ACTA BIOLOGICA CRACOVIENSIA Series Botanica 61/1: 43–51, 2019
DOI: 10.24425/abcsb.2019.127740

Lack of Signals of Selection at Candidate Loci at a Small Geographical Scale along a Steep Altitudinal Gradient in Norway Spruce (*Picea abies* [L.] Karst.)

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Received April 23, 2019; revision accepted June 6, 2019

Local adaptation is a key concept in biology: shift of genetic structures of populations due to differential survival of genotypes is expected to lead to phenotypes providing an advantage in the local environment. Variation of sequences of twelve candidate genes was investigated in 13 Norway spruce (*Picea abies* (L.) Karst.) provenances originating from sites distributed along an altitudinal gradient from 550 to 1300 m a.s.l. Signals of selection were assessed in 103 single nucleotide polymorphisms (SNP). The Bayesian F_{ST}-outlier identification methods as implemented in the programs BayeScan and Arlequin did not identify any SNP with a clear evidence of selection. The approaches relying on SNP-climate associations (spatial analysis method based on logistic regression of allele frequencies with environmental variables, Bayesian method applied in BayEnv2) identified several relationships but none of them remained significant after correction for multiple testing. Gene flow, epigenetic inheritance and former management of the studied populations are discussed as potential reasons for this weak evidence of selection signals.

Keywords: local adaptation, single nucleotide polymorphisms, F_{ST}-outliers, spatial analysis method

INTRODUCTION

The concept of local adaptation is of fundamental importance not only for evolutionary biology but it also has practical implications in nature conservation and forestry. Conservationists frequently focus on populations on marginal or extreme sites, expecting that such populations have developed specific gene pools by adaptation to local environments (Araújo and Williams, 2001; Lesica and Allendorf, 1995; Parsons, 1989). In forestry, local adaptation is actually the basis for the legislation on procuring and transfer of forest reproductive material. The current EU regulations are based on so-called regions of provenance, serving as a guiding framework for the choice of appropriate reforestation material. A region of provenance is defined as 'the area or group of areas subject to sufficiently uniform ecological conditions in which stands or seed sources showing similar phenotypic or genetic characters are found' (European Communities, 1999). This geography-based approach relies on the idea that climate, photoperiod and other factors associated with the geographical location are the main drivers of natural selection, which shapes genetic variation of tree populations. Non-local seed sources are considered risky because of the concerns about potential losses in yield and other forest functions (Hemery, 2008). Even though the ongoing climate change makes such rules of seed transfer questionable, the proposed solutions again rely on the idea of climate-driven local adaptation: assisted migration, i.e., transfer of genetic material from populations, which in the past experienced climatic conditions expected on target sites in the future (Williams and Dumroese, 2013), is also based on the assumption that gene pools of such populations are adapted to local climates.

The patterns of adaptive genetic variation have traditionally been studied by the common-garden approach; this is especially true for forest trees (Mátyás, 1996). On the other hand, in spite of recent rapid developments in forest tree genomics (González-Martínez et al., 2006; Neale and Ingvarsson, 2008), the knowledge of adaptation

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PL ISSN 0001-5296

processes and the resulting variation patterns at the molecular level is by far not sufficient, especially in conifers having large and complex genomes (Prunier et al., 2016). The candidate gene approach still predominates in conifer genomics studies, because of rapid decay of linkage disequilibrium in tree populations, which poses problems for association studies (Neale and Kremer, 2011). Moreover, adaptive variation patterns are also influenced by neutral processes such as gene flow, migration or genetic drift (Savolainen et al., 2007). Experimental designs of local adaptation studies need to reflect this fact.

This study focused on variation patterns at polymorphisms in candidate genes potentially involved in adaptation to temperature and precipitation variations or cold tolerance. We primarily focused on genes showing significant differences between a pair of climatically contrasting spruce provenances in an earlier study (Romšáková et al., 2012). We attempted to verify whether these polymorphisms would show a clinal pattern along an altitudinal (and climatic) gradient within a small territory, where the patterns arising from adaptation are not confused with differences caused by different population history.

