



Present genetic structure is congruent with the common origin of distant Scots pine populations in its Romanian distribution



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ABSTRACT

Owing to the fact that mitochondrial, chloroplast and nuclear genomes are differently affected by evolutionary forces, the comparative study of all three genomes in combination with paleobotanical evidences allows for a more accurate interpretation of present genetic make-up. In this study, we analyzed the *nad1–nad7* mitotype, 14 cpSSR loci and 10 nSSR loci in 13 Romanian Scots pine (*Pinus sylvestris* L.) populations from the Romanian Carpathians and two Hungarian populations. Previous analysis of the macrofossil records supports the presence of Scots pine and Swiss stone pine (*Pinus cembra* L.) during the glacial period in Hungary and Romania. For a better image into the population structure in the studied area we also included in the data analysis two outgroup populations (north and south of Sweden) of known post-glacial origin, which resulted in a more defined genetic structure. Our study reveals that relatively high genetic diversity is preserved at all the Romanian and Hungarian populations, with no distinction among naturally regenerated, managed autochthonous and introduced populations. Furthermore, genetic differentiation analysis fails to distinguish the two introduced populations from the Romanian ones. AMOVA clustering is congruent with the paleobotanical-based evidences of contraction of Scots pine range since the Holocene to its present fragmented distribution.

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1. Introduction

Scots pine (*Pinus sylvestris* L.) currently has a wide distribution across Europe that is largely the result of post-glacial expansion out of southern glacial populations where the species main habitat was located during the last glacial period. During this period, the ice sheet extended south to central Poland, Lithuania and northern Belarus (Huijzer and Vandenberghe, 1998) and multiple studies of fossil records suggest that Scots pine survived in the Iberian, Italian and Balkan peninsulas (Birks and Williams, 1983; Bennett et al., 1991; Willis et al., 2000; Willis and Van Andel, 2004), Baltic region (Heikkilä et al., 2009; Binney et al., 2009), Russian plains (Svenning et al., 2008), and possibly also even west and southwest of Norway (Kullman, 2002). In connection with Central and Eastern Europe, the paleobotanical record clearly support that in the Carpathian Region, in Eastern Romania, Moldavia and in the Ukrainian loess belt, Scots pine was widespread during the last glacial cycle and

it was relatively abundant in the loessic regions at low and hill zones (Feurdean et al., 2007b; Feurdean et al., 2014; Fletcher et al., 2010; Gerasimenko, 2006; Haesaerts et al. 2010; Jankovska et al., 2002; Jankovska and Pokorný, 2008; Magyari et al., 2014a, 2014b; Obidowicz, 1996; Rudner and Sümegi, 2001; Willis et al., 2000).

Despite there are evidences supporting the Romanian territory as one of the main reserves of the boreal flora during the Last Glacial Maxima (LGM) (Feurdean et al., 2014), exhaustive genetic analysis of populations from the Romanian plateau and the Carpathian arc have not yet been conducted in Scots pine. To date, studies of mitochondrial variation in Scots pine have mostly focused their attention on the western Carpathian populations located in Slovakia, Ukraine and the Czech Republic (Naydenov et al., 2007; Pyhäjärvi et al., 2008; Buchovska et al., 2013), which represent less than half of the Carpathians entire range. These three molecular analyses investigated the distribution of haplotypes from two mitochondrial regions (*nad7* intron1 and *nad1* intron B/C: the *nad7–nad1* mitotype) to gain more insight into the location of Scots pine glacial populations. Naydenov et al. (2007) and Pyhäjärvi et al. (2008) suggest the presence of glacial refugia in mid-northern latitudes for the origin for *nad7* intron1

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allele “A”, while the high frequency of *nad7* intron1 allele “B” in northern Finland, St. Petersburg, and Moscow suggests more eastern and northern origin of allele “B” (Naydenov et al., 2007; Pyhäjärvi et al., 2008; Buchovska et al., 2013). Moreover, any interpretation of the post-glacial movements of Scots pine populations based on the *nad7–nad1* mitotype, warrants caution considering that the locus *nad7* is only biallelic and *nad1* is mostly monomorphic.

Pyhäjärvi et al. (2008) already highlighted the need for a denser sampling of the Eastern Carpathians and Russia to identify more precisely the location of Scots pine glacial habitat that have contributed to the subsequent northward colonization. Cheddadi et al. (2006) conducted DNA analysis on Scots pine populations from the Romanian Carpathians and although the name or location of the analyzed populations are not reported in the article, it is possible to infer from Fig. 2 (see Cheddadi et al., 2006) that five Romanian populations were included, at least, in the analysis of the *nad1* mitochondrial region, which however is monomorphic in these populations. DNA analysis of *Pinus cembra* and *Picea abies* L. (H.) Karst. also support eastern and western Romanian glacial populations as sources of expansion for the colonization of the Bohemian massive, eastern Alps and Poland during the Holocene (Gugerli et al., 2001; Bodnariuc et al., 2002; Tollefsrud et al., 2008; Höhn et al., 2009).

