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ORIGINAL PAPER

Genetic structure in *Pinus cembra* from the Carpathian Mountains inferred from nuclear and chloroplast microsatellites confirms post-glacial range contraction and identifies introduced individuals

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Abstract Genetic differentiation of scattered populations at neutral loci is characterized by genetic drift counteracted by the remaining gene flow. Populations of *Pinus cembra* in the Carpathian Mountains are isolated and restricted to island-like stands at high-elevation mountain ranges. In contrast, paleobotanical data suggest an extended early Holocene distribution of *P. cembra* in the Carpathians and its surrounding areas, which has contracted to the currently disjunct occurrences. We analyzed the genetic variation of 11 Carpathian populations of *P. cembra* at chloroplast and, in part newly developed, nuclear microsatellites. Both marker types revealed low levels of genetic differentiation and a lack of isolation by distance, reflecting the post-glacial retraction of the species to its current distribution. Stronger effects of genetic drift were implied by the higher genetic differentiation found for haploid chloroplast than for diploid nuclear markers. Moreover, we found no association between the values of population genetic differentiation for the two marker types. Several populations indicated recent genetic bottlenecks and inbreeding as a consequence of decline in population sizes. Moreover, we found individuals in two populations from the Rodnei Mountains that strikingly differed in assignment probabilities from the remaining specimens, suggesting that they had been introduced from a provenance outside the studied populations. Comparison with Eastern Alpine *P. cembra* and individuals of the closely related *Pinus sibirica* suggests that these individuals presumably are *P. sibirica*. Our study highlights the importance of the maintenance of sufficiently large local population sizes for conservation due to low connectivity between local occurrences.

Keywords Disjunct distribution · Gene flow · Genetic drift · Molecular markers · Population bottleneck

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Introduction

Historically disjunct distribution areas and isolated populations are exceptionally interesting from the biogeographic and population genetic perspective, and they also provide insights into processes that are relevant in conservation genetics. Scattered, reproductively isolated populations may display particular genetic characteristics compared to those in a continuous area due to reduced gene flow, genetic drift, founder events, or population bottlenecks (Ellstrand and Elam 1993), and the reduced migration load may enable the development of locally adapted genotypes (Lenormand 2002). The extent of population genetic differentiation of isolated populations at neutral loci will depend on the existing level of gene flow, genetic drift, and mutations (Latta and Mitton 1997; Allendorf and Luikart 2007). However, on the short evolutionary scale, mutation rate may have a negligible effect compared to the rates of inter-population gene flow at these loci (Petit et al. 1993; Ennos 1994).

Gene flow among plant populations takes place through dispersal of pollen and seed. In coniferous trees, biparentally inherited nuclear genes are transmitted both through long-distance wind pollination and shorter-distance seed transport, whereas chloroplastic genes are paternally inherited in most species and, hence, are dispersed first by pollen and subsequently by seed. Owing to the capacity of long-distance pollen dispersal, coniferous trees generally exhibit high levels of intra-population genetic diversity, but low levels of genetic differentiation between populations (Provan et al. 2008) as long as populations remain in contact by at least occasional gene exchange.

Unlike the nuclear genome, the chloroplast genome behaves as a single haploid locus that does not undergo recombination. The haploid, uniparentally inherited chloroplast genes have lower effective population size compared to diploid, biparentally inherited nuclear genes. Thus, chloroplastic loci are prone to higher stochasticity and have higher susceptibility to genetic drift than nuclear loci (Birky et al. 1989; Petit et al. 1993; Ennos 1994; Hu and Ennos 1997, 1999; Ribeiro et al. 2002). Consequently, population genetic differentiation is expected to be higher for chloroplast loci than for nuclear genes, and their spatial autocorrelation will be a better indicator for genetic drift (Myers et al. 2007).

Populations of *Pinus cembra* L. in the Carpathian Mountains are an example of a naturally disjunct distribution of a coniferous tree species. *P. cembra* is considered a glacial relict species of high-altitude mountains in Central Europe, with a disjunct native range in the Alps and the Carpathian Mountains. While *P. cembra* is widely distributed in the Alps, all of its scattered occurrences in the Carpathian Mountains are restricted to summit areas of the highest mountain ranges (Fig. 1). Despite this currently disjunct range, *P. cembra* may have been much wider distributed in earlier times, as pollen and macrofossil evidence indicates its presence during the full-glacial and late-glacial periods in areas around the Carpathian Mountains (Willis and Van Andel 2004; Jankovská and Pokorný 2008). Moreover, it may have been widely distributed in lowlands, but also present within the mountain range even at higher elevations (e.g. Jankovská 1984; Feurdean et al. 2011; Magyari et al. 2012).

Forest management has become extensive even on remote sites of the Carpathian Mountains in the second half of the nineteenth century, and despite its rarity in the Carpathian Mountains, *P. cembra* was well known for its versatile wood for making furniture. Therefore, intensive logging raised concerns about the total disappearance of *P. cembra* from the Carpathian Mountains already at that time (Rowland 1882; Fekete 1887). However, the use of *P. cembra* in forestry plantations in the Carpathian Mountains has only been documented in the Tatra Mountains. (Western Carpathians) where Alpine *P. cembra* and even Siberian *Pinus sibirica* were planted with the purpose of assisting the recruitment of heavily exploited populations at the end of the nineteenth century (Paryski 1971 *cit. in* Zwijacz-Kozica and Żywiec 2007).

Only a limited number of studies have focused on genetic patterns of disjunctly distributed plant species in the Carpathian Mountains (Ronikier 2011), but the remarkably small and isolated Carpathian *P. cembra* populations have repeatedly attracted the attention of geneticists. Szmidt (1982) reported surprisingly high variation within a population from the Retezat Mountains (Southern Carpathians) and substantial differentiation between the southernmost Retezat Mountains and northernmost Tatra Mountains by analyzing allozyme variation. This genetic differentiation was not confirmed later, neither by allozymes (Belokon et al. 2005) nor by chloroplast microsatellites (Höhn et al. 2005, 2010). In fact, chloroplast microsatellites found low population differentiation and highest similarity among populations from the Retezat and the Tatra Mountains (Höhn et al. 2010). All these surveys as well as Goncharenko et al. (1992) and Krutovsky et al. (1992) reported high intra-population genetic variability in Carpathian populations of *P. cembra*, which was recently found to surpass genetic variability of populations from the contiguous part of the range in the Alps (Höhn et al. 2009). Despite the focus of these studies on genetic patterns of Carpathian *P. cembra* populations, the relationship of populations from different Carpathian mountain ranges could yet not be fully clarified (Höhn et al. 2010). A more detailed perception of the genetic characteristics of the scattered Carpathian *P. cembra* populations may be a valuable element for the general understanding of plant species' phylogeographic patterns in the Carpathian Mountains, and also to better evaluate the needs and possible actions for conservation purposes.

