

Short note: Development of a new set of SNP markers to measure genetic diversity and genetic differentiation of Mongolian oak (*Quercus mongolica* Fisch. ex Ledeb.) in the Far East of Russia

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Abstract

We developed a new set of 25 nuclear (nc), 12 chloroplast (cp) and 7 mitochondrial (mt) SNPs and used it to genotype 371 Mongolian oak (*Quercus mongolica* Fisch. ex Ledeb.) trees from seven locations in a 200 km by 400 km area in the Russian Far East. One of the locations in an area of 15 km by 25 km east of the city Ussuriysk was analyzed more intensively with 188 collected trees. The genetic differentiation at the nuclear SNPs was small to moderate and for the plastid SNPs it was high when considering all trees from the seven locations. The gene pool distances between locations were for 19 out of 21 pairs statistically highly significant. There was no correlation of genetic and spatial distances. Only three different multilocus-haplotypes could be identified and 42 two-loci-combinations of plastid SNPs could be used to identify them. Conclusions for the practical application such as timber tracking and gene conservation are discussed.

Keywords: : *Quercus mongolica*, genetic differentiation, genetic diversity, SNPs, nuclear, plastid, mitochondrial

Introduction

Mongolian oak (*Quercus mongolica* Fisch. ex Ledeb.) is an important timber species in the Far East of Russia, China, and other Asian countries. The species is partly overexploited and subject to illegal logging (Newell and Simeone, 2014). At present, the full scale of illegal harvest on the species is not known,

however, within Far East Russia it is causing rapid declines. Since 2004 the volume of *Quercus mongolica* harvested in the Russian Far East has exceeded the national quota for the species (Smirnov et al., 2013). In 2010, the amount of timber traded was double the official quota but prior to 2009 the volume of timber being harvested over the quota was even greater (Bastow, 2018). This has led to the species being placed on Appendix III of CITES. Additionally, the rapid decline requires *Q. mongolica* timber to be traded across boundaries with the correct permits and documentations. Furthermore, *Q. mongolica* is an important element of the tree species rich ecosystems in the Russian Far East. Measures to ensure sustainable forest management and to reduce illegal logging rely on effective, tamper-proof techniques to trace back the origin of timber (Lowe et al., 2016).

So far, mostly microsatellites have been applied to study the genetic differentiation of Mongolian oak populations and genetic differences to and hybridisation with other Asian white oak species in China and Japan (Lyu et al., 2018; Tamaki and Okada, 2014; Zeng et al., 2015). Because of their even distribution across the genome, higher number, and higher reproducibility during genotyping nuclear and plastid SNPs are increasingly used for population genetic analysis, tracking of origin and species identification for tree species (Blanc-Jolivet et al., 2018; Pakull et al., 2016; Schroeder et al., 2016a).

Here, we describe a new set of nuclear and plastid SNPs and its potential application to measure genetic diversity and genetic differentiation.

Materials and Methods

Species description

Quercus mongolica of the section *Quercus* (white oaks) is an important element of the temperate, mixed, deciduous hardwood forests in Russia, China, Japan, the Republic of Korea and the Democratic Republic of Korea. Oak forests in the Russian Far East cover an area of about 3.5 million hectares. They occupy various ecological conditions: southern insolated slopes, plains and valleys, mountains up to 1000 m altitude. It tolerates various soils, except wetlands, waterlogged and flooded. *Quercus mongolica* is a diploid (2n=24) monoecious, wind-pollinated, highly self-incompatible forest tree species. The species regenerates through both vegetative and seeds. Reproduction from seed can be successful after years with strong fructification (Yang and Yi, 2012). Usually the age structure includes a mixture of seedlings, saplings, mature and senescing trees (Suh and Lee, 1998). The abundance of the oak in a stand is highly variable, depending on the ecological conditions. *Quercus dentata* is the only other white oak tree species in the region. This species is rare. Thus, hybridization of *Quercus mongolica* with other species, as common for other tree species in the region like pines (Petrova et al., 2018), is not influencing the genetic composition of the oaks.

Sampling

We collected cambium of Mongolian oak trees at seven locations (L1 to L7) in the Far East of Russia (Figure 1). The sampled region is a 200 km by 400 km area with a focus area (L6) close to the city Ussurisk. At each place 30 to 32 individuals have been sampled and at the focus area 188 trees in a 15 km by 25 km area have been collected (Figure 1, Table 1).

