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Additional Information

1 **Highly informative SSR genotyping reveals large genetic diversity and limited**
2 **differentiation in European larch (*Larix decidua*) populations from Romania**
3

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20 **Abstract:** European larch (*Larix decidua*) is naturally distributed at high elevations in
21 Central Europe. Romanian populations of *L. decidua* are scattered in different areas of
22 the country. In this study, we used 12 highly informative genomic simple sequence
23 repeat (SSR) markers for genotyping seven populations from different areas of
24 Romania. The SSR markers were highly variable, with up to 11 alleles per SSR locus

and an average polymorphic information content of 0.713. High values of observed (H_o=0.542) and expected (H_e=0.738) heterozygosities were observed. Cluster analysis of populations did not group them according to geographical distance, but some clusters contained populations from a similar altitudinal range. The partition of genetic variation revealed that two-thirds of the genetic variation was found within individuals (due to high H_o levels), while only one-sixth of the total genetic variation corresponded to variation among populations. A population structure analysis identified four genetic clusters, and in most cases individuals from a single population were adscribed to several genetic clusters. A multivariate principal coordinates analysis (PCoA) confirmed the population structure analysis. SSR markers are a powerful tool for evaluating diversity, relationships and genetic structure of Romanian *L. decidua* populations, which have high levels of diversity and low genetic structuration.

Key words: AMOVA, heterozygosity, genetic diversity, *Larix*, SSR markers

1. Introduction

European larch (*Larix decidua* Mill.) is an important deciduous forest tree species from the *Pinaceae* family with high economic interest for its timber (Matras et al., 2008). Because of the high quality of its timber and the fast growth, several breeding programmes have been established to develop improved materials for establishing cultivated commercial stands of European larch (Mihai and Teodosiu, 2009; Marchal et al., 2017). European larch is used as an ornamental species (Torchik et al., 2010) and, among coniferous species, is considered one of the best in terms of coexisting with good understorey pastures for domestic grazing animals (Mosca et al., 2012) European larch mostly grows in mountainous cold areas in Central Europe (Alps, Carpathians, Sudetes)

(Matras et al., 2008) and because of this, it is particularly susceptible to climate change (Mosca et al., 2012; Nardin et al., 2015). Due to higher temperatures caused by climate change, European larch can be displaced from its present altitudinal range to higher elevations by other evergreen conifers that are better adapted to warmer temperatures, which in some regions may limit its occurrence and long-term population viability (Kharuk et al., 2007; Matras et al., 2008; Nardin et al., 2015). At the same time, European larch is colonizing new areas where glacier retreat and warmer temperatures allow the tree line to move up to higher elevations (Pluess, 2011).

In Romania, European larch is distributed in the Carpathians mountains range, in particular in the northern part of the country as well as in some scattered areas in the Center and West of the country (Matras et al., 2008; Vilcan et al., 2017). European larch populations of Romania are geographically isolated from the other main natural distribution areas in the Alps and the Sudetes, are largely fragmented and are situated at the southeastern edge of its natural distribution (Mihai and Teodosiu, 2009). This makes Romanian populations of European larch particularly sensitive to climate change (Hamrick, 2010). A similar concern exists on the effects of climate change in other Romanian forest trees situated in the edge of distribution, like European beech (*Fagus sylvatica* L.) (Budeanu et al., 2016).

Knowledge of the genetic diversity and structure of the Romanian populations of European larch is important in order to devise strategies for their conservation, assessing its potential for adaptation to climate change, as well as for its selection and breeding (Young, 2000; Mihai and Teodosiu, 2009; Marchal et al., 2017; Vilcan et al., 2017). Not much information exists on the genetic structure and differentiation of European larch populations. In this respect, Maier (1992) using allozymes found a low

genetic differentiation between populations from the Alps, Poland, Czech Republic, and Slovakia. In a more recent study, Nardin et al. (2015) using simple sequence repeat (SSR) markers found a low genetic differentiation between altitudinal gradients in a continuous natural population of European larch from the Alps. Little information is available on the genetic diversity and structure of Romanian populations of European larch. In this respect, Mihai and Teodosiu (2009), used isozymes to characterize five Romanian populations of *L. decidua* and found similar levels of diversity to those reported for other studies from other areas using similar methodologies (Lewandowski et al., 2000). More recently, Vilcan et al. (2017) using random amplified polymorphic DNA (RAPD) markers found reduced genetic differentiation among Romanian populations of *L. decidua*, although some provenances could be distinguished at the molecular level.

Genetic characterization of Romanian populations with highly informative molecular markers could provide relevant information on their genetic diversity and structure (Matras et al., 2008). SSR markers present several advantages over other markers, as they are co-dominant, abundant and scattered all over the genome, robust, highly polymorphic, amenable to multiplexing, and have a high inter- and intra-laboratory reproducibility (Kalia et al., 2011). Among SSRs, genomic SSRs are mostly associated with non-coding regions and are generally more polymorphic than EST-SSRs, which are derived from expressed regions of the genome (Kalia et al., 2011; Muñoz-Falcón et al., 2011). SSRs are widely used for evaluating genetic diversity and relationships in many forest tree species (González-Martínez et al., 2006), and being heterozygous allow estimating the real heterozygosity of each of the individuals sampled (Kalia et al., 2011). Genomic SSR markers have been developed and tested in

L. decidua (Isoda and Watanabe, 2006; Wagner et al., 2012). These same markers have been used in other European larch genetic variation studies (Pluess, 2011; Nardin et al., 2015), confirming its utility.