This study explores genetic patterns within the Carpathian range of *P. cembra* at both biparentally inherited nuclear microsatellites (nSSRs) and paternally inherited chloroplast microsatellites (cpSSRs). With the two complementary marker sets and expanded sample size, we aim to more precisely describe the range-wide population history that has previously been inferred by cpSSRs only (Höhn et al. 2009, 2010), considering both natural and human-mediated processes. Besides giving a higher resolution, the additional biparentally inherited nSSRs also allowed us to test if recent population bottlenecks occurred and whether there is inbreeding in the populations. We expect to find low genetic differentiation among populations due to post-glacial fragmentation of a formerly wide distribution and wide-ranging gene flow predominantly through pollen. Alternatively, populations may show high genetic differentiation because they originated from multiple glacial refugia. With a comparison between genetic differentiation and diversity at cpSSRs and nSSRs, we examine effects of gene flow and drift on the population structure. Higher genetic differentiation for cpSSRs may mean limited gene transfer between populations and between regions, while higher genetic variability could be explained by gene flow among regions. We expect to find an

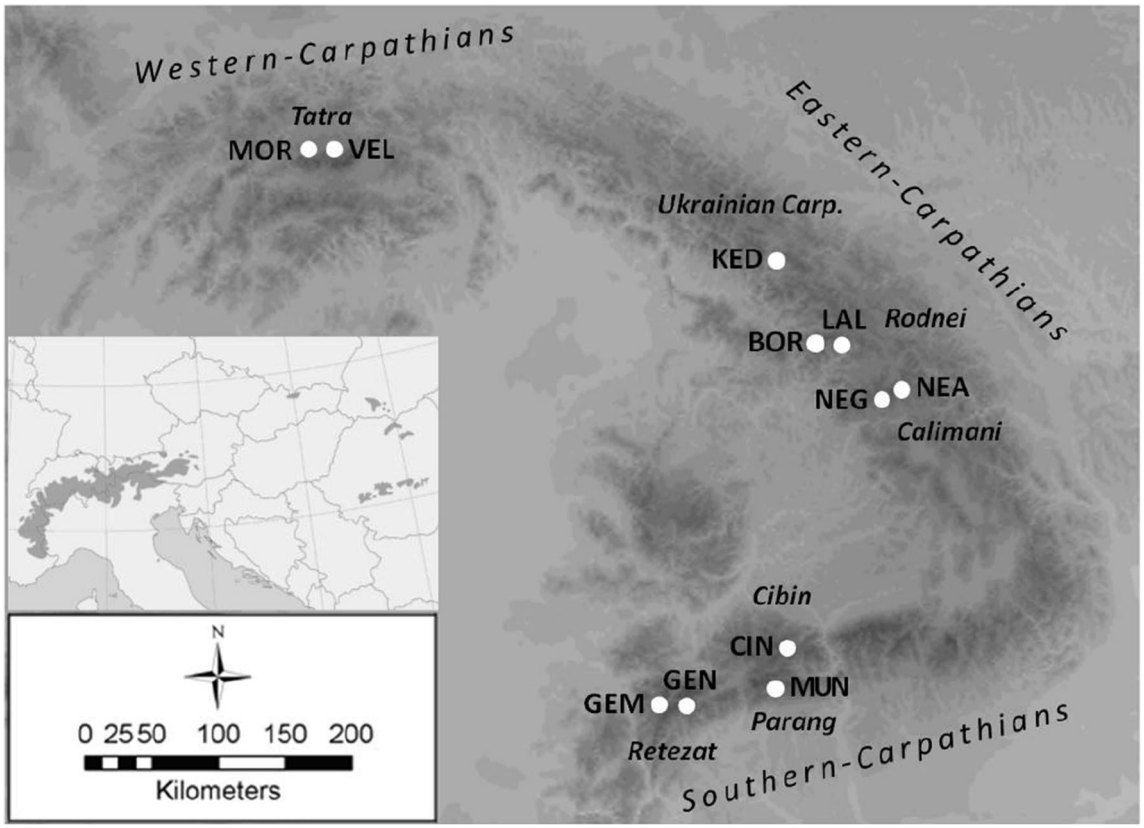

Fig. 1 Location of the *Pinus cembra* populations from the Carpathian Mountains involved in this study with population abbreviations (see Table 1) and Carpathian mountain ranges. The *inset map* shows the total distribution area of *P. cembra* in the Alps and the Carpathian Mountains (source: European Forest Genetic Resources Programme, euforgen.org)

association between population-wise genetic differentiation and diversity of the two marker types. Furthermore, we introduce and apply a set of nuclear microsatellite markers newly developed for *P. cembra*.

Materials and methods

Study species

P. cembra is a species reaching highest elevations among trees of Central Europe: stands occur at the timberline or often beyond as singular trees among dwarf pine. Well adapted to the harsh conditions, individuals can reach ages of several hundred years. Reproduction takes place through wind pollination and distribution of the nutritious seeds by the spotted nutcracker (*Nucifraga caryocatactes*). In a particular interaction with *P. cembra*, this bird collects, carries, and hides seeds in caches mostly within its territory of several hectares, but seed depositions up to 20 km away from the collection sites have been observed (Mattes 1982; H. Mattes, University of Münster; personal communication). Seeds that survive in the caches can create clumped growth of different individuals (Tomback et al. 1993).

P. cembra is closely related to *P. sibirica* Du Tour, a widespread species of the Siberian taiga region. The two species are sometimes even considered as subspecies due to their nearly identical morphology (Meusel et al. 1965; Goncharenko et al.1992; Belokon et al. 2005 and references therein). The high genetic similarity between *P. cembra* and *P. sibirica* has been supported by the high similarity in allozymes (Goncharenko et al. 1992; Krutovsky et al. 1992; Belokon et al. 2005), shared chloroplast haplotypes (Gugerli et al. 2001), and a high proportion of common nSSR alleles (S. Brodbeck, unpublished).

In the Western Carpathians, *P. cembra* exists exclusively in the Tatra Mountains. The closest occurrences are in the Ukrainian Eastern Carpathians at nearly 300-km distance. Other stands in the Eastern Carpathians are the Rodnei (also referred to as Rodna) and Călimani Mountains in Romania. In the Southern Carpathians, small populations exist in the Bucegi, Făgăras, Cindrel, and Lotru Mountains, where stands are found on a few slopes and are often not larger than a few dozen trees (Blada 2008). More extended populations are found only in the westernmost Retezat and Parang Mountains.

Fossil remains of *P. cembra* were recovered from the last full-glacial, late-glacial, or early Holocene periods from various sites neighboring the Carpathian Mountains: the Moravian

basin and Austrian Danube valley in the west, the transition to the Polish plain in the north, and the bank of the Dniester river in Moldavia and the side of the Prut river in Romania in the east, as well as several locations on the Hungarian plain in the Carpathian basin (for references, see Willis et al. 2000; Willis and Van Andel 2004; Jankovská and Pokorný 2008). Besides its frequent persistence at lower altitudes, there are also few examples of *P. cembra* found within mountain ranges in late-glacial and early Holocene sediments. Seeds and pollen were found in the full-glacial period in inter-mountain lowlands and on the southern piedmonts of the Tatra Mountains, and also in late-glacial sediments of a glacial lake close to the site where *P. cembra* presently occurs in the Tatra Mountains, Western Carpathians (Jankovská 1984; Rybníčková and Rybníček 2006; Kuneš et al. 2008). In the Eastern and Southern Carpathians, late-glacial pollen and macro-remains of *P. cembra* were reported from glacial lakes in the vicinity of present-day populations in the Rodnei Mountains, the Călimani Mountains, and the Retezat Mountains, and also in the Gutâiului Mountains, where it subsequently became extinct (Wohlfarth et al. 2001; Feurdean et al. 2011; Magyari et al. 2012). The fossil remains of *P. cembra* suggest that the species may have had a much broader distribution than today, existing both in lowlands around and elevated locations within the range of the Carpathian Mountains during the late-glacial and early Holocene periods. It cannot, however, be ascertained based on the fossils whether these distant extant occurrences previously formed part of a single contiguous area and whether they originated form a common glacial refugial area.

Population sampling and genotyping

Samples were collected from 11 sites covering the major massifs within the range of *P. cembra* in the Carpathian Mountains (Table 1 and Fig. 1). This included subsamples from populations previously studied (Höhn et al. 2009) and two newly collected sites (LAL, MUN). Needles were sampled from mature trees at least 30 m apart from each other, although the scattered occurrence of trees often resulted in much larger inter-individual distances. Sample sizes ranged from 7 to 26. Some of the sample sizes are relatively small, which in part reflects low census sizes of some of the populations. However, our results on the population genetic structure were consistent, whereas we acknowledge that the accuracy of parameter estimates might be critical at least in those cases in which sample size is not integral part of parameter estimation. Therefore, we omitted population BOR from part of the analyses after removing individuals considered non-native (see below).