Table 1
Name, ID given during sampling, geographic position and sample size (N) at the sampling locations in the Far East of Russia

Name	ID sampling	Latitude	Longitude	N
L1	QmLz	45.4623	133.5658	30
L2	WWF8	44.8402	131.7162	31
L3	WWF9	44.4746	132.9432	32
L4	QmCH	44.2656	132.4700	30
L5	QmUS	43.9784	132.0287	30
L6	Concession	43.8017	132.0660	188
L7	WWF2	43.4007	131.4456	30

SNP development

For the identification of SNPs in the chloroplast and mitochondrial genome paired-end sequencing using a Illumina MiSeq system (2x150 bp) has been applied using a pool of *Q. mongolica* containing 20 individuals. The individuals for the pool were chosen from 10 locations in the Far East of Russia with two individuals per location (Schroeder et al., 2018). The reads from the *Q. mongolica* pool were trimmed and then mapped against a nearly complete reference chloroplast DNA sequence of a *Q. mongolica* reference individual (Schroeder et al., 2016a). Read trimming, reference guided read mapping as well as the following polymorphism detection were performed using CLC

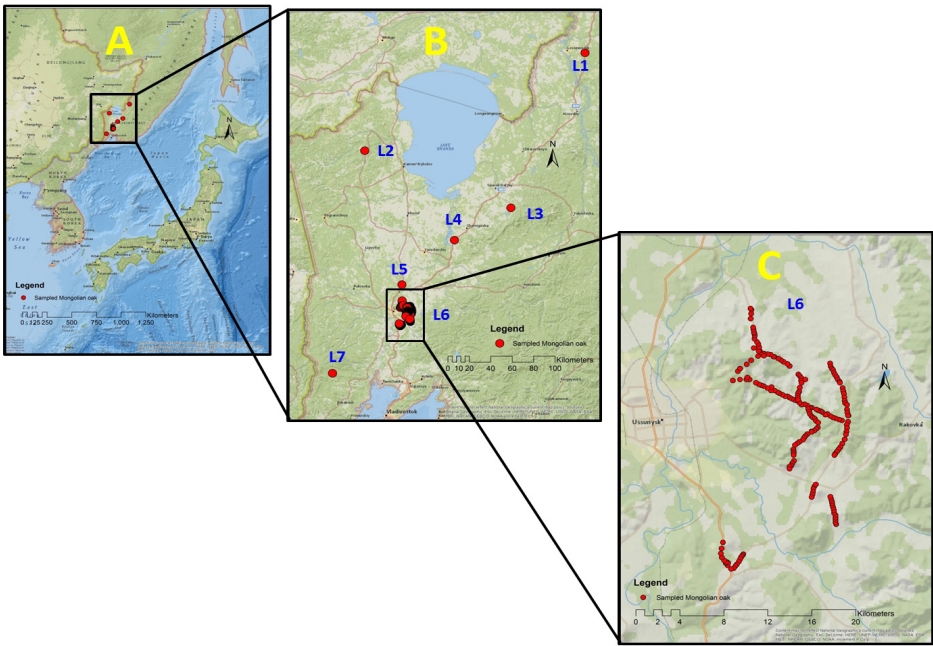


Figure 1
Sampling region (A) in the Far East of Russia, the geographic position of the sampling locations (B) and spatial distribution of the sampled trees in the focal location L6 (C)

Genomics Workbench version 7.5.1 (CLC-bio, a Qiagen company; Aarhus, Denmark). Variants (SNPs and indels) detected by CLC Genomics Workbench were exported to tab-delimited files and processed using an in-house script (*Variant Tools*) to identify intraspecific polymorphisms. Details of the methods are described in Schroeder et al. (2016a). For detection of the best differentiating SNPs between locations a prescreening using 120 chloroplast SNPs and 20 *Q. mongolica* individuals was accomplished. 27 SNPs were then selected by a quality check using the program GDA_NT (Degen, 2008) to determine the potentially most differentiating SNPs between locations. These 27 SNPs have been validated using 380 *Q. mongolica* individuals. From this validation 12 cp and 7 mt SNPs have been selected for analysis (Table 2, supplementary I).

The detection of SNPs in the nuclear genome was based on RAD sequencing (Baird et al., 2008) of two *Q. robur* individuals. The RAD sequencing has been performed using a restriction enzyme with an eight base pair recognition sequence. A set of 233 SNPs from a total of 1500 SNPs identified between the two individuals have been selected to find variations for population/location differentiation within *Q. mongolica*. For this purpose 90 individuals of *Q. mongolica* were chosen from 30 locations in the Russian Far East (Table 2) and genotyped at the selected SNP positions. From this prescreening, 25 nuclear SNPs were selected by a quality check using GDA_NT (Degen 2008). For the quality check the program estimates a) the completeness of the data, b) the amount of heterozygotes compared to a random combination of alleles, c) the genetic diversity and d) the genetic differentiation.

