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Nucleotide polymorphisms associated with climate and physiological traits in silver fir (*Abies alba* Mill.) provenances

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ABSTRACT

Single-nucleotide polymorphisms in seven candidate genes for drought and heat tolerance identified in earlier studies were studied in 13 populations of *Abies alba* Mill. covering the eastern and southeastern half of the distribution range. Trees were planted in a provenance trial plot of the international experiment IUFRO 2005 in Hertník, Slovakia. In parallel, genotyped trees were also scored for physiological traits (PSII thermotolerance, chlorophyll *a* fluorescence kinetics, partly gas exchange, assimilatory pigments, needle osmotic potential). For the detection of natural selection imprints, we used different methods: analysis of F_{ST}-outliers, testing of SNP–climate associations and SNP–phenotype associations. F_{ST}-outlier analysis revealed 2 SNPs with signals of selection. Moreover, three other SNPs showed significant association with latitude or bioclimatic variables; one of them (C/T polymorphism in a gene coding for serine/threonine phosphatase) was significantly associated with needle osmotic potential and marginally with other drought-related physiological traits. That may indicate its role in drought-stress response. In addition, T/C polymorphism in a gene coding for reduced epidermal fluorescence 4 was found to be involved in heat-stress response. However, migration history seems to be a more important driver of differentiation at SNPs in the studied candidate genes than recent adaptation.

1. Introduction

Under the ongoing climate change, adaptation has become a central issue in forestry, especially in relation to collection and transfer of forest reproductive materials. Global climate change leading to an increased incidence of extreme drought events in most of Europe is expected to be one of the greatest challenges facing forestry and nature conservation even in a short-term perspective. This is especially true in mountain forests of Central Europe, which are generally formed of conifers, mainly Norway spruce (Christensen et al., 2007). In addition to being a natural dominant species of subalpine forests in most mountains ranges, spruce as an important commercial species was extensively planted also in lower vegetation belts since the beginning of the 19th century or even earlier, and gradually replaced a substantial part of natural mixed fir-beech forests (Spiecker et al., 2004). Currently, the future of these stands is at stake.

Silver fir (*Abies alba* Mill.) has recently been increasingly considered as a suitable replacement for spruce (Rothe et al., 2011; Vitali et al., 2017; Bošeľa et al., 2018), except on the southern range margin and at low elevations. It is more resistant and resilient to extreme summer droughts and has a positive effect on biodiversity (Castagneri et al., 2014; Vitali et al., 2017). Moreover, as natural old-growth forests with silver fir belong to the most productive forest ecosystems in Europe (Leibundgut, 1982; Korpeľ, 1995; Holeksa et al., 2009), preference of

Abbreviations: A, CO_2 assimilation rate; Car, content of carotenoids (carotenes + xanthophylls); Chla, content of chlorophyll *a*; Chla + b, total content of chlorophyll; Chlb, content of chlorophyll *b*; E, transpiration rate of needles; EndPoint_RLC$_{NPQ}$, non-photochemical quenching at final actinic light intensity of 2969 μmol. $m^{-2}.s^{-1}$; ETR, maximum electron transport rate; FDR, false discovery rate; F_v/F_m, maximum fluorescence quantum yield; F_{ST}, frequency-based fixation index; g_S, stomatal conductance; IBD, isolation by distance; PI, performance index of PSII; R, index of the severity of thermal stress; RC/ABS, density of active photosystem II reaction centers; SAM, spatial analysis method; Slope_RLC$_{YIELD}$, initial slope of the PSII effective quantum yield curve; T_{15}, temperature threshold of PSII resistance; T_c, critical temperature for PSII stability; T_{Wk}, critical temperature at which the variable fluorescence at the K-step abruptly increases; Ψ_S, needle osmotic potential

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Table 1
List of provenances used in the SNP-assay and in the assessment of physiological traits.