The needles were dried on silica gel and genomic DNA was extracted with DNeasy Plant Mini Kit or DNeasy 96 Plant Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Six nuclear microsatellite primer pairs (Pc1b, Pc7, Pc18, Pc22, Pc23, Pc35) were amplified from the *P. cembra* microsatellite primer set designed by Salzer et al. (2009). Multiplex PCRs were carried out in 10-μl volume containing 4.5 μl 2× Multiplex PCR master mix (Qiagen), ~20 ng genomic DNA, and fluorescently labeled primers (Table S2). PCRs started with 95 °C for 15 min followed by 35 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 50 s and a final extension at 72 °C for 30 min.

These nuclear microsatellite loci were complemented with six newly designed markers (Supplementary Table S1). Primer development followed the procedure described in Schoebel et al. (2013), and Mendelian inheritance was confirmed using open-pollinated progenies (S. Brodbeck and F. Gugerli, unpubl. data). Single-tube multiplex PCR reactions were performed in 10-μl volume containing ~20 ng genomic DNA, 4.5 μl 2× Type-it Multiplex PCR master mix (Qiagen), and different amounts of fluorescently labeled primers (Supplementary Table S2). PCRs started with 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 90 s, 72 °C for 30 s and a final extension step of 72 °C for 30 min.

Four chloroplast microsatellite loci (Pt630718, Pt15169, Pt36480, and Pt26081) were amplified from the primer set of Vendramin et al. (1996). Compared with the data in Höhn et al. (2009), locus Pt15169 was additionally considered in this study, and we re-genotyped the previously collected samples. PCR amplification was carried out in single-tube multiplex reactions with fluorescently labeled primers (Supplementary Table S2). PCRs were performed in 10-μl reaction mixtures containing ~10 ng genomic DNA, 4.5 μl 2× Multiplex PCR master mix (Qiagen), additional 10 nmol $MgCl_2$, and optimized amounts of primers (Supplementary Table S2). PCR reactions started with 95 °C for 15 min followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s and a final extension at 72 °C for 20 min.

PCR reactions were performed on a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA), and fragments were separated on an ABI 3130 capillary sequencer (Applied Biosystems) with GeneScan ROX–400 (for cpSSRs and newly designed nSSRs) or LIZ–500 (for nSSRs of Salzer et al. (2009)) as internal size standards. Alleles were scored with GeneMapper v3.7 (Applied Biosystems).

Data analysis

Nuclear microsatellites

Other than the remaining loci, one locus, SWK, showed a significant deviation from Hardy-Weinberg equilibrium (HWE) in several Carpathian populations, suggesting the presence of null alleles or non-neutral behavior; thus, this marker was omitted from all analyses. All other loci largely conformed to the expectations of HWE within single populations (data not shown).

Table 1 Location and number of genotyped samples of *Pinus cembra* populations from the Carpathian Mountains

Population	Abbreviation	Mountain range	Part of Carpathian Mountains	Coordinates	N^a
Morskie Oko	MOR	Tatra Mts.	Western Carp., Poland	49.20° N, 20.08° E	11
Velka Studena Dolina	VEL	Tatra Mts.	Western Carp., Slovakia	49.17° N, 20.20° E	17
Kedryn Forest Reserve	KED	Ukrainian Carpathians	Eastern Carp., Ukraine	48.42° N, 24.00° E	17
Borşa	BOR	Rodnei Mts.	Eastern Carp., Romania	47.58° N, 24.63° E	13
Valea Lala	LAL	Rodnei Mts.	Eastern Carp., Romania	47.53° N, 24.92° E	26
Neagra Şaralui	NEA	Călimani Mts.	Eastern Carp., Romania	47.17° N, 25.28° E	19
Negoiu	NEG	Călimani Mts.	Eastern Carp., Romania	47.10° N, 25.20° E	17
Cibin	CIN	Cindrel Mts.	Southern Carp., Romania	45.58° N, 23.80° E	7
Muntinul Mare	MUN	Parâng Mts.	Southern Carp., Romania	45.36° N, 23.68° E	25
Gentiana	GEN	Retezat Mts.	Southern Carp., Romania	45.38° N, 22.87° E	7
Gemenele	GEM	Retezat Mts.	Southern Carp., Romania	45.37° N, 22.83° E	16

Populations LAL and MUN were newly sampled in addition to the sampling reported in Höhn et al. (2009)

Mts. mountains, *Carp.* Carpathians

[a] *N*=sample size; for chloroplast microsatellites, the number of genotyped specimens was 10 in population MOR and 16 in population KED

A Bayesian non-hierarchical assignment test was performed with STRUCTURE 2.3.4 with admixture model and correlated allele frequencies (Pritchard et al. 2000). Tested numbers of *K* were 1–15 with 10^6 Markov chain Monte Carlo repetitions with 5×10^5 burn-in period, using a computer cluster. Runs were repeated 20 times for each *K*, and an LnP(D) plot was constructed with STRUCTURE HARVESTER (Earl and vonHoldt 2012). The number of *K* best fitting the data was inferred by evaluation of the LnP(D) plot according to the guidelines given in the STRUCTURE manual. Label switching and multimodality among different runs with the selected *K* were adjusted with CLUMPP using the Greedy option (Jakobsson and Rosenberg 2007).

STRUCTURE results identified individuals that remarkably differed from the rest of the samples in both populations from the Rodnei Mountains (Eastern Carpathians). Eight specimens from BOR and seven from LAL were grouped into a unique genetic cluster, leading to the assumption that they were non-native individuals, originating from outside the analyzed Carpathian populations. We therefore tested two hypotheses regarding the possible origin of these individuals by re-running STRUCTURE with an extended dataset. The first hypothesis assumed that these individuals were of Eastern Alpine origin; therefore, genotype data of 436 *P. cembra* specimens from 19 Austrian and 1 Swiss sites were added to our dataset (F. Gugerli et al., unpublished data). The alternative hypothesis assumed that these individuals were planted *P. sibirica*; thus, data of 23 *P. sibirica* individuals from a natural stand in eastern Siberia (Mujakan basin, Severomuisk) and 24 planted individuals surrounding Popradske pleso, Tatra Mountains, were included in STRUCTURE analyses.

STRUCTURE was first run with the settings detailed above, and an additional analysis was performed with the applied model switched to USEPOPINFO, with samples of known origin pooled into four groups: (1) *P. sibirica* from eastern Siberia, (2) *P. sibirica* from the Tatra Mountains, (3) Eastern Alpine *P. cembra*, and (4) presumably native Carpathian *P. cembra*. Individuals of these groups were used as "learning samples," where their population of origin was pre-specified according to their group at $K=4$, while the program was implemented to assign the peculiar individuals of the Rodnei Mountains to the pre-defined groups. This test was repeated 20 times and results were adjusted with CLUMPP.

On the basis of the above analyses, the peculiar individuals from the Rodnei Mountains were omitted from all further analyses. STRUCTURE analysis was performed on the remaining, presumably native Carpathian specimens with the initially described settings. By removing the peculiar specimens, the sample size of BOR was reduced to five. For tests based on population-wise allele frequency differences, such a small sample size may lead to imprecise results due to sampling bias (Leberg 2002); hence, the entire BOR population was excluded from population-wise analyses.