760 individuals from 40 *Q. mongolica* locations were then screened with the 44 selected SNPs (cp, mt, nuclear) for validation of the markers. For the final analysis 25 SNPs were selected from this validation step (Table 2, supplementary I).

SNP-genotyping

Genotyping of 371 samples was conducted by the MassARRAY technology using the iPLEX chemistry (Agena Biosciences, Hamburg) according to the manufacturer's protocol. The MassARRAY technology is conducted on a first amplification of a fragment of ~100 bp containing the SNP of interest in the middle. As a next step a mini-PCR with only one extension primer is conducted, consisting of a single-base extension and termination. Fragments are differentiated on a MALDI-TOF Mass Spectrometry Instrument (Agena Biosciences, Hamburg) and alleles can be identified because of mass variation among nucleotides.

Statistical analysis

As has been shown for other tree species, the success of genetic assignment depends on the level of genetic diversity, genetic differentiation among the groups and the similarity of the genotype frequencies to Hardy-Weinberg-Proportions (Chaves et al., 2018). As a measure for genetic diversity (effective number of alleles) we computed the mean allelic diversity v of group r (Gregorius, 1987). To measure the genetic difference

between groups, we calculated the gene pool distance d_0 between two groups (Gregorius, 1984). Based on the gene pool distance, the complementary compositional differentiation δ_{SD} among the gene pools was used as a measure of differentiation (Gregorius, 1987). For comparison, we also calculated the commonly used Wright's F_{ST} (Wright, 1978) which is a measure of fixation (monomorphism) and not of the genetic difference among groups (Gregorius et al., 2007).

As an indicator for the departure from Hardy-Weinberg proportions we computed the inbreeding coefficient F_{IS} (Wright, 1950). To visualize the genetic differences between locations, cluster analysis based on the pairwise gene pool distances d_0 between locations was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as implemented in the software PAST (Hammer et al., 2001).

Results and Discussion

Diversity and genetic differentiation at the SNPs

As for many other species the chloroplast and mitochondrial SNPs had generally clearly lower within population diversity v and higher genetic differentiation δ and population fixation F_{ST} than the nuclear SNPs (Table 2). For the plastid SNPs, the diversity was in most cases close to one. Thus the locations were nearly all fixed to one haplotype. The differentiation δ varied for the nuclear SNPs between 0.011 and 0.171 and the haplotype differentiation of cp- and mt-DNA had values of 0.476 and 0.548, respectively. The population fixation F_{ST} was for the plastid SNPs nearly complete with again only two different values of 0.920 and 1.000. A value of 1 means that at least one location was fixed at that gene marker compared to another haplotype. In contrast nuclear SNPs were far away from population fixation with F_{ST} values between 0.001 and 0.186.

Eight nuclear SNPs (e.g. RAD_6513_111) had high F_{IS} values ($F_{IS} > 0.45$), whereas nine nuclear SNPs had F_{IS} values between -0.1 and +0.1 (Table 2).

There was no spatial pattern for the distribution of mean diversity of the locations (Figure 2a). The highest genetic diversity was found in location L5 and not in location L6, as expected by large sample size in L6.

Haplotypes

The 19 mt and cp-SNPs combine to only 3 different haplotypes (Figure 2b) numbered as haplotype HT2, HT3 and HT4 (Schroeder et al., 2016b; Schroeder et al., 2018). There was a high level of redundancy because of the strong linkage disequilibrium observed between cpDNA and mtDNA (Table 3). The three haplotypes can be identified by 42 combinations of two SNPs (e.g. cp_QM_4317 & cp_QM_5832) (Table 3). Those loci that can be combined are also those with the two different values for the differentiation δ in Table 2. The spatial distribution fits

to the pattern found by Schroeder et al. (2016b; 2018): The haplotypes HT3 and HT4 are more common in China and the central part of the Russian Far East and HT2 is rare in China but typical for the Northern distribution range of the species and a bit scarcer in the Southern part.