In the present study, we used genomic SSRs developed by Isoda and Watanabe (2006) and by Wagner et al. (2012) to characterize European larch individuals from seven Romanian populations from different areas. The information on the genetic diversity and structure and genetic relationships of these populations will be useful to devise measures for the present and future conservation of genetic diversity of Romanian *L. decidua* as well as for designing strategies for selection and breeding of this timber forest species.

2. Materials and Methods

2.1. Plant material

Seven populations of *Larix decidua* from different parts of Romania were sampled for the present study (Table 1, Figure 1). Five of the populations are situated in the central part of Romania (Latorita, Brasov Valea Cetatii, Sacele, Brasov Valea Popii, and Sinaia), one in the western part of Romania (Anina), and one in the North of Romania (Gura Humorului). The populations had a stand area of between 1.8 ha (Anina) and 9.6 ha (Gura Humorului) and were comprised an altitudinal range between 520-570 m (Gura Humorului) and 1000-1200 m (Latorita) (Table 1). Distal parts of side branches having young light green leaves were picked for DNA extraction of three adult trees (estimated age between 80 and 125 years) situated in different parts of the tree stand of each population.

1 **2.2. DNA extraction**

2 Total genomic DNA was isolated from young needle-like leaves, according to the
3 CTAB protocol (Doyle and Doyle, 1987) with slight modifications. The extracted DNA
4 was dissolved in Milli-Q water and its concentration and quality (230/260 and 260/280
5 nm ratios) were initially checked with a NanoDrop ND-1000 spectrophotometer
6 (Thermo Fisher Scientific, Waltham, USA). For samples not having high-quality
7 parameters, the extraction was repeated. Quality of these samples was confirmed in
8 agarose gel at 0.8% and with Qubit® 2.0 Fluorometer (Thermo Fisher Scientific,
9 Waltham, USA). DNA was diluted with Milli-Q water to a concentration of 30 ng/μL
10 for PCR amplification of SSRs.

11 **2.3. SSR genotyping**

12 Twelve genomic SSR markers (Table 2) developed by Isoda and Watanabe (2006) and
13 Wagner (2012) were used to screen the *L. decidua* individuals. SSRs were organized in
14 two multiplexes according to the expected allele range size (Wagner et al., 2012) (Table
15 2). The SSR amplification was performed by PCR in a total volume of 12 μL including
16 7.21 μL water, 1.2 μL 1× PCR buffer, 0.6 μL MgCl2 50 mM, 0.24 μL dNTPs 10 mM,
17 0.3 μL reverse primer 10 μM, 0.06 μL forward primer with M13 tail 10 μM, 0.24 μL
18 fluorochrome (FAM, VIC, NED and PET) 10 μM, 0.15 μL Taq DNA Polymerase
19 (5U/μL), 2 μL DNA template 30 ng/μL. The PCR program used was the following: 95
20 °C for 3 min for a denaturation, 30 cycles of 30 s at 95 °C followed by 30 s at 60 °C and
21 of 30 s at 72 °C and finally 72 °C for 5 min for the last step of extension. The PCR
22 products were diluted in formamide and sequenced by capillary electrophoresis through
23 an ABI PRISM 3100-Avant sequencer (Thermo Fisher Scientific, Waltham, USA)
24 using a 600 LIZ GeneScan size standard (Thermo Fisher Scientific, Waltham, USA).

PCR fragments were analyzed using the GeneScan software (Thermo Fisher Scientific, Waltham, USA) to obtain the electropherograms. Polymorphisms were analyzed with the Genotyper DNA Fragment Analysis software (Thermo Fisher Scientific, Waltham, USA).

2.4. Data analysis

Software packages PowerMarker (Liu and Muse, 2005) and GenAlEx 6.503 (Peakall and Smouse, 2012) were used for the molecular marker analysis of SSRs. For each SSR locus, the following parameters were calculated using the PowerMarker package: number of alleles (N_a), major allele frequency (*f*), number of effective alleles (N_e), number of genotypes (N_g), polymorphic information content (*PIC*) values calculated as $PIC = \mathbf{1} - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2 p_j^2$ (where *n* is the total number of alleles detected, p_i the frequency of the ith allele, and p_j the frequency of the jth allele) (Botstein et al., 1980), observed heterozygosity (H_o), calculated as the number of heterozygous alleles/number of alleles, expected heterozygosity (H_e), calculated as $H_e = \mathbf{1} - \sum_{i=1}^{n} p_i^2$ (where p_i is the frequency of the ith allele) (Nei, 1972), and fixation index (F_{is}), calculated as $F_{is}=1-(H_o/H_e)$ (Wright, 1965). A consensus tree was obtained in PowerMarker with genetic similarity data, using Nei's genetic distance (Nei, 1972), to evaluate the relatedness among populations using the neighbor-joining method (Saitou and Nei, 1987). Branch support on the phenogram was tested by bootstrap analysis with 1000 replications using PowerMarker and visualized by PHYLIP version 3.67 software (Felsenstein, 2007). An analysis of molecular variance (AMOVA) was performed using GenAlEx 6.503 (Peakall and Smouse, 2012) software using the data of the 12 SSR loci. Total molecular variation was partitioned in the following sources of variation: among populations, among individuals, and within individuals. The genetic distance matrix