Code	Provenance	Country	Long (°)	Lat (°)	Ele (m)	T_{MEAN} (°C)	T_{59} (°C)	P_{YEAR} (mm)	P_{59} (mm)	SNP	Ph1	Ph2
AT	Koetschach	AT	13.18	47.10	1300	0.0	6.2	1368	700	x	x	x
CZ	České Švýcarsko	CZ	14.37	50.88	270	7.3	14.4	605	329	x	x	
PL01	Dobkowa	PL	18.87	49.70	570	5.9	13.3	1000	581	x	x	
PL03	Bukowa-2	PL	18.97	49.72	550	6.8	14.4	932	547	x	x	
PL17	Bieśnik	PL	20.63	49.75	420	8.5	16.6	697	429	x	x	
PL21	Berest-1	PL	20.95	49.55	690	6.1	13.8	839	486	x	x	x
PL41	Kadlubiska	PL	22.17	50.30	250	7.5	15.8	597	357	x	x	x
SK01	Staré Hory	SK	19.75	48.75	500	6.0	13.5	829	471	x	x	x
SK02	Bardejov	SK	21.25	49.42	900	6.1	13.8	817	465	x	x	x
TC01	Nidže *	MK	21.73	41.00	1500	7.1	14.1	681	246	x	x	
TC02	Slavjanka *	BG	23.52	41.33	1450	11.9	19.5	523	192	x	x	
TC05	Zarnasti *	RO	25.68	45.69	920	8.0	16.2	623	383	x	x	
TC06	Valiug *	RO	22.46	45.20	900	5.2	12.2	983	552	x	x	

Long longitude (°), *Lat* latitude (°), *Ele* elevation (m a.s.l.), T_{MEAN} mean annual temperature (°C), T_{59} mean temperature during the vegetation season (°C), P_{YEAR} annual precipitation (mm), P_{59} precipitation of vegetation season (mm), * provenances originating from the Balkan refugium, SNP populations used in the SNP assay, Ph1 populations analyzed for PSII performance and thermostability, Ph2 populations analyzed for the remaining physiological traits.

silver fir is justified also from the point of view of commercial forestry.

Genetic structures of silver fir were largely shaped by the Holocene history. Most of the current distribution range was colonized from two refugial areas, located in the southern part of the Balkan Peninsula and in the Apennines, with relatively sharp boundaries between maternal lineages (Liepelt et al., 2009; Gömöry et al., 2012). History of populations is still recognizable in their adaptive properties. The decline of silver fir in the second half of the past century was substantially less pronounced in populations originating from the Balkan refugium compared to the Apennine one (Larsen, 1986). A detailed analysis of diameter-increment patterns across the Carpathian arc also revealed differences between the Holocene lineages in growth and climatic responses (Bošeľa et al., 2016). Such findings underline the necessity of taking the historical context into consideration in the search for signals of adaptation.

Although silver fir is often regenerated naturally in managed stands (Dobrowolska et al., 2017), the climate-change-associated shift into higher elevations will unavoidably require artificial planting. Currently, the choice of the appropriate reproductive materials mostly relies on field experiments. However, provenance recommendations are frequently based on performance at juvenile age, and not necessarily confirmed by later practical experience. Moreover, growth or survival are summary indicators of fitness; the detailed causes of poor performance or high mortality are not always evident from field observations alone. A responsible choice of reproductive materials appropriate for future climates needs a more detailed knowledge of the adaptive variation both on the physiological and genetic levels.

In spite of recent progress of forest tree genomics (Neale and Ingvarsson, 2008; Plomion et al., 2016), there is limited information on geographic patterns of adaptive genetic variation at the molecular level. In genomic studies of forest trees, the candidate-gene approach has been preferred because of a rapid decay of linkage disequilibrium in tree populations making association studies difficult (Neale and Kremer, 2011). Among the targeted genes, those underlying phenology, tolerance of drought and high temperatures predominate; i.e. genes associated with climatic adaptation.

Single-nucleotide-polymorphism associations with climate or climate-related phenotypic traits were mostly tested on a local or regional scale (transects or climatically contrasted sites) in *Abies* or other Pinaceae (Roschanski et al., 2016; Mosca et al., 2016; Goto et al., 2017), while studies on a broader or even range-wide scale are rare (Wachowiak et al., 2009; Eckert et al., 2010; Mosca et al., 2014; Scalfi et al., 2014). Even less frequent are studies on the genetic background of physiological traits (Romšáková et al., 2012), probably because of logistic problems: traits like parameters of gas exchange, water regime or photosynthesis require specific equipment and should be scored under controlled conditions (climatic chambers, phytotrons) or at least in common gardens to avoid environment-induced phenotypic variation.