Table 2

Type of genome, locus name, mean inbreeding coefficient F_{IS} , mean allelic diversity v over all seven locations and genetic differentiation δ and population fixation F_{ST} for all 44 SNP loci

Type of Genome	Locus	F_{IS}	v	δ	F_{ST}
Nucleus	RAD_159_295	0.097	1.629	0.067	0.028
Nucleus	RAD_361_430	0.472	1.432	0.061	0.032
Nucleus	RAD_858_482	-0.040	1.804	0.171	0.099
Nucleus	RAD_869_363	0.207	1.392	0.069	0.044
Nucleus	RAD_1468_344	-0.091	1.078	0.026	0.021
Nucleus	RAD_1744_292	0.196	1.467	0.039	0.011
Nucleus	RAD_1792_305	0.175	1.361	0.097	0.083
Nucleus	RAD_1803_395	-0.119	1.173	0.044	0.033
Nucleus	RAD_2056_363	1.000	1.087	0.022	0.015
Nucleus	RAD_3360_135	0.064	1.381	0.098	0.106
Nucleus	RAD_3759_228	0.059	1.130	0.027	0.012
Nucleus	RAD_3995_238	-0.022	1.250	0.136	0.186
Nucleus	RAD_4226_222	-0.065	1.672	0.076	0.023
Nucleus	RAD_4279_533	0.259	1.100	0.025	0.018
Nucleus	RAD_5156_367	0.204	1.446	0.033	0.008
Nucleus	RAD_5747_495	0.540	1.863	0.067	0.024
Nucleus	RAD_5885_366	-0.050	1.402	0.068	0.032
Nucleus	RAD_6137_303	0.673	1.128	0.024	0.011
Nucleus	RAD_6458_424	-0.054	1.227	0.011	0.001
Nucleus	RAD_6487_308	0.688	1.853	0.140	0.087
Nucleus	RAD_6513_111	0.782	1.081	0.028	0.019
Nucleus	RAD_6731_338	0.195	1.128	0.031	0.017
Nucleus	RAD_6850_331	0.547	1.358	0.045	0.013
Nucleus	RAD_7034_164	0.450	1.162	0.057	0.064
Nucleus	RAD_8473_483	-0.009	1.865	0.115	0.055
Chloroplast	cp_Qm_4317		1.000	0.476	1.000
Chloroplast	cp_Qm_5832		1.055	0.548	0.920
Chloroplast	cp_Qm_20138		1.055	0.548	0.920
Chloroplast	cp_Qm_32153		1.055	0.548	0.920
Chloroplast	cp_Qm_32884		1.055	0.548	0.920
Chloroplast	cp_Qm_51353		1.000	0.476	1.000
Chloroplast	cp_Qm_62975		1.000	0.476	1.000
Chloroplast	cp_Qm_69074		1.000	0.476	1.000
Chloroplast	cp_Qm_72548		1.055	0.548	0.920
Chloroplast	cp_Qm_76468		1.000	0.476	1.000
Chloroplast	cp_Qm_123128		1.000	0.476	1.000
Chloroplast	cp_Qm_130306		1.055	0.548	0.920
Mitochondrion	mt_Qm_19049		1.000	0.476	1.000
Mitochondrion	mt_Qm_27289		1.000	0.476	1.000
Mitochondrion	mt_Qm_35092		1.000	0.476	1.000
Mitochondrion	mt_Qm_43122		1.000	0.476	1.000
Mitochondrion	mt_Qm_60606		1.055	0.548	0.920
Mitochondrion	mt_Qm_66263		1.000	0.476	1.000
Mitochondrion	mt_Qm_77774		1.000	0.476	1.000

Table 3

SNP composition of the three haplotypes (HT2, HT3, HT4) at the 12 chloroplast and 7 mitochondrial SNPs. Twelve loci (dark grey) distinguish between HT3 and the group of HT2 and HT4, and seven loci (light grey) distinguish between HT2 and the group of HT3 and HT4.

Type of Genome	Locus	HT2	HT3	HT4
Chloroplast	cp_Qm_4317	A	C	A
Chloroplast	cp_Qm_5832	G	T	T
Chloroplast	cp_Qm_20138	G	C	C
Chloroplast	cp_Qm_32153	G	A	A
Chloroplast	cp_Qm_32884	C	T	T
Chloroplast	cp_Qm_51353	T	C	T
Chloroplast	cp_Qm_62975	A	C	A
Chloroplast	cp_Qm_69074	T	C	T
Chloroplast	cp_Qm_72548	G	A	A
Chloroplast	cp_Qm_76468	T	G	T
Chloroplast	cp_Qm_123128	T	C	T
Chloroplast	cp_Qm_130306	G	T	T
Mitochondrion	mt_Qm_19049	A	G	A
Mitochondrion	mt_Qm_27289	C	A	C
Mitochondrion	mt_Qm_35092	A	G	A
Mitochondrion	mt_Qm_43122	G	T	G
Mitochondrion	mt_Qm_60606	C	A	A
Mitochondrion	mt_Qm_66263	T	A	T
Mitochondrion	mt_Qm_77774	A	G	A