(Nei, 1972) among the different accessions was calculated with the GenAlEx 6.5 software package. The relationship between pair-wise genetic distances and geographical distances among individuals was calculated using linear regression analysis. Population structure was estimated using a model-based Bayesian structure implemented in the software STRUCTURE 2.3.4 (Pritchard, 2000). Twenty runs of STRUCTURE were performed by setting the number of clusters (K) from 1 to 7 (number of locations of the sampled accessions). Each run consisted of a length of burn-in period of 5,000 steps followed by 50,000 Monte Carlo Markov Chain (MCMC) replicates, assuming an admixture model and uncorrelated allele frequencies. No prior knowledge of the population of origin was introduced. ΔK method (Evanno et al., 2005) was used to identify the most likely number of clusters (K) using STRUCTURE HARVESTER 0.6.94 software (Earl, 2012). Based on the estimation of the best K of the latter, an additional run of STRUCTURE was performed using the following parameters: K3 to K5, length of burn-in period of 500,000, number of MCMC reps after burn-in of 750,000, number of iteration of 20. The ultimate most likely K was found running again Structure Harvester software. Each individual was assigned to its corresponding group based on maximum membership probability, as indicated by Remington et al. (2001). A principal coordinates analysis (PCoA) to graphically represent genetic relationships among individuals was performed using GenAlEx 6.5 software.

3. Results

3.1. SSR characterization and diversity

No overlap in the ranges of marker sizes was detected within the two multiplexes for the SSR loci tagged with the same dye (Table 2), and therefore the 12 SSR markers could be unambiguously scored. For the 12 SSR markers, the size ranges we obtained were included within the size ranges described by Wagner et al. (2012). These 12 SSR markers amplified in all individuals, except in one individual for each of the markers bcLK26, Ld30, Ld50, and Ld101, and in two individuals for Ld58, resulting in a missing data rate of 2.38%. In this way, only six out of the 21 individuals genotyped had one missing SSR marker, while 15 had no missing data.

All the SSR loci scored were polymorphic (Table 3), and all the individuals genotyped had a different genetic profile. The number of SSR alleles (N_a) detected ranged between four (for Ld42) and 11 (for bcLK253 and bcLK263). The major allele frequency (f) ranged between 0.214 (bcLK228) and 0.750 (Ld101), while the number of effective alleles (N_e) varied between 1.73 (Ld101) and 7.74 (bcLK253), and the number of genotypes (N_g) between six (Ld101) and 14 (bcLK253) (Table 3). The polymorphic information content (PIC) for SSR loci ranged between 0.397 (Ld101) and 0.858 (bcLK253), with an average value of 0.713, and all loci but one (Ld101) having PIC values above 0.5. The observed and expected heterozygosities (H_o and H_e, respectively) had average values of H_o=0.542 and H_e=0.738, resulting in an average fixation index (F_{is}) of F_{is}=0.256 (Table 3). Although wide ranges of variation were found among SSR loci for H_o (0.300 to 0.857) and H_e (0.421 to 0.871) the correlation between both parameters was reduced (r=0.327) and non-significant (P=0.2989). This resulted in a wide variation among SSR loci for the F_{is}, with values from -0.194 (Ld56) to 0.621 (bcLK263) (Table 3).

3.2. Multivariate cluster analysis of populations

The multivariate neighbor-joining cluster analysis of the seven populations studied shows that population 3 (Gura Humorului) from the north of Romania and from the lowest altitude (520-570 m) is basal to the other populations from central Romania (Figure 2). Among these, two clusters are clearly differentiated, with a bootstrap value of 100%, one containing population 6 (Brasov Valea Popii) which is the one ranking second in low altitudinal range (670-700 m) and the other one consisting of the remaining populations. Among these, bootstrap values above 50% are only detected for the node separating population 7 (Sinaia) from populations 1 (Anina) and 4 (Brasov Valea Cetatii), which in turn are also differentiated by a bootstrap value of 96% (Figure 2). These three latter populations are from altitudes of 800 m or lower, while the two other populations, i.e., populations 2 (Latorita) and 5 (Sacele) are from altitudes above 850 m. Amazingly, with the exception of population 3, no clear geographical pattern is observed in the clustering of populations (Figure 2).