MATERIALS AND METHODS

The study relies on a local nursery provenance experiment comprising 13 provenances originating within the natural range of Norway spruce in Slovakia, distributed along an altitudinal gradient from 550 to 1500 m a.s.l. (Table 1). Seeds were received from the gene bank of forest trees of Slovakia (OZ Semenoles Liptovský Hrádok), sown in a forest nursery in 2014 and replanted in 2016.

In 2018, branches of ~5 cm length were taken in the nursery from 10 seedlings per provenance. Total genomic DNA was isolated from 10 mg of silica-dried needles per seedling using a modified CTAB protocol following Doyle and Doyle (1987). DNA concentration was measured spectrophotometrically. Twelve loci reported in two different studies as adaptive were sequenced. The loci M002, M007B2, M007C2 and M007D1 (Lamothe et al., 2006) were identical with those studied by Romšáková et al. (2012). They were complemented by the loci 09870a, 16364e, 03870a, 04312b, 06340a, 05811e, 09644m and 08398a (Prunier et al., 2011). Primer sequences and the thermal cycling profile for PCR followed Lamothe et al. (2006) and Prunier et al. (2011). The PCR mixtures for all markers were done in volume 20 μL consisting of 1 × PCR buffer, 3 mM $MgCl_2$, 0.2 μM of primer, 0.3 μM dNTP, 0.5 U Taq DNA polymerase (Solis), 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. The obtained raw data were evaluated using SeqScape v.2.5. Sequences were reduced to sites exhibiting single nucleotide polymorphisms (SNPs).

Climatic data of the sites of origin of the studied populations were taken from the WorldClim high-resolution interpolated climate database (Fick and Hijmans (2017); variables are derived from meteorological data within the period 1960–1990 at a 1 km resolution), and were complemented by variables generated with the ClimateEU v4.63 software (http://tinyurl.com/ClimateEU, 1 km resolution) based on the methodology described by Hamann et al. (2013).

To obtain basic information on genetic structure of the studied populations, the following indices of genetic diversity were calculated for each population: mean number of alleles per SNP (A; as sample size was constant, no rarefaction was done), expected heterozygosity (H_e) and within-population fixation index (F_{is}). The significance of F_{is} was tested using 100,000 permutations. Analysis of molecular variance (AMOVA; Excoffier et al., 1992) was carried out; the significances of variance components attributed to populations and individuals were tested using 100,000 random permutations. Calculations were done using the PopGene 1.3 (Yeh et al., 1999) and Arlequin 3.5.1.3 (Schneider et al., 2000) computer programs. To account for potential population genetic substructure, we used the Bayesian clustering approach implemented in the program STRUCTURE v.2.3 (Pritchard et al., 2000) to infer individual membership to one or more genetic clusters. The procedure was run ten times for each K = 1–10, with a burn-in period of 200,000 and subsequent 1,000,000 iterations without prior information on the population of origin to determine the number of clusters. The optimum number of clusters was determined using the procedure of Evanno et al. (2005).

We used a combination of several methods to detect single-nucleotide polymorphisms (SNPs) that exhibit signs of selection, as recommended by Di Pierro et al. (2016). The first method relying on the F_{ST}-outlier approach is implemented in BayeScan (Foll and Gaggiotti, 2008), and uses population differentiation of the loci to search for those affected by selection. Version 2.1 of BayeScan was used with 20 pilot runs and burn-in with 5,000 iterations and final 50,000 iterations to estimate the posterior distributions. Prior odds for the neutral model were set to 10 (default). The evidence of selection was based on Bayes factors, measuring odds for the

TABLE 1. Localization of the studied populations and the planting site (forest nursery).