Effective number of alleles, number of unique alleles, and unbiased genetic diversity (uHe) were calculated for each population in GenAlEx 6.5 (Peakall and Smouse 2012). Inbreeding (F_{IS}) was calculated for each population, and its significance was tested in Fstat 2.9.3.2 assuming no Hardy-Weinberg equilibrium within the samples and using 1,000 permutations (Goudet 1995, 2002). BOTTLENECK 1.2.02 was used to test if recent bottlenecks affected the genetic structure of the populations (Piry et al. 1999). In this analysis, the infinite allele model (IAM) was implemented and significance of heterozygosity excess or deficiency was tested with Wilcoxon two-tailed tests included in the software.

Analysis of molecular variance (AMOVA) without grouping was performed with 9,999 permutations, and values of

pairwise population differentiation (F_{ST}) were calculated with their significance through 9,999 randomization steps.

Isolation by distance was tested with Mantel tests (Mantel 1967) using 9,999 permutations with two different assumptions. First, we assumed long-distance connectivity between populations through wind pollination; thus, we measured population distances as Euclidean distances throughout the range. The distances were logarithmically transformed as suggested by Rousset (1997). Secondly, we assumed only shorter gene flow between populations with connectivity approximating a one-dimensional stepping-stone model (Kimura and Weiss 1964). Accordingly, between-population geographic distances were measured along a line connecting the sites through the arch of the Carpathian Mountains. For this method of distance measurement, pairwise population F_{ST} values were linearized with the $F_{ST}/(1-F_{ST})$ transformation following Rousset (1997). These calculations were performed in GenAlEx 6.5.

Spatial variation in genetic differentiation of the populations was analyzed with spatial analysis of genetic variation (SAMOVA), a method based on Voronoï polygons created from the geographic location of the populations (Voronoï 1908). The software uses a simulated annealing approach to establish maximally differentiated (F_{CT}) groups of populations with regard to their geographic position for each number of groups set by the user (Dupanloup et al. 2002). Runs were conducted with the number of groups set from two to five.

Chloroplast microsatellites

The four chloroplast microsatellite loci were concatenated into chloroplast haplotypes. Effective number of haplotypes, number of unique haplotypes, and unbiased haploid genetic diversity (uh) were estimated for each population. Pairwise population differentiation values were calculated as Φ_{PT}. Analyses of AMOVA, Mantel test, and SAMOVA were carried out by using the Φ_{PT} values with the same settings as described for the nSSRs.

To further investigate the cpSSR population structure of the presumably native 158 individuals, we ran the Bayesian assignment algorithm implemented in BAPS version 6.0 (Corander et al. 2003; Corander and Tang 2007). We applied mixture analysis with linked loci for individuals grouped according to populations. To identify the most probable number of K genetic groups based on the maximum log(marginal likelihood) values, the number of K was set from 2 to 10, and the analysis was run ten times for each K. Additional individual runs were performed for K values from 2 to the most probable cluster number, with 100 repetitions in each run.

Comparison between nuclear and chloroplast microsatellites

Mantel tests were performed to assess the correlation of estimates of pairwise population differentiation (F_{ST} for nSSRs and Φ_{PT} for cpSSRs) with 9,999 permutations. Furthermore, mean values of the genetic diversities and pairwise population genetic differentiation estimates were compared between the two marker types with a t test for paired samples.

Intra-population variability was estimated through Shannon diversity index (H), a measure that is easy to calculate and allows comparing among different types of genotypic data (Sherwin et al. 2006). H was calculated as implemented in GenAlEx 6.5 with the formula $\sum p_i \ln p_i$, where p_i is the proportion of the ith allele in the case of nSSRs or haplotype in the case of cpSSRs. Correlation was tested between Hs calculated for the nSSR and cpSSR data sets. The Mantel test was performed in GenAlEx 6.5; Pearson correlation and paired t tests were carried out with Statistica 11.0 (Statsoft Inc., Tulsa, OK, USA) with pre-checking the assumption of normal distribution.

Results

Nuclear microsatellites

Among the 175 Carpathian individuals, eight specimens from BOR and seven from LAL were assigned to a cluster virtually absent in other samples of these populations in the results of STRUCTURE when $K>2$ (Supplementary Fig. S1). These samples were again assigned to a unique cluster when samples from 20 Eastern Alpine *P. cembra* populations, 1 native, and 1 planted *P. sibirica* populations were included in the analysis (Supplementary Fig. S2). With the applied model in STRUCTURE switched to USEPOPINFO, the peculiar samples showed high assignment rates to the introduced *P. sibirica* population from the Tatra Mountains (Fig. 2).

STRUCTURE results indicate that the most likely number of populations is $K=3$ when the dataset of the Carpathian populations were re-analyzed excluding the 15 peculiar individuals (Fig. 3a). Geographic structuring of the clusters was weak, and only individuals from MUN formed a unique cluster. The remaining ten populations contained two genetic groups that were highly admixed; only individuals from NEA and NEG were largely homogeneous (Fig. 3b).

The mean number of effective alleles per locus ranged between 2.22 (GEN) and 3.37 (LAL; Table 2). Unique alleles were present in all but the NEA, CIN, and GEN populations, with the highest number of six found in LAL. Unbiased heterozygosity ranged between 0.48 (GEN) and 0.61 (GEM). Five of the populations had F_{IS} values significantly higher than 0, suggesting inbreeding in half of the populations (Table 2). The Wilcoxon test implied heterozygote excess due to recent bottlenecks in populations LAL, CIN, MUN, and GEM.

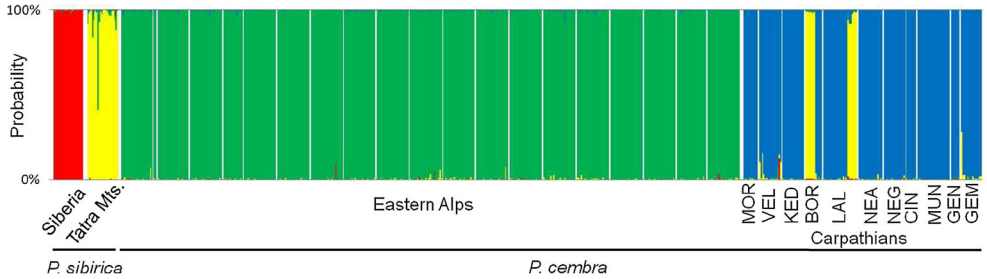

Fig. 2 Assignment of the non-native individuals from BOR and LAL populations using the USEPOPINFO model in STRUCTURE (Pritchard et al. 2000). Population of origin is predefined for the following groups: (1) *P. sibirica* from eastern Siberia, (2) *P. sibirica* from the Tatra Mountains, (3) *P. cembra* from the Eastern Alps, and (4) *P. cembra* from the Carpathian Mountains. The non-native individuals (*yellow*) were assigned to the cluster of *P. sibirica* from the Tatra Mountains (Color figure online)

Pairwise population F_{ST} values were significantly higher than 0 in 39 of 45 cases (Supplementary Table S4). The AMOVA revealed an overall F_{ST}=0.055, i.e., almost 6 % of the total genetic variance resided among populations, while nearly 94 % of the variance was attributed within populations: 17 % among individuals within populations and 77 % within individuals.

No isolation by distance was discovered among the populations by Mantel tests neither performed using Euclidean distances (r_{xy}=0.049, p=0.345) nor assuming restricted gene flow (r_{xy}=0.128, p=0.234).

SAMOVA first separated MUN from the rest of the populations. In the consecutive steps, GEM, GEN-CIN, and NEA-NEG created individual groups, leaving the populations from the Western Carpathians and northern part of the Eastern Carpathians together as a group (Table 3).