3.3. Genetic structure of populations

The AMOVA analysis (Table 4) reveals that around one-sixth of the genetic variance observed is caused by the differences among populations, while another one-sixth is accounted by genetic differences among individuals. Two-thirds of the genetic variation observed is accounted by within individual genetic variation (Table 4), due to heterozygosity. The lack of population structure is confirmed when the relationship between genetic distance and geographical is studied (Figure 3). In this respect, no significant correlation exists between both distances ($r=-0.010$; $P=0.8806$).

The ΔK statistic had a maximum peak at $K=4$ (Figure 4), indicating the presence of four genetic clusters in the plants of the seven populations of *L. decidua* sampled. With the exception of individuals from populations 2 (Latorita) and 4 (Brasov Valea Cetatii), in

which all the individuals of each of these populations clearly belonged to the same genetic cluster (Figure 5), individuals of each population were adscribed to more than one genetic cluster.

In three individual plants, corresponding to two individuals from population 3 (Gura Humorului) and one from population 6 (Brasov Valea Popii), no clear adscription could be made to any of the genetic clusters and were considered as admixed between two or three genetic clusters (Figure 5). A first genetic cluster (I) is composed of one individual of population 1, the three individuals of population 4 and one of population 7; a second cluster (II) is the largest and is made of by two individuals of population 1, the three individuals of population 2, one from population 5 and two from population 6; the third genetic cluster (III) is made by just two individuals of population 5; and the fourth genetic cluster (IV) by two individuals of population 7 (Figure 5). Two individuals of population 3 correspond to an admixture of clusters II, III and IV, while one individual of population 6 is an admixture of genetic clusters II and IV (Figure 5).

3.4. Multivariate PCoA analysis of individuals

The four genetic clusters are confirmed in the multivariate PCoA analysis (Figure 6). In this analysis, the first and second principal coordinates account, respectively, for 20.6% and 12.3% of the SSR genetic variation. The first coordinate basically separates genetic cluster I, with positive values, genetic cluster II, which plots in the middle part of the graph, and the genetic clusters III and IV, which have negative values for this first coordinate (Figure 6). These two latter clusters are separated in the third component, with cluster III having positive values and cluster IV negative ones for the second principal component. The admixed individuals plot intermediate between the clusters of which they are an admixture (Figure 6).

1

2 4. Discussion

3 The multiplexing strategy developed by Wagner et al. (2012) proved effective in
4 genotyping Romanian *L. decidua* populations. In this way, as occurred in other works
5 using these same markers in *L. decidua* (Pluess, 2011; Nardin et al., 2015), no overlap
6 in the range of marker sizes was observed for SSR markers labelled with the same dye,
7 allowing an efficient use of multiplexing. The size ranges obtained for individual SSRs
8 matched the ones obtained by Wagner et al. (2012), although the range of variation was
9 narrower in our case probably due to the fact that these authors sampled a broader
10 geographical area (several countries *vs*. Romania in our case) of *L. decidua* populations.
11 The interpretation of SSR data is particularly sensitive to missing data (Reeves et al.,
12 2016); however, in our case the missing data rate for individual SSR markers was zero
13 or very low and therefore did not justify removing any of the SSR markers from the
14 analyses (Nardin et al., 2015; Reeves et al., 2016).
15 The diversity statistics obtained for the genomic SSR markers reveals that a large
16 genetic variation exists in the materials scored. The number of alleles (N_a) per marker is
17 somewhat lower than those obtained by (Wagner et al., 2012; Nardin et al., 2015),
18 although these authors sampled more individuals. However, the number of effective
19 alleles (N_e) does not differ significantly from the values obtained by Nardin et al.
20 (2015). The fact that the number of genotypes (N_g) is generally different and larger than
21 the number of alleles (N_a) is an indication of the presence of heterozygous loci (Guo and
22 Thompson, 1982). PIC values above 0.5 are considered as high (Pandey et al., 2012),
23 and in our case all SSR loci except one had values above this threshold, which indicates
24 that a reduced number of highly informative genomic SSR loci, like the ones we used,

1 provides relevant information for the reliable evaluation of genetic diversity and
2 structure in *L. decidua* (Pluess, 2011; Wagner et al., 2012; Nardin et al., 2015). In this
3 respect the PIC values obtained by us are higher than those obtained for genic SSRs in
4 *L. kaempferi* (Chen et al., 2015), and similar to highly informative SSR markers in other
5 conifer species, like those from genus *Picea* (A'Hara and Cottrell, 2009; Shi et al.,
6 2014).
7 The high values for observed heterozygosity (H_o) were expected, given the allogamous
8 reproduction of *L. decidua* (Lewandowski and Burczyk, 2000) and large sizes of the
9 populations. Expected heterozygosity (H_e) values were also high, with an average value
10 above 0.7, and similar to the values observed for this species (Pluess, 2011) and for the
11 related *L. kaempferi* (Isoda and Watanabe, 2006) providing further evidence of the
12 utility and highly informative nature of the SSRs used. As observed in *L. decidua*
13 (Pluess, 2011; Wagner et al., 2012; Nardin et al., 2015) and in other conifer species
14 (Mosca et al., 2012; Leonarduzzi et al., 2016), H_o and H_e values were quite similar
15 revealing a high degree of allogamy and low genetic isolation in the materials evaluated,
16 as observed with RAPD markers in these same populations (Vilcan et al., 2017). As a
17 result, the fixation index (F_{IS}) values for the SSR markers were low and as occur in both
18 *Larix* (Nardin et al., 2015; Vilcan et al., 2017) and other conifers (Leonarduzzi et al.,
19 2016), in some cases negative, revealing an excess of heterozygotes over those
20 predicted by the Hardy-Weinberg equilibrium for these markers (Wright, 1965).
21 Overall, because of its co-dominant nature, high degree of amplification, efficient
22 multiplexing, robustness, reduced rate of missing data, high polymorphism and high
23 informativeness, the markers used here, which were developed by Isoda and Watanabe