Our study focuses on signs of local adaptation within a set of provenances represented in the Slovak trial plot of an international provenance experiment. Our objectives were i) testing which particular single-nucleotide polymorphisms in candidate genes, identified in earlier studies responsive to heat and drought stress, are affected by selection, ii) identification of their relationships with climatic variables, and iii) identification of their potential associations with physiological traits.

2. Materials and methods

2.1. Experimental material

The study was based on the Slovak trial plot of the 2nd international provenance experiment with silver fir IUFRO 2005 established in 2005 with 5-years-old plants (Tabel, 2000), planted close to the village Hertník in eastern Slovakia (49.217 °N, 21.271 °E; 390 m a.s.l.). The trial comprises 17 provenances covering the eastern half of the distribution range of silver fir, and was established under a randomized complete block design with three blocks, each plot containing initially 35 plants planted at 2 m × 2 m spacing. Out of them, 13 provenances were chosen for the study (Table 1) with 10 trees per provenance being sampled. Population codes follow the labelling in the common database of the provenance experiment. The collection of material and measurements of physiological parameters were done during the vegetation seasons 2015 and 2016.

Climatic variables of the sites of the provenance origin were taken from the WorldClim high-resolution interpolated climate database. In addition to basic climatic descriptors (mean annual temperature, annual precipitations), the bioclimatic variables derived from meteorological data within the period 1960–1990 at the 30” resolution were considered. For details, see Hijmans et al. (2005).

2.2. Physiological traits

The choice of the assessed parameters was oriented on physiological processes and phenomena related to drought and heat stress. As some measurements are time-consuming and labour-intensive, they were performed only on a subset of provenances (Table 1). Details of assessment procedures and biological relevance of the scored traits can be found in Appendix A in the Supplementary materials, Konôpková

(2017), and Konôpková et al. (2018). The following parameters were measured:

Fast kinetics of chlorophyll *a* fluorescence measured under non-stressing conditions (13 provenances): F_v/F_m – the maximum fluorescence quantum yield; *PI* – photosynthetic performance index; and *RC/ABS* – density of active photosystem II reaction centers.

Thermal stability of photosystem II (13 provenances): *R* – the index of the severity of thermal stress expressed as the ratio of F_v/F_m measured at 51 °C and a control measurement at 30 °C; T_{15} – the temperature threshold of PSII resistance expressed as temperature at which F_v/F_mdeclines 15% from the maximum value (Froux et al., 2004); T_c – critical temperature for PSII stability expressed as temperature at which the basal fluorescence F_0 abruptly increases (Bigras, 2000; Froux et al., 2004); and T_{Wk} – critical temperature at which the variable fluorescence at the K-step abruptly increases.

Assimilatory pigments concentration in dry mass (5 provenances): *Chla* – the concentration of chlorophyll *a*; *Chlb* – the concentration of chlorophyll *b*; *Chla* + *b* – the concentration of chlorophyll *a* + chlorophyll *b*; *Car* – the concentration of carotenoids (carotenes + xanthophylls) (all $mg.g^{-1}$).

Free proline concentration (*proline* (μmol. g^{-1}); 5 provenances).

Needle osmotic potential (Ψ_S (MPa); 5 provenances).

Gas exchange (5 provenances): *A* – CO_2 assimilation rate (μmol CO_2. $m^{-2}.s^{-1}$); g_S – stomatal conductance (mol H_2O. $m^{-2}.s^{-1}$); and *E* – needle transpiration rate (mmol H_2O. $m^{-2}.s^{-1}$).

Rapid light curves of chlorophyll *a* fluorescence under field conditions (5 provenances, 12 measurements per provenance): $Slope\_RLC_{YIELD}$ – initial slope of the PSII effective quantum yield curve (°); $EndPoint\_RLC_{NPQ}$ – non-photochemical quenching at final actinic light intensity (photosynthetic photon flux density) of 2969 μmol. $m^{-2}.s^{-1}$; and *ETRmax* – maximum electron transport rate (μmol e-. $m^{-2}.s^{-1}$).

The thermal stability of PSII was studied after the simulated heat stress in June 2016 in the laboratory. The other parameters were measured after the natural moderate heat and drought period in August 2015 in field conditions during a cloudy day.

2.3. DNA extraction and sequencing

As a rule, twigs with one-year-old needles were collected from ten trees per population in March 2016. Genotyping for SNPs were conducted on the same set of trees as for physiological measurements. Total genomic DNA was extracted from the silica-dried leaves using a modified CTAB protocol following Doyle and Doyle (1987).