In this study we focus in the largest Carpathian area, the Romanian range. Romanian forests have been recognized of particular interest for their location in the region of contact between the sub-continental, Mediterranean and steppic regions (Bodnariuc et al., 2002), where Scots pine, despite not being a dominant species, is of great ecological importance (Banarescu and Boscaiu, 1973). We characterized the status of genetic diversity and population structure in the Romanian Scots pine populations in an effort to provide relevant information for the present and future Scots pine conservation and management in Romania.

We have performed a dense sampling of Scots pine in 13 populations across its present Romanian range, which in this area can be referred as an interglacial refugium considering that this cold-adapted tree species had a larger distribution than it shows today in the area of study (see Stewart et al., 2010). Two of the sampled populations are unmanaged reservation areas (naturally regenerated). Other nine populations are managed through assisted regeneration with seeds of autochthonous origin and two were replanted with non-Romanian seeds, and therefore, those are of non-autochthonous origin. We also sampled two populations from the nearest Scots pine populations, two Scots pine naturally regenerated forests from the Hungarian plain whose potential glacial origin has been debated (Willis et al., 2000; Cheddadi et al., 2006).

The history of Scots pine forest fluctuations in composition and structure as a result of last glacial climate changes and anthropomorphic activities differs across regions in Romania, which is reflected in the present day populations, which vary in size, density, isolation, habitat quality and level of management (i.e., from naturally regenerated to non-autochthonous) (Borlea et al., 2006; Veen et al., 2010; Magyari et al., 2014b). Our study is the first attempt to estimate genetic diversity, inbreeding and genetic structure in present day Scots pine populations represented by natural, managed autochthonous and introduced populations (non-autochthonous). Our hypotheses are: (a) genetic diversity and inbreeding have been altered following management activities; (b) population structure analysis based on mitochondrial loci (maternally inherited) could reveal the non-autochthonous origin of some of the introduced populations, whereas, chloroplast- and nuclear-based loci should mostly reflect present population structure characterized by extensive pollen exchange of chloroplast and nuclear genes among autochthonous and introduced trees; and (c) even under dramatic population size reduction due to human

activities in the last few centuries, the outbred and high heterozygotic nature of Scots pine should have preserved the genetic imprint of populations glacial history of expansion and posterior shrinkage, as revealed by the paleobotanical records. In this study, we have gained insights in present day Scots pine populations, which are the raw material for the future development and conservation of Scots pine forest in Romania. Successful strategies for the maintenance of this species require understanding the levels and distribution of genetic diversity in the relic, autochthonous and also in non-autochthonous Scots pine forests for their potential impact in the conservation of this economically and ecologically important species. To test our hypotheses we have analyzed the *nad1* intron1 (exonB/C) and *nad7* intron1 mt regions as well as 14 cpDNA and 10 nuclear SSR loci. To improve clustering of our samples into well-defined entities we also incorporated two Swedish outgroup populations of known post-glacial origin into our analyses (Willis et al., 1998).

2. Material and methods

2.1. Plant material and sites selection

Dormant buds were sampled from 16 to 20 Scots pine (*P. sylvestris* L.) trees per population. The sampled trees were separated by a minimum of 50 m from each other to avoid sampling related individuals.

The 13 Romanian populations sampled span the entire Carpathian mountain range in Romania (Table 1).

All Romanian populations are administrated by the RNP (National Administration of Forests) and the Romanian Ministry of Agriculture and Rural Development. An extensive description of each population is available in the supplementary file “Populations description” (Supplementary material). Populations VL, SV, and both Hungarian populations are naturally regenerated. VL is located between the Cozia (Vâlcea) and Cindrel (Sibiu) National Parks. SV is located in the Poiana Stampei National Park (Suceava). The SV and VL populations are both affected by abiotic stress: SV is located on swampy ground that causes root anoxia (oxygen deficiency) and VL is located on a rocky hill (1250 m altitude), where only few scattered trees have survived the strong winds and where soil availability is limited. G, R, S, T and B are managed through assisted regeneration from autochthonous seeds (i.e., Seeds collected from local forests) and are located at a distance between 25 and 50 km from National Parks (see Populations description, Supplementary material). A, HD, BV and VR are managed through assisted regeneration with autochthonous seed, however, there is no record about these activities (A. Colisar, Assistant professor at the University of Agricultural Sciences and Veterinary Medicine, Department of Forestry, personal communication). Those four populations are located at distances between 1 and 30 km from National Parks. TM and CS are introduced from non-autochthonous seeds. In the case of TM the seeds are of Austrian origin, and in the case of CS there is no record on the origin of the seeds utilized for its plantation. TM population is being displaced by hard wood species and there is hardly any tree left, however, the exact reason for the low performance of Scots pine in this area is not known. TM is located at 50 km from Retezat National Park (Hunedoara). CS is located between the Cheile Caraşului (Caraş-Severin) and Cheile Nerei-Beuşniţa (Caraş-Severin) National Parks.

The Hungarian trees were sampled from two Natural protected reservations (https://msw.botanika.hu/meta/2_terkepek_2.0/kV_EH_n13_elterjedes_2.0.htm). The Western population, Vas, Velemér, extends over 1100 hectares and it connects to the Austrian Eastern forest. This natural population was managed in the

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