Chloroplast microsatellites

The four cpSSRs resulted in 33 chloroplast haplotypes in the 153 Carpathian samples after excluding the genetically peculiar individuals based on the results of STRUCTURE and the remaining five individuals from BOR. The number of haplotypes present in each population varied between 4 (CIN and GEN) and 11 (VEL); the effective number of haplotypes ranged from 3.27 (CIN and GEN) to 9.32 (VEL; Table 2). Unique haplotypes were found in 7 out of 11 populations; the highest number of four was found in populations VEL and GEM. Unbiased haploid genetic diversity varied between 0.73 (MUN) and 0.95 (VEL).

The AMOVA resulted in Φ_{PT}=0.095, i.e., 10 % of the total genetic variance residing among, and 90 % within populations. The Mantel tests revealed no isolation by distance among the populations, neither with Euclidean distances (r_{xy}=0.147, p=0.144) nor assuming the stepping-stone model (r_{xy}=0.049, p=0.390). Φ_{PT} values were found significantly higher than 0 for 29 out of 45 population pairs (Supplementary Table S4).

No clear geographic pattern of population grouping resulted from SAMOVA at K=2 (Table 3). By increasing the number of groups, populations CIN and MUN became separated, and then populations NEA and NEG created a separate group.

The Bayesian assignment test with BAPS identified K=4 as the most probable number of genetic clusters (98.4 % probability). Population MUN was separated from all other populations at K=2 and remained distinct at higher K values. The remaining populations did not form geographically distinct groups at any value of K tested (Fig. 4).

Comparison between nuclear and chloroplast microsatellites

Pairwise population genetic differentiation was generally higher for the cpSSRs than for the nSSRs (mean values 0.097 and 0.057, respectively; t=0.013, p<0.001). The Mantel test revealed no significant association between pairwise population genetic differentiation measured by cpSSRs and nSSRs (r=0.134, p=0.275, Fig. 5a).

Shannon diversity indices per population were found to be higher for the cpSSRs (mean value 1.71) than nSSRs (1.01; t=7.606, p<0.001). Correlation was strong between the diversities measured by the different markers (r=0.738, p=0.015, Fig. 5b).

Discussion

The aim of the present study was to examine genetic structure of scattered *P. cembra* populations from the Carpathian Mountains on the basis of both biparentally inherited nSSRs and paternally inherited cpSSRs. While we detected certain coincidence, and also discrepancies, between the outcomes of various analyses using the two marker types, our findings suggest that the effects of genetic drift dominate over those of historical gene flow in these populations. The low level of population genetic differentiation suggests post-glacial contraction of the species' range to its currently restricted, disjunct

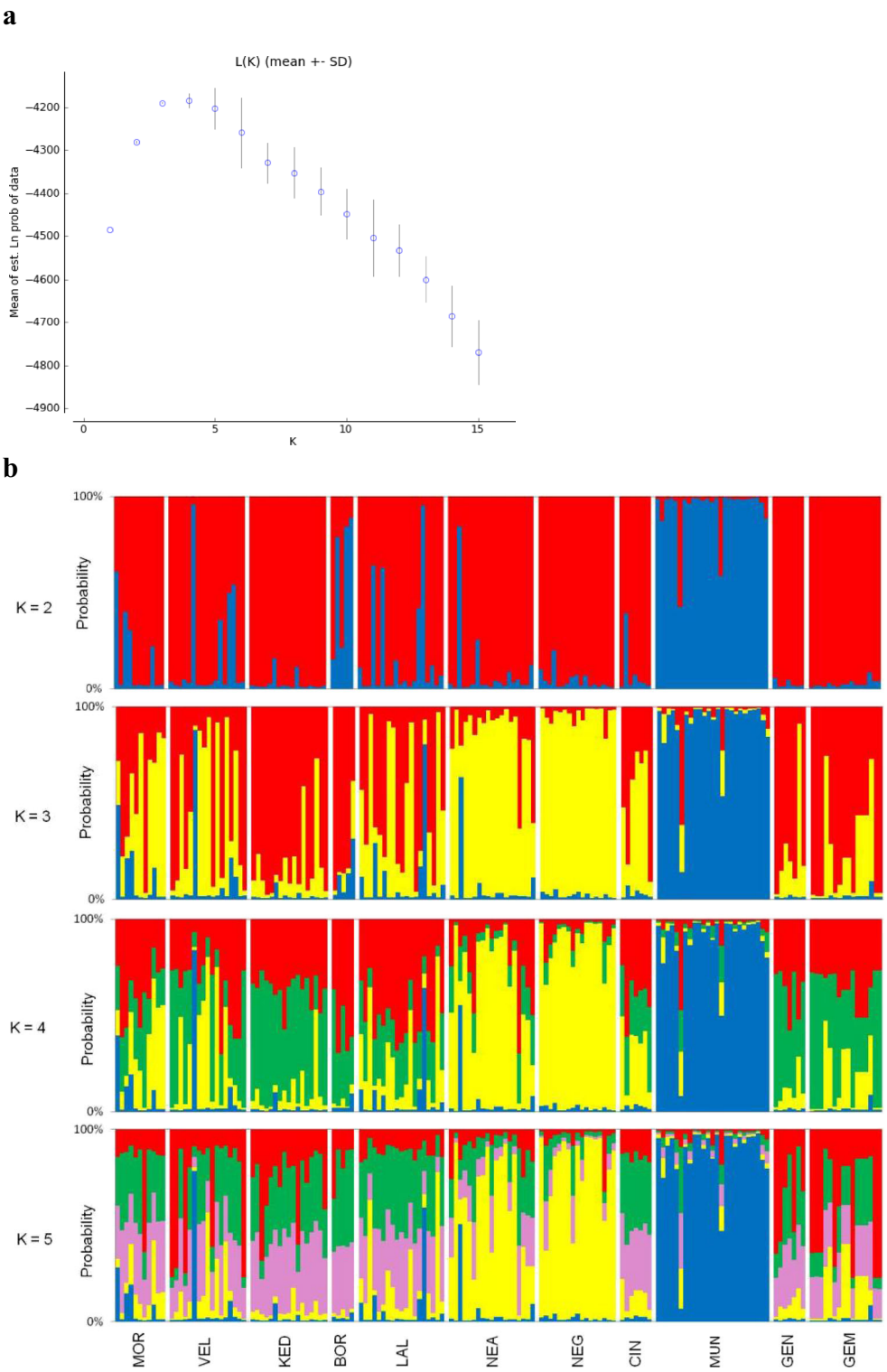

Fig. 3 STRUCTURE (Pritchard et al. 2000) results of 160 Carpathian *Pinus cembra* individuals from 11 populations: **a** Ln of the probability of data plotted against number of *K* clusters and **b** *bar plots* showing individual assignment for *K*=2–5

distribution, which is in line with paleobotanical data. Recent genetic bottlenecks and respective inbreeding were observed in several populations, likely as a consequence of decline in population sizes. Moreover, our molecular-genetic markers allowed us to identify putatively non-native individuals in two populations from the Rodnei Mountains, which were likely associated to *P. sibirica*. Offspring in these populations will possibly experience introgression between the two species, which should be taken into account in conservation strategies.