Genetic distances among locations

We used only the 25 nuclear SNPs, and the two chloroplast SNPs cp_QM_4317 & cp_QM_5832, for the further analysis of the genetic differentiation. These two cpSNPs were one of the 42 possible combinations sufficient to differentiate among the three haplotypes. The gene pool distances varied with the minimum of $d = 0.032$ (L1 / L6) and the maximum of $d = 0.197$ (L2 / L3) largely among the different locations (Table 4). All gene pool distances with exception of the distances among L1 and L5 and L6 were statistically highly significant with 100 % of the distances in the permutations smaller than the observed values. The UPGMA cluster-analysis based on the gene pool distances (Figure 3) visualises the pattern for all locations. L1 and L6 were grouped closely together followed by L5 and L3.

Locations L4, L2 and L7 built another cluster. The location L7 had the largest difference to all other locations. There was no correlation of gene pool distances and geographic distances ($r = -0.10$).

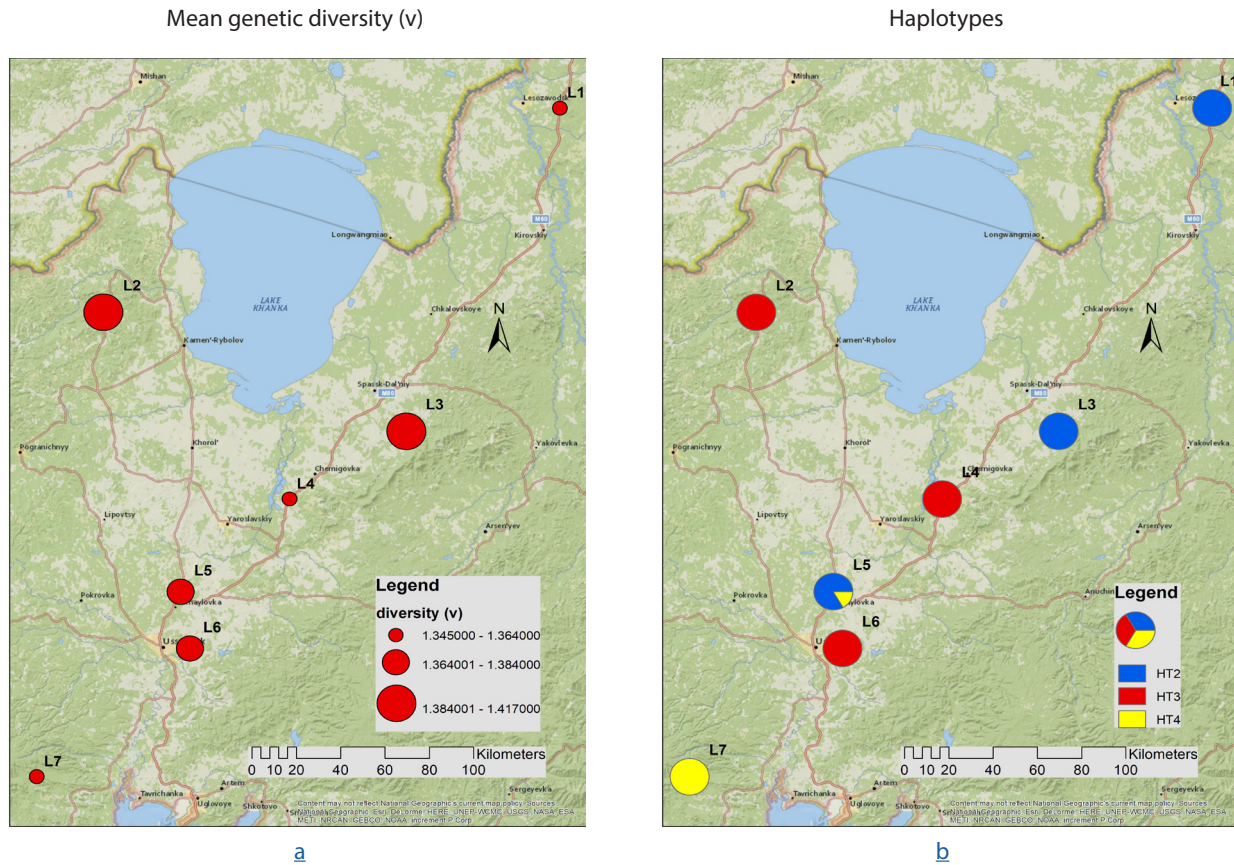


Figure 2

a) Spatial distribution of mean genetic diversity (v) at nuclear SNPs, b) Spatial distribution of the three cp-mt haplotypes (HT2, HT3, HT4) in the seven locations