Code	Alt (m a.s.l.)	Long	Lat	Forest unit	Locality	Gene bank no.
pab225CA-004	550	49°24’	18°42’	Čadca	Zákopčie	2003/009
pab225CA-003	550	49°24’	18°42’	Čadca	Husáre	2003/011
pab214BB-188	650	48°46’	19°24’	Slovenská Ľupča	Pohronský Bukovec	2003/008
pab235BR-062	750	48°50’	19°45’	Beňuš	Hrobcovo	2003/018
pab215RK-867	870	49°09’	19°25’	Liptovská Teplá	Prosečné	2010/026
pab235BR-250	910	48°42’	19°30’	Hronec	Hrončecký grúň	2010/029
pab216TS-840	920	49°15’	19°39’	Habovka	Žriedla	2010/034
pab216TS-106	1050	49°16’	19°43’	Habovka	Zadná Kremenná	2010/041
pab216LM-039	1060	48°59’	19°48’	Malužiná	Tajch	2010/035
pab216LM-028	1100	49°09’	19°41’	Liptovský Mikuláš	Žiar	2010/037
pab217BR-169	1280	48°50’	19°25’	Slovenská Ľupča	Jasenie	2010/046
pab217TS-110	1335	49°14’	19°13’	Habovka	Zverovka	2010/033
nn	1500	48°57’	19°27’	Partizánska Ľupča	sedlo Ďurkovej	nn
Nursery	860	48°40’	19°01’	VšLP TU Zvolen	Mláčik	

Code – registration code of the approved seed stand, **Alt** – altitude, **Long** – longitude, **Lat** – latitude, nn – not an approved stand

selection model versus the neutral model derived from posterior probabilities of each of the models (Foll and Gaggiotti, 2008). The second method based on the F_{ST}-outlier approach is that of Excoffier et al. (2009), which relies on obtaining the distribution of F_{ST} across loci as a function of heterozygosity between populations by performing coalescent simulations. We used Arlequin 3.5.1.3 to perform 10,000 simulations under the finite island model.

Two other methods were based on the search for SNP-environmental variable relationships. The spatial analysis method (SAM) as implemented in Samβada (Stucki et al., 2017) is based on logistic regression of allele frequencies with environmental variables. SAM needs presence/absence data; therefore, SNP genotypes were coded as suggested by Joost et al. (2007), considering the effect of the SNP allele dominant. Markers with minor allele frequency of less than 10% were removed. Both Wald test and *G*-test implemented in Samβada were taken into account when examining the significance of the results, while Benjamini-Hochberg procedure was used to correct both for multiple testing. Since multiple redundant tests would reduce the power of this approach, some markers were removed so that no pair of markers had Spearman correlation index higher than 0.9. A similar criterion was used to prune environmental variables: the order of priority that guided removal of correlated environmental variables was the following: geographic coordinates > WorldClim bioclimatic variables > other WordClim variables > ClimateEU. At the end, 15 variables were retained out of the original 189: latitude, longitude, elevation, WorldClim bioclimatic variables BIO2 (mean diurnal range), BIO3 (isothermality), BIO6 (minimim temperature of the coldest month), BIO13 (precipitation of the wettest month), BIO14 (precipitation of the driest month) and BIO15 (precipitation seasonality), solar radiation average in January and October, vapor pressure in December, degree-days > 18 °C (DD18), and Hargreaves climatic moisture deficit (CMD). In addition, Bayesian factors for the support for the models in which SNP frequencies covary linearly with environmental variables over models in which SNPs vary according to neutral expectation were assessed using the program BayEnv2 (Günther and Coop, 2013). For each SNP-environmental variable combination, the procedure was run with 100,000 iterations.

RESULTS AND DISCUSSION

In total 393 SNPs were identified, out of which 290 were discarded from further evaluations because of too many missing data or overall minor allele frequency below 10%.

The levels of within-population genetic variation were quite similar in all populations (Table 2). In spite of a relatively small sample size, the proportion of monomorphic SNPs was small, as documented by high mean numbers of alleles per SNP, which exceeded 1.8 in all populations. Except the population Hrobcovo with a slight excess of homozygotes, the populations were at Hardy-Weinberg equilibrium. AMOVA showed that the interpopulation differentiation is negligible (0.56% of the total variation; Table S1 in Supplementary material).

The Bayesian clustering analysis done by the procedure STRUCTURE revealed a certain divergence of the high-elevation population 13 (sedlo Ďurkovej), which corresponds to $K = 2$ as the optimum number of clusters indicated by the ΔK measure of Evanno et al. (2005) (Fig. S1). The outcomes of the analyses for $K = 3$ and $K = 4$ confirmed the distinctness of the population 13, and did not reveal any potential hidden substructure (Fig. 1). The reason for the divergence of the population 13 is unclear: it is a population growing in extreme climatic conditions at the upper tree limit (isolated trees alternating with patches of *Pinus mugo* krummholz). Both climate-driven selection and marginality may be responsible for its specific structure.