1 (2006) and Wagner et al. (2012), are of great utility for evaluating the diversity and
2 genetic structure of *L. decidua* populations.
3 The cluster analysis of the seven populations basically grouped them according to
4 altitudinal ranges rather than by geographical proximity. In a study of the genetic
5 differentiation of *L. decidua* along four different altitudinal ranges in the French Alps,
6 Nardin et al. (2015) found a low, but significant genetic differentiation signal among
7 them. A similar result, this time with two altitudinal ranges, was obtained in *L. decidua*
8 from the Swiss Alps [40] and in *L. kaempferi* from Mount Fuji in Japan (Nishimura and
9 Setoguchi, 2011).
10 The AMOVA results reveal a low level of genetic structure among the seven
11 populations, even though they are situated at distances of up to 358 km. In fact, genetic
12 variation among individuals within a population were similar to the differences among
13 populations. These results are in agreement with those obtained for *L. decidua*
14 populations from the Alps, Poland, Czech Republic and Slovakia (Maier, 1992), and a
15 low level of genetic differentiation among plots and among individuals within plot in a
16 population of European larch from the Alps (Nardin et al., 2015). Most of the genetic
17 variation corresponded to within-individual variation, which is due to the presence of
18 different alleles at individual loci (i.e., heterozygosity). This situation is common in
19 forest trees, where high levels of heterozygosity are commonly found (González-
20 Martínez et al., 2006; Carabeo et al., 2016; Tóth et al., 2017). In our case, the low levels
21 of genetic structure have important implications for selection and breeding purposes,
22 indicating that a wide genetic diversity can be found in the offspring of a single
23 individual, which would be resulting from the high level of heterozygosity combined
24 with the allogamous nature of *L. decidua* (Lewandowski and Burczyk, 2000). The low

genetic structuration is confirmed by the lack of correlation between genetic and geographical distances, which was also observed with RAPD markers in these same populations (Vilcan et al., 2017). This provides an indication that despite fragmented habitats, as occurs in other forest trees (Kremer et al., 2012), long distance extensive genetic flow takes place in *L. decidua*.

The number of genetic clusters is lower than that of populations and, except for two populations, individuals from the same population belong to different genetic clusters. This again provides support for extensive migration and gene flow in *L. decidua* (Lewandowski and Burczyk, 2000; González-Martínez et al., 2006). One major genetic cluster is distributed across five out of the seven populations, suggesting that this genetic background may confer broad adaptation. On the other side, the fact that two small genetic clusters (III and IV) are confined to some individuals of a single population may be the consequence of adaptive advantage to specific environmental conditions (Hamrick, 2004). This would allow the maintenance of these genetic clusters despite extensive gene flow, migration and hybridization.

In conclusion, despite the limited sample size per population used, highly informative genomic SSR markers (Isoda and Watanabe, 2006; Wagner et al., 2012) have proved useful for evaluating the diversity and assessing the genetic structure of *L. decidua* populations from Romania. The results reveal that a large genetic diversity of highly heterozygous individuals exists in the European larch populations from Romania and that, despite significant geographic distances and altitudinal ranges in the population's samples, there is a limited genetic differentiation. This has important implications for the conservation of European larch genetic diversity in a climate change scenario, as well as for selection and breeding of this important timber species (Matras and Pâques,

2008; Marchal et al., 2017; Vilcan et al., 2017). The high genetic diversity also may facilitate adaptation, through selection, of European larch to climate change.

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4 **Table 1.** Geographical data of the seven *Larix decidua* populations studied. Three

5 individuals were sampled per population. Trees were aged between 85 and 125 years

6 old.

Population	Provenance	Stand area (ha)	Latitude	Longitude	Altitude (m.a.s.l.)
1	Anina	1.8	45°54' N	21°51' E	680-710
2	Latorita	7.8	45°23' N	23°50' E	1000-1200
3	Gura Humorului	9.6	47°33' N	25°53' E	520-570
4	Brasov Valea Cetatii	4.8	45°38' N	25°36' E	800
5	Sacele	5.0	45°37' N	25°41' E	870-940
6	Brasov Valea Popii	6.4	45°25' N	25°19' E	670-700
7	Sinaia	3.0	45°19' N	25°33' E	750

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1 **Table 2**. SSR markers used in the present study, including the multiplex in which they
2 were included, their repeat motif, dye, size range obtained in this study, and the original
3 source of the SSR.