Polymorphisms in seven candidate genes for drought and heat tolerance, taken from the study of Mosca et al. (2012a), were studied, as they are expected to respond to climatic contrasts (Appendix B.1 in the Supplementary materials). Primer sequences (Appendix B.2 in the Supplementary materials) and thermal cycling profile for PCR followed Mosca et al. (2012a). The PCR mixtures for all markers were done in a volume of 15 μl consisting of 1 × PCR buffer, 3 mM $MgCl_2$, 0.2 μM of primer, 0.3 μM dNTP, 0.04 U *Taq* DNA polymerase (GeneCraft), 0.8 μg/μl of BSA, and 25 ng of template DNA. The PCR products were checked on 1.5% agarose gel and afterwards they were sent to IGA Technology services (Udine, Italy) for sequencing. For all primer pairs, both DNA strands were sequenced. Obtained raw data were evaluated using SeqScape v.2.5.

2.4. Data analysis

Sites exhibiting single nucleotide polymorphisms (SNPs) were extracted from complete sequences, and for all later evaluations, each polymorphic site was treated as separate locus. As we worked with diploid biological materials, phase (i.e., combination of alleles present on each of the two chromosomes) was reconstructed using the Bayesian MCMC algorithm of Stephens and Donelly, (2003) using the program DnaSP v5 (Librado and Rozas, 2009).

The annotation of candidate genes with notable results was checked by nucleotide blast (blastn) against the nucleotide collection at NCBI (including the GenBank database) and translated blast (Blastx) against the UniProtKB/Swiss-Prot database (including CDD: Conserved Domain database). Only plant sequences were searched for. In addition, the ConGenIE database (congenie.org) was searched using translated blast.To identify a potential population substructure, the Bayesian clustering algorithm was applied as implemented in the program STRUCTURE v. 2.3.4 (Pritchard et al., 2000). Individuals were assigned to *K* clusters employing the admixture model with correlated allele frequencies. *K* values ranging from 1 to 8 were tested. A burn-in period of 500,000 iterations was followed by 1,000,000 iterations for estimation of the membership coefficients. Ten independent runs were performed for each *K*. The choice of the true number of clusters *K* relied on the method of Evanno et al. (2005) as implemented in STRUCTUREHARVESTER (Earl and vonHoldt, 2012) and the run with the highest posterior probability for the selected *K* was chosen (Appendix C.1 and C.2 in the Supplementary materials).

Isolation by distance (IBD) was tested using the regression method of Rousset (1997). Geographical WGS84 coordinates were converted to orthogonal coordinates using Lambert conformal conic projection (http://twcc.free.fr/). IBD calculation was done under a two-dimensional stepping-stone model: estimates of F_{ST} / (1–F_{ST}) were regressed against logarithm of distance. The significance of the regression slope was tested by a Mantel test with 99,999 permutations (program Genepop; Rousset, 2008). Exact tests of allelic differentiation among populations were also performed in Genepop using 10,000 dememorization steps followed by 1000 batches of 5000 iterations each, and the resulting probabilities were corrected using sequential Bonferroni correction.

Signals of selection were identified by several approaches. First, frequency-based detection of outlier SNPs was done using Bayescan v.2.1 (Foll and Gaggiotti, 2008). F_{ST} outliers were identified in 20 pilot runs of 5000 iterations each and a burn-in of 50,000 iterations followed by 50,000 iterations for the estimation of the posterior distributions with a thinning interval of 10. Prior odds for the neutral model were set to 10 (default). The evidence of selection was based on Bayes factors, measuring odds for selection model versus neutral model derived from posterior probabilities of each of the models. False discovery rate (FDR; the expected proportion of false positives among outlier markers) was controlled through the *q*-value, quantifying the minimum FDR at which this SNP may become significant (*q*-value is defined in the context of multiple testing across all polymorphic sites).