Neither of the two approaches aimed at the detection of adaptive variation found any reliable evidence of selection. F_{ST} values ranged between 0.032 and 0.037, which is slightly higher than reported for strictly neutral markers such as nuclear microsatellites in studies covering similarly small areas (Máchová et al., 2018; Scotti et al., 2006) but indicates negligible differentiation among populations anyway. The highest value of the logarithm of posterior odds for the selection model against the neutral model as calculated by BayeScan was -0.875, which actually means that the neutral model was more probable than the selection one (Fig. 2, Table S2 in Supplementary material). Simulations under the finite island model in Arlequin yielded the same result: for none of the SNPs the outlier F_{ST} value remained significant after correction for multiple testing (Table S3 in Supplementary material).

In the case of Samβada, no reliable evidence for a marker–climatic variable relationship was found either. Without correction for multiple testing, one SNP on the 9644 gene (G/T polymorphism at site 24, Table 3, Table S4 in Supplementary material) showed significant association with several climatic variables, related to both

TABLE 2. Basic characteristics of the population genetic structure of the studied populations.

	Population	*A*	H_e	F_{is}	*P*
1	Zákopčie	1.9223±0.3032	0.2816±0.1553	0.0480	0.283
2	Husáre	1.8252±0.3816	0.2496±0.1783	-0.0288	0.270
3	Pohronský Bukovec	1.9223±0.3032	0.2771±0.1639	-0.1386	0.089
4	Hrobcovo	1.8447±0.3900	0.2522±0.1600	0.1404	0.042
5	Prosečné	1.8932±0.3405	0.2507±0.1550	0.0306	0.357
6	Hrončecký grúň	1.9223±0.2690	0.2703±0.1511	-0.1000	0.219
7	Žriedla	1.8350±0.3730	0.2537±0.1648	0.0237	0.390
8	Zadná Kremenná	1.8932±0.3104	0.2814±0.1618	-0.0433	0.331
9	Tajch	1.8641±0.3718	0.2683±0.1752	0.0438	0.286
10	Žiar	1.8835±0.3224	0.2618±0.1709	0.0017	0.497
11	Jasenie	1.8932±0.3104	0.2721±0.1673	-0.0988	0.138
12	Zverovka	1.8835±0.3224	0.2872±0.1618	-0.0224	0.393
13	sedlo Ďurkovej	1.8544±0.3811	0.2630±0.1686	-0.0040	0.474

A – mean number of alleles, $\boldsymbol{H_e}$ – expected heterozygosity, $\boldsymbol{F_{is}}$ – fixation index, ***P*** – significance of H_0: $F_{is} = 0$

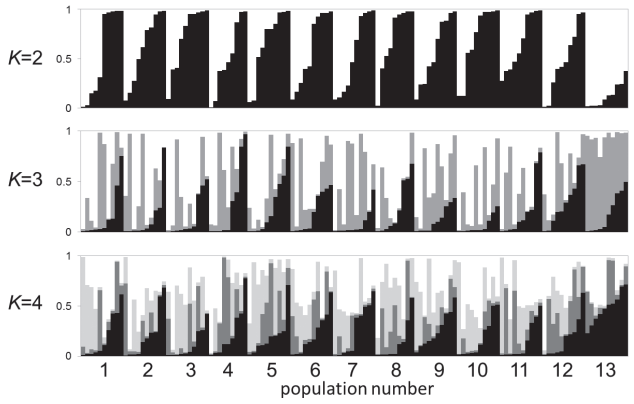

Fig. 1. Bar plots of the Bayesian clustering analysis by STRUCTURE for the numbers of groups K = 2–4.