Table 2 Population genetic parameters, calculated for eleven nuclear microsatellite (nSSR) and four chloroplast microsatellite (cpSSR) loci in populations of *Pinus cembra* in the Carpathian Mountains

Population	nSSRs					cpSSRs		
	Ne	Nu	uHe	F_{IS}	Bottleneck	Ne	Nu	Uh
MOR	2.90±0.42	2	0.60±0.07	0.05	0.139	4.17	2	0.84
VEL	2.65±0.32	4	0.57±0.07	0.16*	0.161	9.32	4	0.95
KED	2.56±0.34	4	0.56±0.06	0.04	0.207	3.77	0	0.78
LAL	3.37±0.77	6	0.58±0.08	0.09*	0.007*	5.23	3	0.85
NEA	3.28±0.67	0	0.58±0.08	0.04	0.065	6.12	2	0.88
NEG	2.60±0.35	3	0.55±0.07	0.12*	0.188	4.31	1	0.82
CIN	2.61±0.44	0	0.55±0.09	−0.05	0.002*	3.27	0	0.81
MUN	2.70±0.40	1	0.56±0.07	0.29*	0.027*	3.31	2	0.73
GEN	2.22±0.36	0	0.48±0.09	0.03	0.116	3.27	0	0.81
GEM	2.94±0.39	2	0.61±0.07	0.16*	0.002*	7.11	4	0.92

Sample sizes are given in Table 1

Ne average number of effective alleles, *Nu* cumulative number of unique alleles, *uHe* unbiased heterozygosity, F_{IS} inbreeding coefficient, *Bottleneck*: *p* values of significance for heterozygosity excess calculated by Bottleneck software (Piry et al. 1999)

*Significant *p* values ($p<0.05$) for F_{IS} and Bottleneck

Common origin of distant populations

We detected shallow geographic structuring of genetic groups in our sample of *P. cembra* from the Carpathians. Bayesian assignment of multilocus nSSR genotypes with STRUCTURE discovered highly mixed genetic clusters shared among eight populations from five mountain ranges, including those of the Western, Eastern, and Southern Carpathians. The exceptional population was MUN from the Parâng Mountains, and populations NEA and NEG from the Călimani Mountains. NEA and NEG were genetically more homogeneous and were mainly assigned to a genetic cluster present, but not dominant, in other populations. In turn, MUN was characterized by a totally distinct genetic cluster, which was absent in other regions. The Bayesian assignment of the populations based on linked cpSSR loci with BAPS found no strong geographic structuring throughout the range of the Carpathians but corroborated the peculiarity of MUN (Fig. 4).

For the nSSRs, also SAMOVA first separated MUN and, with increasing numbers of groups, populations from the Southern Carpathians. In turn, no distinct geographic structuring was found for the cpSSRs, with Southern Carpathian populations becoming separated when increasing the number of groups. Populations MOR-KED-LAL and NEA-NEG assembled into the same groups for both markers at all group numbers. The higher effect of genetic drift for uniparentally inherited cpSSRs compared to biparentally inherited nSSRs may cause higher stochasticity, resulting in shallower geographic structure and common grouping of distant populations, e.g., the northern VEL together with southern GEN and

Table 3 Population groups identified by spatial analysis of molecular variance (SAMOVA; Dupanloup et al. 2002) for 11 Carpathian populations of *Pinus cembra* with both nuclear and chloroplast microsatellite markers

Marker type	Number of groups	F_{CT}/Φ_{PT}	Population grouping
nSSRs	2	0.058	{MUN} {GEM/GEN/CIN/NEA/NEG/VEL/MOR/KED/LAL}
	3	0.053	{MUN} {GEM} {GEN/CIN/NEA/NEG/VEL/MOR/KED/LAL}
	4	0.054	{MUN} {GEM} {GEN/CIN} {NEA/NEG/VEL/MOR/KED/LAL}
	5	0.057	{MUN} {GEM} {GEN/CIN} {NEA/NEG} {VEL/MOR/KED/LAL}
	6	0.061	{MUN} {GEM} {GEN/CIN} {NEA/NEG} {VEL} {MOR/KED/LAL}
cpSSRs	2	0.071	{CIN/MUN/GEN/GEM/VEL/NEA} {NEG/MOR/KED/LAL}
	3	0.085	{CIN} {MUN/GEN/GEM/VEL/NEA} {NEG/MOR/KED/LAL}
	4	0.085	{CIN} {MUN} {GEN/GEM/VEL} {NEA/NEG/MOR/KED/LAL}
	5	0.091	{CIN} {MUN} {GEN/GEM/VEL} {NEA/NEG} {MOR/KED/LAL}
	6	0.095	{CIN} {MUN} {GEN/GEM} {VEL} {NEA/NEG} {MOR/KED/LAL}

Predefined number of groups range from two to six

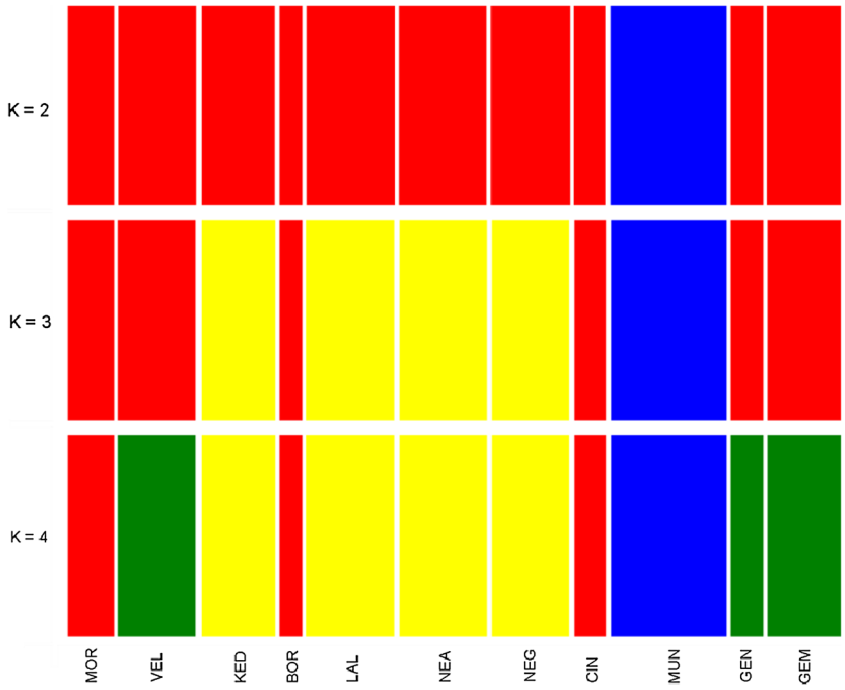

Fig. 4 Bayesian assignment of the 11 Carpathian *Pinus cembra* populations based on chloroplast microsatellites using BAPS (Corander and Tang 2007) at group numbers K=2–4

GEM—as also found in the BAPS results of cpSSRs—or the immediate separation of CIN with an extremely small census size (Höhn et al. 2009). The overall low geographic congruity reflects the preponderance of drift over gene flow and a lack of isolation by distance (Provan et al. 2008). The clustering of MUN, NEA-NEG, and the remaining populations into three groups of populations, as found in the STRUCTURE results, might also indicate three evolutionary lineages that have recolonized the Carpathians from separate refugia during the Last Glacial Maximum. However, this explanation seems less likely given the inconsistent grouping found among different analytical approaches and marker types, and also the genetic admixture of the majority of populations. Hence, we presume that common ancestry combined with genetic drift better explains the patterns observed.

The genetically distinct population MUN occurs on a remote, rocky north facing slope in the Parang Mountains, Southern Carpathians. Our results indicate a long-term reproductive isolation of MUN from the rest of the sampled populations, which is corroborated by a significantly positive F_{IS}, indicative of inbreeding, and a significant test statistic for a historical bottleneck (Table 2). Therefore, this population might have descended from a distinct refugial population, with restricted connectivity to surrounding populations through pollen and seed dispersal.