Second, geographical coordinates (longitude, latitude, elevation) and climatic variables (basic climatic descriptors and all bioclimatic variables provided by WorldClim) were tested for correlations with SNP allele frequencies employing multiple univariate logistic regression models with the spatial analysis method (SAM), as implemented in the Samβada v0.5.1 software (Joost et al., 2007). The significance of correlations was assessed using the likelihood-ratio test and the Wald test implemented in SAM, applying an initial 95% confidence interval. As the SAM requires input of presence / absence data, genotypes were coded as suggested by Joost et al. (2007) (the effect of a particular SNP allele controlling putative environment-related traits is expected to be dominant). Sequential Bonferroni correction for multiple testing was applied; correlations that remained significant after correction for both test statistics were retained. In addition, associations identified by SAM as significant were tested by univariate linear regressions between population allelic frequencies and environmental variables (Narum et al., 2010).

Associations between SNPs and the phenotypic traits were tested using TASSEL v. 5.0 (Bradbury et al., 2007) under the general linear model (GLM):

$$y = Xb + e,$$

where **y** is the vector of phenotypic data, **X** is the design matrix, **b** is a vector of fixed effects (SNP and the population structure) and **e** is the vector of random residues. The population structure (Q matrix) was deduced by the principal component analysis using the covariation method implemented in TASSEL; missing data were estimated using unweighted Manhattan distance (Price et al., 2006). Sequential Bonferroni correction was applied to the resulting significance values.

3. Results

In the seven studied genes, 31 SNPs were identified out of the total sequence length of 3085 bp. Subsequently, polymorphic sites with the frequency of the rare allele lower than 2% were excluded, what resulted in 17 SNPs, i.e. in average 2.43 SNPs per studied gene. Most SNPs were found in CL4354 and CL866 (4 SNPs each), least in the genes 8852 and 15036 (1 SNP each).

The annotation of candidate genes check mostly confirmed the annotation given by Mosca et al. (2012a) (Appendix B.3 in the Supplementary Materials). In all searches at GenBank, the best hits were unspecified putative protein coding sequences from related conifer species, mostly from genus *Pinus*, but also *Abies* and *Larix*. The annotations for the loci CL4354 (serine/threonine-protein phosphatase), CL866 (dihydrolipoamid-S-acetyltransferase), 15036 (RING-type zinc-finger protein), 8852 (galactokinase 1) and CL1148 (dihydrolipoamide S-acetyltransferase) were confirmed by our search. Locus 2937 as a gene coding ATP:ADP antiporter protein was also confirmed, but only by GenBank and ConGenie. UniProt and CDD found no hits. The gene 1528 turned out relatively low amount of hits in the databases. UniProt found only 3 hits, but confirmed its putative coding of protein reduced epidermal fluorescence 4, with alternative protein name being "mediator of RNA polymerase II transcription subunit". No conserved domains were identified. Result of the ConGenIE search was also rather short, two out of three hits pointing to GO terms for "epidermal cell differentiation" and "cellular component organization".

Isolation by distance proved to be significant and quite strong (Spearman's rank correlation coefficient between F_{ST} / (1–F_{ST}) and log (distance) was 0.643, P = 0.0014; regression slope was 0.126 with 95% CI 0.053–0.172). However, when only populations from the Apennine refugium (AT, CZ, PL, SK) are considered, IBD is significantly weaker (Spearman's rank correlation coefficient of 0.138, P = 0.0201; slope of 0.0032). Differentiation tests show identical pattern: if all provenances are considered jointly, differences of allelic frequencies across SNPs are significant at 11 out of 17 sites. However, a look on pairwise differences between populations reveals that this outcome relies on differentiation between refugia: none of the pairwise differences within the Central European group and within the Balkan group (TC01 to TC09) was significant, while all pairwise differences between populations belonging to different groups were significant (Appendix D.1 and D.2 in the Supplementary materials).

In the analysis of the SNP dataset under the STRUCTURE procedure, the ΔK measure (Evanno et al., 2005) rendered K = 2 as the most probable number of groups (Appendix C.1 and C.2 in Supplementary material). The distribution of cluster proportions corresponds well with the origin of populations from different Pleistocene refugia (Fig. 1). Significant correlations of cluster frequencies with geographical and climatic variables (Table 2) are thus likely statistical artefacts resulting from the southeastern position and generally higher elevation of provenances originating from the Balkan refugium.