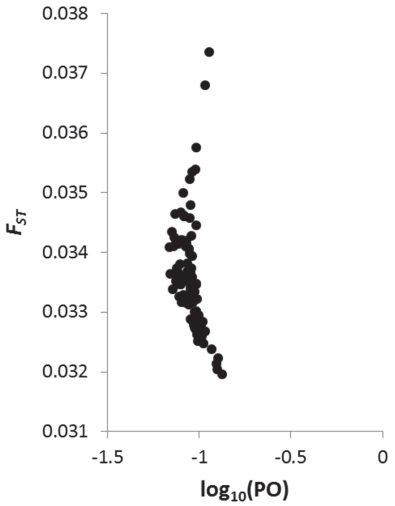

Fig. 2. Results of BayeScan: F_{ST}-values plotted against the decadic logarithm of posterior odds for the selection model (PO).

precipitation (BIO13) and temperature (BIO2, BIO6, DD18). Nevertheless, after Benjamini-Hochberg correction for multiple testing, none of these relationships remained significant: even the association with precipitation of the wettest month (WorldClim bioclimatic variable 13), which showed a relatively low P-value of 0.0000770 in the G-test, would need a P-value below 0.0000758 to be statistically significant even at the 10% significance level. On the other hand, most of these relationships were confirmed by the BayEnv2 analysis: the Bayes factor for the association 9644_2.024.G/BIO13 was 16.96, which means strong support for the selection model. In the case of the associations of the polymorphism at this site with the other above-mentioned climatic variables, Bayes factors also exceeded 3, meaning substantial support, and the same applies to several other SNP-climate associations (Table 4, Table S5 in

TABLE 3. SNP-climatic variable relationships significant at $P < 0.01$ without correction for multiple testing.

SNP	Enviro	G	P_G	Wald	P_{Wald}	McFadden R^2	β_0	β_1
9644_2.024.G	BIO13	15.631	0.000077	9.971	0.001590	0.1251	6.1824	-0.0633
9644_2.024.G	radiation	12.997	0.000312	10.944	0.000939	0.0967	-83.4101	0.0076
9644_2.024.G	BIO2	9.154	0.002482	7.869	0.005028	0.0554	-16.9328	1.7393
9644_2.024.G	BIO6	8.692	0.003197	8.287	0.003992	0.0505	22.4807	2.5028
9644_2.024.G	CMD	8.503	0.003545	8.783	0.003040	0.0484	-2.1451	0.0552
9644_2.024.G	DD18	7.016	0.008078	6.995	0.008175	0.0324	-2.2298	0.0246
9644_2.047.G	BIO6	8.376	0.003801	7.919	0.004892	0.0261	16.9089	1.7869
M007B2.376.A	CMD	7.851	0.005079	8.683	0.003213	0.0460	-2.6919	0.0575
M007B2.361.A	BIO15	8.815	0.002988	7.227	0.007182	0.0437	-7.5250	0.1691
16364.200.C	BIO6	7.831	0.005136	7.357	0.006681	0.0219	16.3363	1.7017

SNP – designation of marker, site and the dominant base at a particular SNP, **Enviro** – climatic variable, G – G-test score, P_G – significance of the G-test, **Wald** – Wald test score, P_{Wald} – significance of the Wald-test, **McFadden R^2** – adjusted McFadden goodness-of-fit measure, β_0, β_1 – intercept and slope of the linear logistic regression model, respectively.
BIO2 – mean diurnal range of temperatures, BIO6 – minimum temperature of the coldest month, BIO13 – precipitation of the wettest month, BIO15 – precipitation seasonality , CMD – climatic moisture deficit, DD18 – degree-days > 18°C, radiation – yearly average of solar radiation.

TABLE 4. Bayes factors (BF ≥ 3.0) for the support of selection model over the neutral model in the SNP-climatic variable relationships.

Locus	longitude	BIO2	BIO3	BIO6	BIO13	BIO14	radiation	vapour	DD18	CMD
9644_2.024.G		4.52		6.81	16.96	4.09	11.39	3.14	3.50	6.23
M007B2.376.A										3.03
M007B2.275.A		9.42			3.50	6.52				3.36
16364.232.A										3.00
5811.397.G								3.28	4.07	
5811.397.A								3.80	5.33	
8398_2.126.G	3.44		3.36						3.19	
8398_2.266.A	6.94		3.62						4.66	
8398_2.410.C	3.99								3.70	

Supplementary material). However, Bayes factors cannot be corrected for multiple assessments as easily as probabilities; therefore, these results need to be regarded with caution.

