of genetic differentiation in the Carpathian region might be related to the contact zone that has been established as a consequence of the migration of diverged lineages that survived glaciation in separated refugia and marked the geographical barrier detected.

In conclusion, despite former findings, our microsatellite study of peripheral Scots pine populations from central-eastern Europe have revealed genetic discontinuities along the Carpathian arch. Genetic differentiation of the Eastern Carpathians based on both marker types indicates different origin of these populations and supports the hypothesis of a once existed Pleistocene refugia within the region, also evidenced by some recent palynological records. The genetic

structure between the Western and the Eastern Carpathians was revealed by former phylogeographical studies of tree species native to the Carpathians. The fact that the populations in the Western Carpathians are separated from the Eastern Carpathian populations and cluster with the Hungarian populations of the Pannonian Basin indicates another genetic lineage that might have originated from earlier reported refugia of the surroundings of the Eastern Alps and the Hungarian Plain. As natural Scots pine populations from the Carpathians still harbour high gene stocks yet unaffected by isolation and genetic erosion, the populations represent valuable genetic resources of the species. Enhanced by their peripheral position, in addition to the evidence provided by their particular phylogeographic pattern, these stands can also provide insights into the species' adaptive genetic variation, enriching our understanding of population genetic processes and allowing us to better assess the impact of ongoing climate change.

**Acknowledgements** The authors are grateful to Tamás Pócs (Hungary), Ivan Iliev (Bulgaria) and Tibor Baranec (Slovakia) for the assistance they offered with the collection of plant material and for the valuable discussions in the course of which they shared their insights. We also wish to thank the Associate Editor for helpful comments and the Anonymous Reviewers who helped us improve the manuscript. We also thank EUFORGEN database for providing the distribution map of Scots pine. This work was supported by National Research, Development and Innovation Office, Hungary, by a grant of the Hungarian Scientific Research Fund [OTKA K101600].

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