Bayescan identified two polymorphic sites in two different genes showing a significant deviation from the null model and thus a signal of selection. In the site 2937_121, the logarithm of posterior odds for selection (in comparison to the neutral model) was 1.525, which according to Foll and Gaggiotti (2008) is a very strong evidence of selection, while a low q-value of 0.029 (minimum false-discovery rate) suggests that this SNP is not a false positive. For the second site CL1148_28 the evidence is much weaker, $\log_{10}$(odds) was only 0.595,

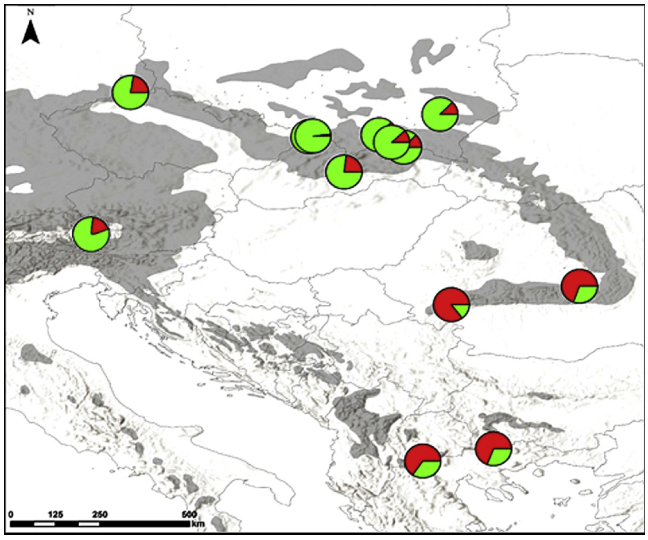

Fig. 1. Ratio of assignment to cluster 1 (red/dark colour) or to cluster 2 (green/bright colour) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Parameters of linear regression of cluster 1 frequency on geographic and climatic variables.

Variable	R^2	P	slope
Lat	0.677	0.001	−0.0708
Long	0.320	0.044	0.0472
Ele	0.333	0.039	0.0004
BIO3	0.314	0.046	0.7394
BIO9	0,307	0,049	0,0199

Lat latitude, *Long* longitude, *Ele* elevation, *BIO3* isothermality, *BIO9* mean temperature of driest quarter.

Table 3
SNPs identified as F_{ST}-outliers.

Gene	Site	SNP	F_{ST}	$\log_{10}$(PO)	Evidence of selection[1]	q value	P
2937	121	A/C	0.068	1.525	Very strong	0.029	0.971
CL1148	28	T/C	0.099	0.595	Substantial	0.116	0.798

[1] according to Foll and Gaggiotti (2008).

which is interpreted as 'substantial' evidence of selection, but the q-value of 0.116 is high (Table 3).

SAM revealed the SNPs at two genes as correlated with geographical coordinates and temperature variation. Most significant associations were found for a T/C polymorphism in the gene 1528, which is a fragment of a homologue of the reduced epidermal fluorescence 4 (*ref4*) gene of *Arabidopsis thaliana*, participating in the phenylpropanoid metabolic pathway. The other SNP showing association with latitude is a C/G polymorphism in the CL4354 gene coding for serine/threonine phosphatase (Table 4). Univariate linear regressions of SNP frequencies on the respective environmental variables also revealed generally close associations (Appendix E in Supplementary material).

Table 5 summarizes associations between SNPs and physiological traits at the individual level. Separate tests rendered a broad spectrum of physiological variables as associated with SNPs including PSII thermostability (T_c and T_{Wk}), non-photochemical quenching of chlorophyll *a* fluorescence (*EndPoint_ RLC*$_{NPQ}$) and proline concentration; however, after Bonferroni correction, only a C/T polymorphism at site of 167 in the CL4354 gene (serine/threonine phosphatase) remained significantly associated with needle osmotic potential.

Table 4
Summary of single-nucleotide polymorphisms exhibiting significant associations with environmental characteristics at the sites of origin.

Gene	Site	SNP	Variable	*G*-score	Bonf	Wald-score	Bonf	McFadden pseudo-R^2	β_0	β_1
1528	366	T/C	*Lat*	33.76***	***	24.76***	***	0.251	18.456	−0.411
1528	122	C/A	*Lat*	24.46***	***	20.32**	**	0.178	14.676	−0.331
CL4354	215	C/G	*Lat*	23.14***	**	19.42**	**	0.153	13.750	−0.307
1528	366	T/C	*BIO3*	22.37***	**	17.78**	*	0.166	−16.640	5.241
1528	366	T/C	*Long*	31.78***	***	17.10**	*	0.236	−12.879	0.562
1528	366	T/C	*Ele*	20.03***	**	16.94**	*	0.149	−2.976	0.002
1528	366	T/C	*BIO2*	20.54***	**	16.18**	*	0.153	−11.520	1.162

Lat latitude, *Long* longitude, *Ele* elevation, *BIO2* mean diurnal range of temperature, *BIO3* isothermality Statistical significance: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ β_0 and β_1: intercept and slope of the logistic regression model, respectively; Bonf: significance of the logistic regression model after Bonferroni correction.