SSR locus	Motif	Dye	Size range (bp)	Source
Multiplex 1				
bcLK189	$(AG)_{17}AT(AG)_6$	NED	150-172	Wagner et al. (2012)
bcLK211	$(CT)_{16}$	FAM	188-222	Wagner et al. (2012)
bcLK228	$(AG)_{18}$	VIC	177-215	Wagner et al. (2012)
bcLK253	$(AG)_{17}$	NED	195-227	Wagner et al. (2012)
lardec001529 (Ld30)	$(AC)_{18}$	VIC	114-132	Isoda and Watanabe (2006)
lardec022835 (Ld50)	$(CA)_{18}$	PET	157-185	Isoda and Watanabe (2006)
Multiplex 2				
bcLK263	$(TC)_{20}$	PET	195-227	Wagner et al. (2012)
lardec023929 (Ld42)	$(TG)_{14}$	VIC	179-185	Isoda and Watanabe (2006)
lardec024823 (Ld45)	$(CA)_{13}$	FAM	202-212	Isoda and Watanabe (2006)
lardec023228 (Ld56)	$(AC)_{16}$	NED	231-243	Isoda and Watanabe (2006)
lardec022359 (Ld58)	$(AC)_{15}$	FAM	139-171	Isoda and Watanabe (2006)
lardec025807 (Ld101)	$(AC)_{12}$	NED	189-197	Isoda and Watanabe (2006)

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1 **Table 3.** Genetic diversity statistics of SSR markers in *Larix decidua* populations, including size range, number of alleles (Na), major allele

2 frequency (f), number of effective alleles (Ne), number of genotypes (Ng), polymorphic information content (PIC), observed

3 heterozygosity (Ho), expected heterozygosity (He), and fixation index (Fis).

SSR locus	N_a	f	N_e	N_g	***PIC***	H_o	H_e	F_{is}
bcLK189	9	0.333	4.79	7	0.764	0.429	0.791	0.458
bcLK211	10	0.500	3.42	10	0.685	0.714	0.707	-0.010
bcLK228	10	0.214	7.06	12	0.843	0.667	0.858	0.223
bcLK253	11	0.238	7.74	14	0.858	0.857	0.871	0.016
bcLK263	11	0.375	4.79	11	0.769	0.300	0.791	0.621
Ld30	8	0.325	5.19	10	0.784	0.550	0.808	0.319
Ld42	4	0.500	2.67	7	0.558	0.381	0.626	0.391
Ld45	5	0.333	4.26	10	0.727	0.571	0.765	0.253
Ld50	6	0.425	3.54	9	0.675	0.300	0.718	0.582
Ld56	6	0.500	3.11	8	0.684	0.810	0.678	-0.194
Ld58	9	0.289	5.78	12	0.806	0.526	0.827	0.363
Ld101	6	0.750	1.73	6	0.397	0.400	0.421	0.050
Mean±SE	7.92±0.70	0.399±0.043	4.51±0.51	9.67±0.68	0.713±0.037	0.542±0.055	0.738±0.036	0.256±0.072

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1 **Table 4.** Molecular analysis of variance (AMOVA) among populations. among

2 individuals within population and within individuals (due to heterozygosity) for seven

3 *Larix decidua* populations evaluated. Results are based on 12 SSR markers.

Source of variation	d.f.	S.S.	M.S.	Variance components	Percentage of variation
Total	41	188.83			
Among populations	6	56.33	9.389	0.785	16.63
Among individuals	14	65.50	4.679	0.744	15.77
Within individuals	21	67.00	3.190	3.190	67.60