The high similarity found between the *P. cembra* populations from the Tatra Mountains and the Retezat Mountains is in agreement with results presented by Höhn et al. (2010). However, the genetic structure observed does not follow the trend of deep phylogeographic separation found in many other high-mountain plant species from the Carpathians, where long-term isolation and restricted gene flow is typical between different mountain ranges (Ronikier 2011). Instead, patterns in *P. cembra* are mostly analogous to two high-elevation woody species with scattered populations in the Carpathian Mountains. In *Pinus mugo* occurring in ecologically similar habitats as *P. cembra*, Wachowiak et al. (2013) found low genetic variation among Carpathian populations. Their study revealed a common origin of *P. mugo* in Central Europe, with a gradual retreat to higher elevations from post-glacial lowland distribution. Similarly, the wind-dispersed dwarf shrub *Salix herbacea* showed low genetic differentiation in the Carpathian Mountains, suggesting extensive gene flow even after the last full glaciation (Alsos et al. 2009). In fact, even higher genetic similarity was found between populations from the Western and Southern Carpathians (Tatra and Bucegi Mountains, respectively) than between these regions and the Eastern Carpathians (Rodnei Mountains), which coincides with our findings. The modeled distribution of *S. herbacea* showed that it could have persisted in a large area around and within the Carpathian Mountains during the Last Glacial Maximum. Similar patterns and inferred underlying processes were described in the herbaceous alpine plants *Carex curvula* (Puşcaş et al. 2008) as well as *Geum montanum* and *Geum reptans* (Thiel-Egenter et al. 2009). Hence, these comparisons underline our assumption that extant occurrences of *P. cembra* represent remnants of a common, previously more widespread gene pool from the surrounding lowlands, which has shifted to higher elevations and has lead to a concurrent increase in fragmentation in response to climate warming during the Holocene.

Late-glacial and early Holocene fossils of *P. cembra* were found in glacial lakes of the Tatra, Rodnei, Călimani, and

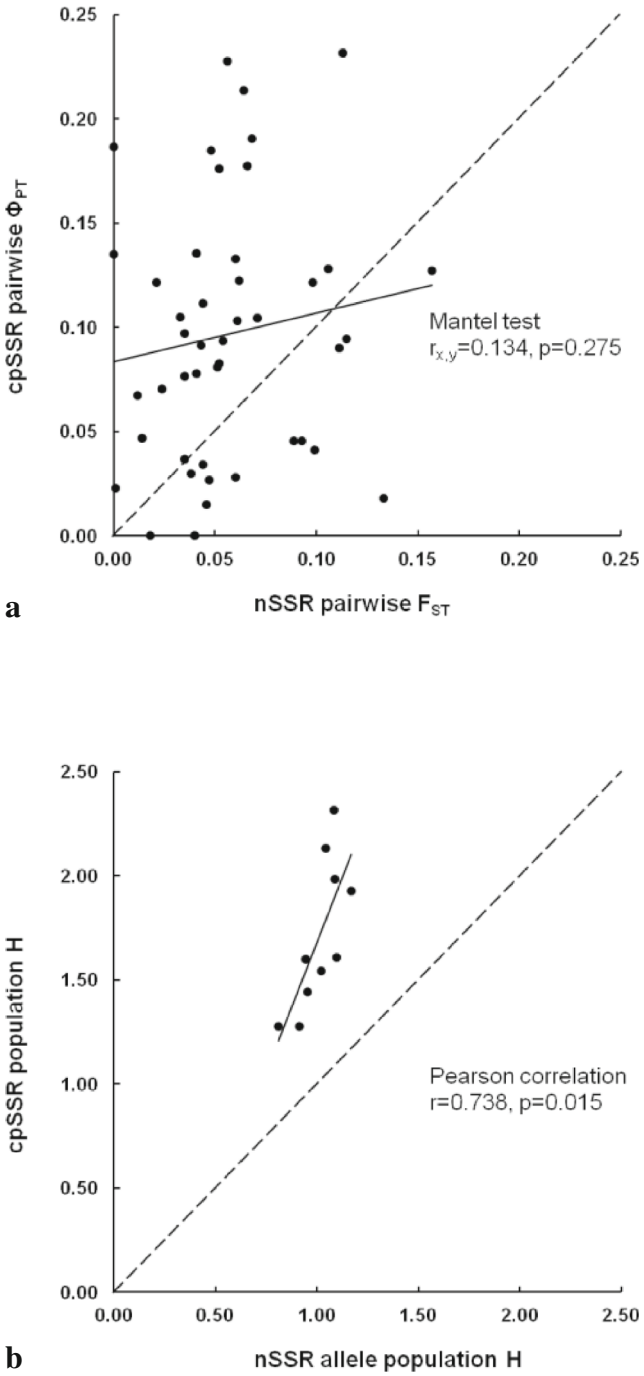

Fig. 5 Comparison between the biparentally inherited nuclear and parentally inherited chloroplast microsatellites in ten *Pinus cembra* populations from the Carpathian Mountains. **a** Pairwise population genetic differentiation measured by F_{ST} and Φ_{PT} for the nuclear and chloroplast data, respectively; **b** genetic diversity measured by Shannon diversity index (*H*) for both marker types. The *dashed line* indicates a 1:1 ratio, the linear trendline is fit on the data points, and the results of correlation statistics (see text) are displayed

Retezat Mountains, suggesting that the species may have been present in the vicinity of these mountains during the last glaciation. From these regions, only the two populations from the Călimani Mountains (NEG, NEA) appeared somewhat different from the others in their nSSR patterns, as they comprised individuals that were mainly assigned to a single genetic cluster (Fig. 3b). This may mean that *P. cembra* had a permanent common gene pool until its recent post-glacial retraction, and insufficient numbers of generations have passed to allow for genetic drift to induce strong genetic differentiation. Alternatively, local genotypes of independent glacial refugia were swamped during a possible post-glacial range expansion, except for MUN. However, both scenarios remain hypothetical.

Population genetic comparison of nSSR and cpSSR markers

For both marker types, rates of genetic variation among populations were slightly lower than the average found in conifers (Petit et al. 2005). For cpSSRs, 9.5 % inter-population genetic variation was found, while the average in paternally inherited markers for conifers is 16.5 %. For nSSRs, the 5.5 % inter-population genetic variation is about half of the 11.6 % average for biparentally inherited markers reported in conifers. However, such comparisons should not be taken too strictly because values depend on the sampling scheme. The lack of isolation by distance throughout the range, independent of marker type and method of gene flow estimation, also implies that even distant populations may either be within gene flow distance and/or have not substantially diverged from their common ancestry. Alternatively, populations originating from distinct lineages may be arranged so that genetic differentiation does not gradually change over distance.

Genetic differentiation was significantly different from zero in the majority of population pairs for both marker types (Supplementary Table S3). Except for MUN, the high number of significantly positive F_{ST} and Φ_{PT} values can to some extent be attributed to the low sample sizes, as there is a trend of larger average genetic differentiation of populations with smaller sample sizes (data not shown). Between populations from the same mountain ranges, population pairs NEA–NEG (Călimani Mountains) did not significantly differ from each other with both marker types, while MOR–VEL (Tatra Mountains) and GEN–GEM (Retezat Mountains) population pairs were not significantly different for the nSSRs and cpSSRs, respectively. This means that moderate rates of gene flow may still exist between adjacent populations, or genetic drift has not yet been sufficiently strong and long-lasting to invoke significant genetic differentiation. At the same time, low genetic differentiation between these neighboring population pairs substantiates that the genetic estimates are fairly representative despite low sample sizes.

According to theoretical expectations, population genetic differentiation should be higher for chloroplast than for nuclear genes because of uniparental inheritance and lower effective number of chloroplast genes when gene exchange is limited (Birky et al. 1989; Petit et al. 1993; Ennos 1994; Hu

and Ennos 1997, 1999). Similar levels of genetic differentiation of nuclear and chloroplast markers in conifers were found only in cases of extensive pollen dispersal (Latta and Mitton 1997; Ribeiro et al. 2002). In the present study, population genetic differentiation values were considerably higher for cpSSRs than for nSSRs. This finding suggests restricted gene flow, which may be due to the fact that extant populations of *P. cembra* in the Carpathians are often separated by dozens or hundreds of kilometers. Accordingly, we presume that the levels of genetic differentiation mirror levels of connectivity, either because of current gene flow or as a result of the formerly contiguous occurrence of *P. cembra* in and around this mountain range.