Table 5
Summary of SNPs polymorphisms exhibiting significant associations with physiological traits.

Gene	Site	SNP	Variable	*F*	*P*	R^2	Bonf
1528	122	C/A	T_C	9.387	0.003**	0.078	–
1528	366	T/C	T_{Wk}	4.616	0.034*	0.040	–
2937	121	A/C	*EndPoint_ RLC*$_{NPQ}$	5.878	0.025*	0.213	–
2937	348	A/G	*EndPoint_ RLC*$_{NPQ}$	6.423	0.020*	0.228	–
15,036	396	T/A	T_C	4.346	0.040*	0.037	–
CL4354	119	C/T	*A*	10.816	0.004**	0.230	–
CL4354	167	C/T	Osmotic potential	10.285	0.002**	0.081	*
CL4354	167	C/T	Proline	5.978	0.025*	0.161	–
CL4354	274	C/T	*A*	10.782	0.004**	0.229	–
CL866	118	A/G	*EndPoint_ RLC*$_{NPQ}$	5.819	0.026*	0.211	–
CL866	290	G/A	Proline	9.020	0.008**	0.215	–
CL866	351	G/T	T_{Wk}	5.421	0.022*	0.042	–
CL866	468	C/T	*EndPoint_ RLC*$_{NPQ}$	8.517	0.009**	0.276	–

Statistical significance: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ Bonf: significance of the linear regression model after Bonferroni correction.

4. Discussion

Bayesian analysis of population structure revealed substantial genetic differentiation between central Europe and the Balkans, clearly associated with the origin from different glacial refugia. This is not an exception: a similar effect of population history on SNP variation in candidate genes was recorded in *Pinus monticola* and *Pinus strobus* (Nadeau, 2014) as well as in silver fir itself (Brousseau et al., 2016). However, this complicates the interpretation of outcomes, as it remains unclear whether the observed differentiation resulted from demography or adaptation in response to various macroenvironmental pressures.

Bayescan identified only one F_{ST}-outlier in the 2937 gene showing a clear signal of selection. The orthologue of this gene (ATP:ADP antiporter) was sequenced in several bacterial and plant taxa. The gene product is a protein localized on the inner side of the plastid membrane transporting ADP from the plastids into the cytosol and ATP in the opposite direction (Winkler and Neuhaus, 1999). In *Arabidopsis*, this gene is mainly expressed in root tips and cotyledons; consequently, the role of this gene in root growth, chlorophyll synthesis and seedling development was suggested (Spetea et al., 2012). Studies of knock-out mutants support this hypothesis, as expression inhibition of this gene led to disruption of rhizogenesis and a decreased amount of chloroplast thylakoids in early ontogenetic stages (Reiser et al., 2004). The SNP in this gene was also significantly associated with the variation of non-photochemical quenching of chlorophyll *a* fluorescence, which reflects the ability of heat dissipation of excess excitation energy and protection of photosynthetic apparatus against damage by high insolation levels (Müller et al., 2001). On the other hand, the logistic-regression approach did not reveal any significant association between SNPs at this gene and climatic variables. This may, however, be related to the choice of climatic proxies. First, the precision of climatic data received by interpolation among weather stations is limited, whatever sophisticated mathematical model is used. Second, aggregates such as mean temperature of the year or vegetation season may not properly reflect true drivers of thermal or radiation stress.

Significant relationships were found between the frequency of polymorphisms in the gene 1528 coding reduced epidermal fluorescence 4 and geographical coordinates (namely, latitude) and / or bioclimatic indicators of temperature fluctuations. There seems to be an association with PSII thermostablility parameters (although remaining only marginally significant after the correction for multiple testing). This may indicate its role in maintaining cell membrane integrity under thermal stress, because the protein coded by this gene participates in the phenylpropanoid pathway, which produces important cell-wall components such as flavonoids, lignin precursors, and also pigments like anthocyanins and hydroxycinnamate esters (Stout et al., 2008). These metabolites generally play a role in abiotic stress response including the response to supraoptimal temperatures (Wahid, 2007; Anderson et al., 2015).