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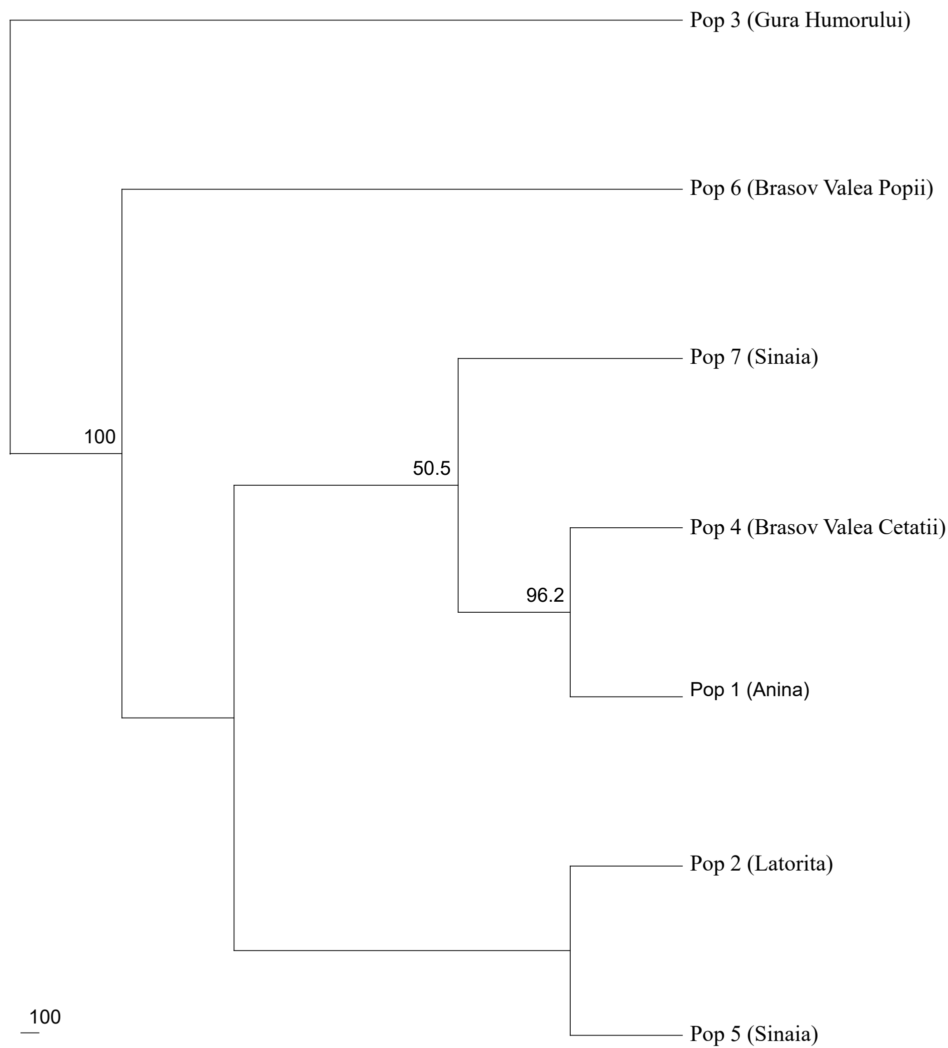
2 **Figure 1.** Map of Romania displaying the situation of each of the seven populations of

3 *Larix decidua* sampled.



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2 **Figure 2.** Neighbor joining clustering phenogram based on Nei (1972) genetic distances
3 for seven populations of *Larix decidua*. Bootstrap values (in percentage; based on 1000
4 replications) greater than 50% are indicated at the corresponding nodes.

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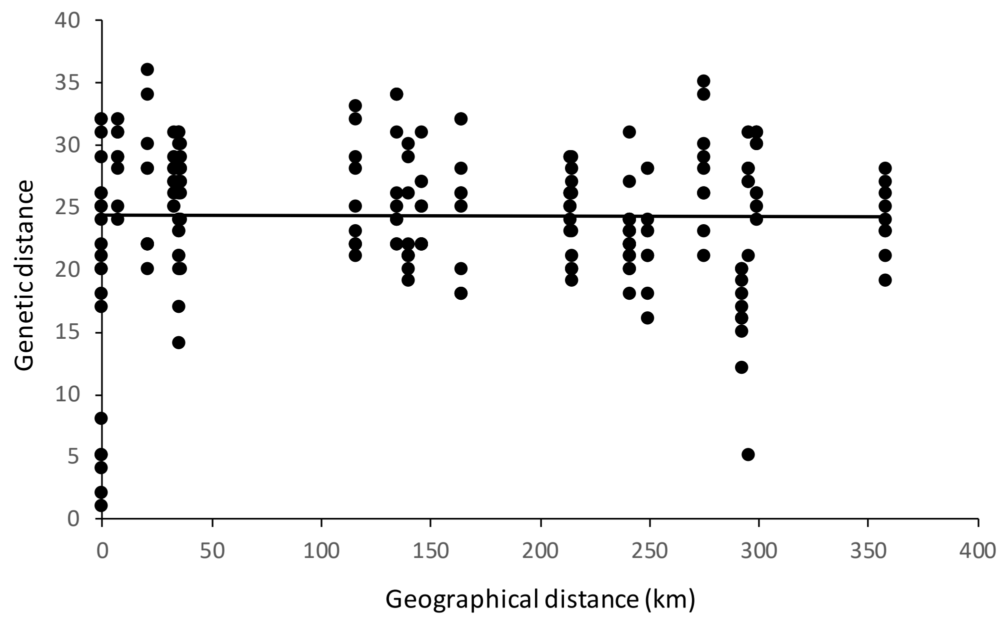

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2 **Figure 3.** Relationships between geographical and Nei (1972) genetic distances based

3 on 12 SSR markers of 21 *Larix decidua* individuals belonging to seven populations

4 (three individuals per population).