Values of pairwise population genetic differentiation are expected to be proportional when measured with different marker types under high levels of gene flow (Ribeiro et al. 2002). However, we found only weak correlation between pairwise population genetic differentiation for the cpSSRs and nSSRs (Fig. 5a). A possible explanation for this phenomenon is that the cpDNA genome behaves as a single locus and the spatial autocorrelation of chloroplast haplotypes is subject to higher stochasticity than the multi-locus nSSRs. Thus, drift may have a stronger effect on the pattern of genetic differentiation of the chloroplast haplotypes than on that measured by nuclear markers (Myers et al. 2007). Nevertheless, we cannot completely rule out that partly low sample sizes contributed to the overall variance in values of genetic differentiation.

Unlike in the case of genetic differentiation, diversity values will depend not only on the extent of gene flow but also on the ploidy level and mutation rate of the molecular markers (Ribeiro et al. 2002). Theoretically, diversity is expected to be similar or higher for biparentally inherited diploid than for paternally inherited haploid markers (Birky et al. 1989), and generally nSSRs have higher mutation rates than cpSSRs (Provan et al. 1999). However, Shannon diversity of non-recombining chloroplast haplotypes depends on the number of loci genotyped, and a higher number of markers will inevitably result in a higher number of haplotypes. In the present case, although the values of population genetic diversity have high correlation between cpSSRs and nSSRs, mean values are markedly higher for the cpDNA haplotypes. Further studies comparing diversity at paternally and biparentally inherited markers may elucidate if this unexpected result is extraordinary or a common trend for species with long life span, wide-ranging gene flow, and disjunct occurrence.

When a population experiences a bottleneck after its size sharply declines, mating between related individuals will occur even under random mating. This may result in substantial inbreeding promoted by the bottleneck event, which may lead to a reduction in genetic diversity if measured in terms of heterozygosity. From the ten *P. cembra* populations studied, four and five were identified as showing a bottleneck effect and inbreeding, respectively. Three populations LAL, MUN, and GEM indicated both bottleneck and inbreeding. This may be an effect of the decline of *P. cembra* stands due to logging and deforestation for creating pastures in past centuries (Feurdean et al. 2011). One population (CIN) showed a significant test for bottleneck effects, but no inbreeding, which may be the case when the reduction in population size was too recent for a detectable increase in inbreeding. In turn, populations VEL and NEG revealed significantly positive inbreeding parameters without apparent bottleneck effects. Such a situation may arise in expanding populations. A similar situation has been documented for the disjunct remnant populations of *Pinus radiata*, which show inbreeding despite signatures of recent population decline (Karhu et al. 2006). Even though we acknowledge that our sample sizes might be at the lower end of the sensitivity range of the bottleneck test, this test statistic takes the sample size into consideration. However, the ultimate reason for these results requires further analyses.

Identification of non-native *P. sibirica* in the Rodnei Mountains

Population BOR from the Rodnei Mountains (Eastern Carpathians) has previously been identified as the most distinct from all other populations based on cpSSRs (Höhn et al. 2009). Our analyses confirmed the genetic distinctiveness of BOR as well as a newly sampled population nearby (LAL). The idiosyncrasy of these populations was unraveled by the Bayesian assignment test using nSSRs that discovered individuals forming a genetic cluster otherwise missing in these populations and only scarcely present elsewhere (Supplementary Fig. S1). Moreover, all but one individual shared an identical chloroplast haplotype. The genetically peculiar trees originated from easily accessible valley sides, unlike the rest of the specimens in both BOR and LAL (M. Höhn, personal observation). These results suggested that a fraction of BOR and LAL individuals had been introduced. We tested two alternatives as likely origins of these presumably non-native individuals. Our first hypothesis was that *P. cembra* may have been introduced to the Rodnei Mountains from Eastern Alpine locations. The alternative hypothesis assumed that individuals of the closely related *P. sibirica* might have been planted in or close to natural *P. cembra* stands, like in some places in the Tatra Mountains (Paryski 1971 *cit. in* Zwijacz-Kozica and Żywiec 2007). The STRUCTURE analysis including Eastern Alpine *P. cembra*, native *P. sibirica*, and planted *P. sibirica* from the Tatra Mountains could not elucidate the origin of the non-native specimens at basic parameter settings, as they still formed their unique genetic cluster not present elsewhere (Supplementary Fig. S2b). As the comprehensive sample from the Eastern Alps covered a wide range (from the easternmost Alpine populations to the central Alps at longitude 7.83° E), we rejected the hypothesis of an Eastern Alpine provenance. In the same line, samples from the native eastern

Siberian population were assigned to a cluster not present in any Carpathian samples, which is not surprising given the distant location of this population and strong genetic differentiation across the range of *P. sibirica* (Krutovsky et al. 1992). Individuals of *P. sibirica* introduced to the Tatra Mountains showed mixed assignment to the genetic groups of the native Carpathian *P. cembra* samples. Thus, the non-native individuals of the Rodnei Mountains seemed to be genetically differentiated from either of the *P. sibirica* populations studied. However, by applying the USEPOPINFO model, all non-native specimens grouped to the cluster of *P. sibirica* from the Tatra Mountains. We hence conclude that the non-native individuals in the two populations of the Rodnei Mountains are *P. sibirica* trees from a different gene pool than the ones planted in the Tatra Mountains or the eastern Siberian provenance included here, e.g., from a western Siberian provenance. The strong differentiation of the population BOR found in cpSSRs by Höhn et al. (2009, 2010) may thus be attributed to the prevalence of an otherwise rare chloroplast haplotype in the *P. sibirica* individuals.

Critchfield (1986) listed *P. sibirica* and *P. cembra* among the inter-fertile species within *Pinus* section *Strobus*; accordingly, crossing of the native and introduced individuals may be forecasted for future generations. Such "genetic pollution" as mixture between the locally adapted and foreign individuals may have a negative effect on the population's fitness through outbreeding depression (McKay et al. 2005) and should be considered in conservation efforts. Genotyping juvenile cohorts could elucidate to what degree introgression has already occurred locally.

Conclusions

Populations of *P. cembra* are considered to need active conservation management for their survival due to the upslope shift of its climate envelope and a respective loss of suitable habitats during the ongoing climate change (Casalegno et al. 2010). Höhn et al. (2009) found higher genetic variability within *P. cembra* populations from the Carpathians compared to those in the central Alps, emphasizing the importance of these populations for species conservation. The present study shows that these isolated populations, in general, have not yet evolved to become genetically unique. However, the effect of current gene flow is weak, and genetic drift predominates in characterizing the genetic variation. This means that population persistence will depend on single populations, suggesting that sustaining sufficiently high population sizes is recommended. Also, quantitative genetic differentiation, as found in provenance tests (e.g., Blada 1997), substantiates the relevance of local populations in conserving genetic diversity in Carpathian *P. cembra* as a whole. The genetic differentiation of population MUN suggests that this population should receive special attention because of its peculiar gene pool among Carpathian populations of *P. cembra*. In turn, the genetic composition of the populations in the Rodnei Mountains, which is influenced by presumably non-native *P. sibirica*, should be observed for possible introgression. This has to be kept in mind in forest management and conservation actions in the region, and further genetic studies may help to elucidate the exact origin and amount of *P. sibirica* individuals introduced in the Rodnei Mountains.

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Data archiving statement Sequence data of nuclear microsatellite loci are retrievable from NCBI GenBank; cp/nSSR data are uploaded on the TreeGenes database.

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