Admittedly, the dataset used in this study was relatively modest in terms of the sample size and geographic coverage. Nevertheless, Prunier et al. (2011), one of the sources of candidate genes used in this work, used 156 individuals in 26 populations to search for signs of selection within a larger pool of candidate genes. Our study (using identical genes) relied on a comparable sample: 128 seedlings from 13 populations. It is thus improbable that our failure in finding convincing evidence for selection was caused by the insufficiently small sample size. There was an obvious difference in the geographic extent of the sampled populations (for instance, the longitudinal span of populations studied by Prunier et al. (2011) was 16° compared to 1°22' in our study). We attempted to keep the sampled territory as small as possible to avoid detecting false positives associated with neutral processes such as colonization and recolonization of the current range during the Quaternary (Kupryjanowicz et al., 2018; Tollefsrud et al., 2008). On the other hand, we tried to make the climatic gradients as steep as possible: the range of average temperatures of the populations was 4.26°C and yearly precipitation 492 mm, compared to the ranges of 4.28°C and 553 mm, respectively, in the study of Prunier et al. (2011). There is obviously enough environmental variation to allow adaptation. Very probably, gene flow among relatively closely located populations in this study counteracted selection and prevented differentiation. This is supported by the findings of Scalfi et al. (2014), who studied adaptive genetic variation in Norway spruce on both macrogeographic and microgeographic scale and indeed found only very few SNPs associated with environmental variables on the microgeographic scale: they detected 2 possibly adaptive loci within altitudinal transects, compared to 38 loci on the range-wide scale. Of course, the levels of gene flow are not exclusively a matter of geographical proximity; as the studied populations are located at different elevations, their flowering times differ. Nevertheless, the temperature gradient underlying this phenological shift is continuous, and there is a considerable overlap in the timing of flowering among neighboring altitudinal zones, which may allow a spread of genes across the whole gradient in a few generations. On the other hand, the studies of Di Pierro et al. (2016, 2017) found signals of climatic selection in Norway spruce populations distributed over areas of a similar size. Apparently, the effects of gene flow counteracting selection depend on a particular geographical situation.

The research on differentiation in growth traits, cold tolerance and phenology in Norway spruce populations distributed along altitudinal gradients at small geographic scales revealed phenotypic climate-related clines (Chmura, 2006; Oleksyn et al., 1998). The question is, whether the basis of these heritable differences is necessarily genetic. Epigenetic effects induced by temperature and photoperiod during seed development have been demonstrated in Norway spruce, affecting budset, flushing and cold acclimation (Johnsen et al., 2005). Yakovlev et al. (2010) found micro-RNAs, which are one of the known epigenetic mechanisms, to have different transcription levels in individuals

from the cold and warm environments. Gömöry et al. (2015) revealed that the early-growth environment also induces changes in budburst phenology of conifers. Such epigenetic effects may hamper adaptive responses by selection, as they decrease the selection pressure.

Finally, Norway spruce is among the most intensively managed tree species in Slovakia and Central Europe. Of course, collections for gene banks (from where we received the materials) focus on indigenous approved seed stands. However, in the case of a species, which has been extensively planted and transferred across the whole region, historical records need not always be completely reliable and autochthony can never be guaranteed (Jansen et al., 2017). Even if we had made sampling in nature reserves, expected to represent virgin forests, human interventions including planting could not be excluded (Sabatini et al., 2018). Theoretical population models predict that several generations are needed to substantially change the frequency of an allele under selection unless selective pressure is very strong (Wright, 1931). Therefore, if our materials included non-indigenous populations, the generated random noise may have obscured the signal. The more populations are included in a study, the higher this risk is; this may be the reason why the same set of SNPs yielded significant results in the study of Romšáková et al. (2012) comparing only two climatically contrasting populations, while our study failed to verify them.

This study demonstrates that the adaptive value of particular polymorphisms depends on the context of the species and environment, and the experimental design may also play a role. Any generalizations require that signals of selection are verified by several independent studies.

AUTHORS' CONTRIBUTIONS

DG designed the experiment and wrote the first draft, DK performed molecular analyses, MH made the mathematical treatment of the data, all authors prepared the final version of the manuscript.

ACKNOWLEDGEMENTS

The study was supported by a grant of the Slovak Research and Development Agency no. APVV-16-0306. Technical assistance of Mrs. G. Baloghová is greatly appreciated.

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