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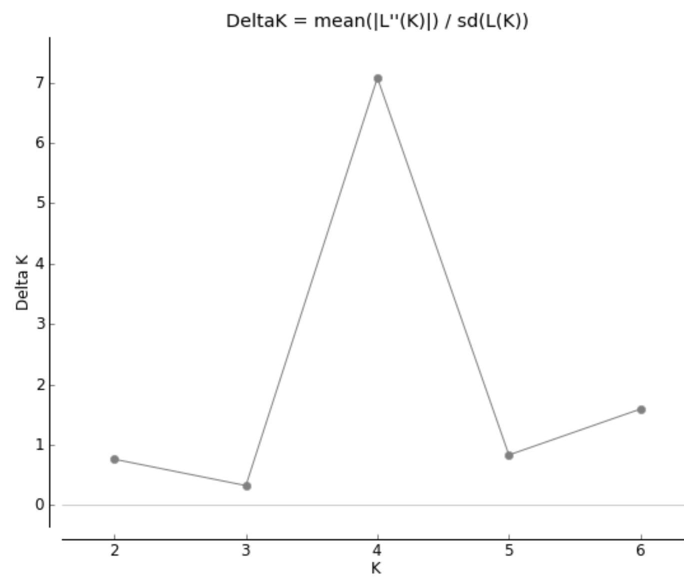

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2 **Figure 4**. Delta K values for 2 to 6 genetic clusters for 21 individuals of *Larix decidua*
3 collected from seven populations. Delta K was calculated according to Evanno et al.
4 (2005).

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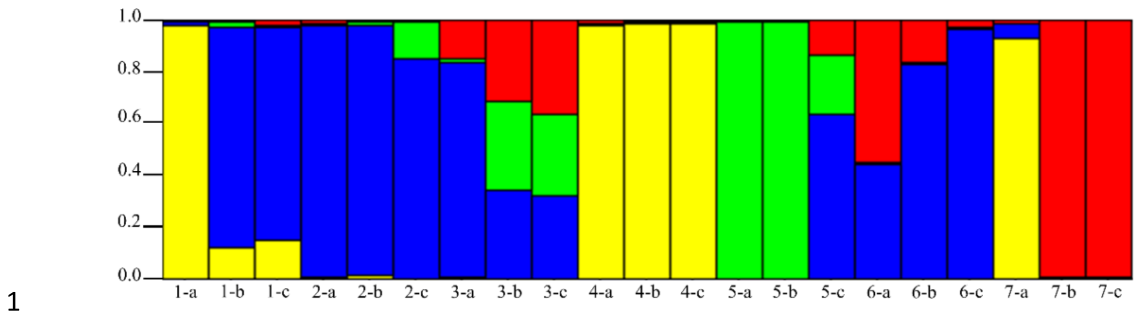

2 **Figure 5**. Estimated population structure for 21 plants of seven *Larix decidua*
3 populations based on a number of clusters (*K*) *K*=4. Each individual plant is represented
4 by a vertical bar. which is partitioned into coloured segments that provide an estimate of
5 the membership fraction in each of the 4 genetic clusters. Each bar is marked with the
6 population number (1-7) and individual (a-c).

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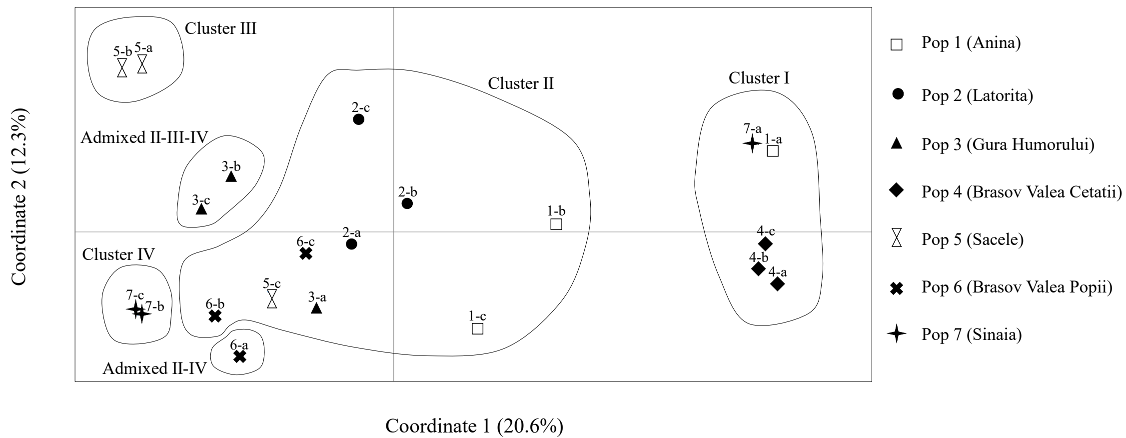

Figure 6. Principal coordinates analysis (PCoA) similarities based on 12 polymorphic SSR markers for *Larix decidua* individuals corresponding to seven populations based on the first (PCo1) and second (PCo2) principal coordinates. PCo1 and PCo2. and PCo3 account for 20.6% and 12.3% of the total variation, respectively. Each point is marked with the population number (1-7) and individual (a-c). The four genetic clusters (I to IV) identified with the population structure analysis as well as the admixed individuals (Figure D) are indicated.