Table 4

Gene pool distance among the 7 locations using the 25 nuclear SNPs and the two chloroplast SNPs cp_QM_4317 & cp_QM_5832 (above diagonal) and proportion of simulated gene pool distances < than the observed values in 1000 permutation tests (below diagonal).

	L1	L2	L3	L4	L5	L6	L7
L1		0.162	0.083	0.112	0.057	0.032	0.108
L2	1.000		0.197	0.104	0.161	0.162	0.118
L3	1.000	1.000		0.159	0.089	0.076	0.154
L4	1.000	1.000	1.000		0.126	0.109	0.108
L5	0.751	1.000	1.000	1.000		0.063	0.112
L6	0.116	1.000	1.000	1.000	1.000		0.103
L7	1.000	1.000	1.000	1.000	1.000	1.000	

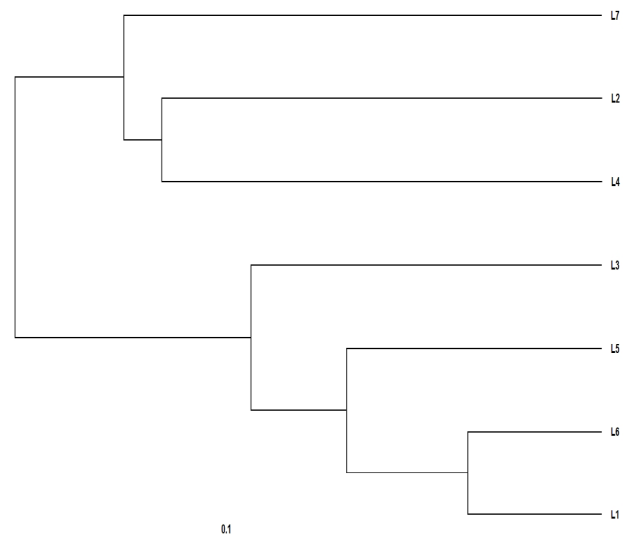


Figure 3

Dendrogram for the cluster analysis (UPGMA) based on gene pool distances among the 7 locations and using the 25 nSNPs + 2 cpSNPs.

Differences in genetic differentiation between nuclear and plastid gene loci

We found a higher genetic differentiation among locations for the plastid loci compared to the nuclear loci. This is explained by the maternal inheritance mode of the chloroplast and mitochondrion. This part of the genome gets only distributed via the seeds. And the seed dispersal is for oaks substantially more limited than for pollen (Buschbom et al., 2011; Chybicki and Burczyk, 2010).

Excess of homozygotes at some loci

The observed excess of homozygotes could be explained by the presence of null alleles. Null alleles could be the result of genetic variation in the primer region of the SNPs. Only a low number of individuals were included in the initial SNP identification using the RAD sequencing approach. The MassArray approach for genotyping includes PCR-reactions. Hence SNPs in the flanking regions that were selected as primers could remain undetected.

High level of redundancy at chloroplast and mitochondrial loci

Also this observation is common since the chloroplast and mitochondrial genomes are haploid, considerable well conserved, non-recombinant, and they have a lower effective population size than the nuclear genome. There was high linkage disequilibrium between mt- and cp-DNA markers. These factors increase the rate of fixation of haplotypes within populations and species (Pham et al., 2017 and citations therein).

Practical application

The SNPs and the presented data could be used as tools for law reinforcements to assign the location of origin for traded timber and forest reproductive material (Lowe et al., 2016). But we did some tests on the success rate of self-assignment (supplementary II) and found that the presented set of SNPs would not be sufficient for an accurate assignment of the material back to the location of origin. A clearly higher number of SNPs with a sufficient genetic differentiation would be needed for this purpose (Ogden and Linacre, 2015). Another field of application is the selection of priority areas of gene conservation (Carabeo et al., 2017). Good candidates as gene conservation units are those stands like our location L5 (Figure 2a) with a high level of genetic diversity.

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