Similarly, genes coding for RING-type zinc-finger protein (15036) and dihydrolipoamid-S-acetyltransferase (CL866) also contained SNPs associated with PSII thermostablility, the latter also with free proline content and non-photochemical quenching of chlorophyll *a* fluorescence. Zinc-finger domains also simultaneously bind ubiquitination enzymes and their substrates and hence function as E3-type ligases, which catalyze ubiquitine binding to the target substrate (Sharma et al., 2013). Microarray assays of the expression of E3-type ligase-coding genes in *Arabidopsis* indicate that their overexpression is induced by a wide spectrum of abiotic stresses, such as salt or osmotic stress, drought, excessive UV-B irradiation or heat stress, which corresponds with our observation. E3-type ligases were suggested to mediate ubiquitine-controlled protein degradation as adaptation response to various environmental stresses (Yee and Goring, 2009).

A significant association was also recorded for the CL4354 gene coding for serine/threonine phosphatase and the osmotic potential (marginally also with other physiological traits related to water use such as free proline or CO_2 assimilation rate). This gene was also detected as F_{ST} outlier in a study of Italian *Abies* populations by Mosca et al. (2014). Several recent studies documented the role of serine/threonine phosphatase in adaptive stress responses to drought (Tóth et al., 2000; Xu et al., 2007; Blakeslee et al., 2008). This protein is a heterotrimeric holoenzyme complex containing a bound with two regulatory subunits. Xu et al. (2007) recorded increased tolerance of water deficit in transgenic tobacco plants overexpressing the catalytic subunit TaPP2 Ac-1. Similar outcomes were recorded in *Arabidopsis* and *Medicago sativa*, where the expression of the catalytic subunit was suggested to be induced by abscisic acid. This is also a strong indication of a specific function of this protein in the response to water deficit (Tóth et al., 2000; Blakeslee et al., 2008; País et al., 2009).

Our study did not reveal SNPs with unambiguous signal of selection evidenced across different methodological approaches, in spite of choosing candidate genes expressed in proteins, which based on their known function in model plants are expected to play a role in physiological functions associated with the response to thermal or drought stress in fir, and are thus expected to be responsive to climate.

Nevertheless, the lack of such finding is not exceptional. Incongruence among different methods of detection of traces of selection appeared in other studies on silver fir as well (Mosca et al., 2014; Roschanski et al., 2016). Partly it may be related to the factors mentioned above, i.e., low precision of climatic data and a loose association between aggregate climatic indicators and the true stress factors. The choice of candidate genes is another factor. The candidate genes we used were originally developed in *Pinus taeda* and successfully transferred to *Abies alba* by Mosca et al. (2012a). For our study, we chose those exhibiting a signal of selection detected by at least one method in earlier studies. However, these studies were performed in different climatic zones (Italy; Mosca et al., 2012b) or under strongly different experimental setups (population pairs with contrasting elevation; Roschanski et al., 2016), which may have contributed to differences in selective responses.

In any case, the principal force driving differentiation at SNPs in the studied candidate genes seems to be historical factors rather than recent adaptation. This does not mean that they need not be relevant for adaptive properties of populations. As shown by Bošeľa et al. (2016), populations belonging to different refugial lineages may exhibit strongly different adaptive stress responses even when they are separated by short geographic distances and are currently exposed to similar climates. For the forestry practice adaptive behaviour is important without regard to the background mechanisms. Nevertheless, designing assisted migration or assisted gene flow as measures for the mitigation of climate change effects requires knowledge of climate-responsive genes and their variation patterns within species' ranges.

Authors' contributions

DG, RL, ĽD conceived the ideas and designed the study; JKm, ĽD, DG planned and coordinated the experiment; AK, DKu, EP, JKu carried out the measurements of physiological traits; AK, DKr, MH carried out the genetic analysis; AK, DKr, DKu, MH, DG processed and interpreted data; AK, DG wrote the first version of manuscript, and all of the authors contributed critically to the drafts and gave final approval for publication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.flora.2018.11.012.

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