

Nucleotide polymorphisms related to altitude and physiological traits in contrasting provenances of Norway spruce (*Picea abies*)

Ivana ROMŠÁKOVÁ¹, Elena FOFFOVÁ², Jaroslav KMEŤ¹, Roman LONGAUER²,
 Marian PACALAJ² & Dušan GÖMÖRY^{1*}

¹ Technical University in Zvolen, Faculty of Forestry, TG Masaryka 24, SK-96053 Zvolen, Slovakia; e-mail: gomory@vsl.d.tuzvo.sk

² National Forestry Centre, Forestry Research Institute, TG Masaryka 22, SK-96092 Zvolen, Slovakia

Abstract: Variation of sequences of six EST-derived markers was investigated in three Norway spruce (*Picea abies* [L.] Karst.) provenances originating from different altitudes growing at two contrasting trial plots in Slovakia (Veľký Lom 450 m a.s.l., Mútne-Zákamenné 1,250 m a.s.l.) within a spin-off experiment of the IUFRO 1964/68 Inventory Provenance Experiment with Norway spruce. Single nucleotide polymorphisms (SNP) were identified and differences in allele frequencies at polymorphic sites were tested against altitude or associated with physiological and growth traits (chlorophyll *a* fluorescence, frost resistance, height, diameter, budburst phenology).

Overall, 5.1% of sites (190 in total) were polymorphic in the studied material. Although there were no differences in nucleotide diversity among provenances, the differentiation was highly significant (the overall between-population variance component assessed by the AMOVA based on both extreme populations P1 and P49 was 6.53%). Only 4 polymorphic sites differed significantly between populations after Bonferroni correction. Four sites showed significant association with phenotypic traits (breast-height diameter, stem volume, chlorophyll fluorescence). In contrast to earlier analyses of growth and physiological traits based on the same material, significant associations with polymorphic sites indicate the effect of local adaptation.

Key words: *Picea abies*; single nucleotide polymorphisms; IUFRO 1964/68; provenance research; cold hardiness; chlorophyll fluorescence

Introduction

Local adaptation is a key concept of evolutionary ecology (Kawecki & Ebert 2004; Savolainen et al. 2007). Shift of genetic structures of populations due to differential survival of genotypes is expected to lead to phenotypes providing advantage under the local environment. Local adaptation in plants may be provoked by many natural and human-induced environmental factors, such as climate, soil, biotic agents (mycorrhizal symbionts, pollinators, parasites), industrial pollution etc., and may be reflected by different selection-responsive traits (Geburek 2000; Linhart & Grant 1996; Mátyás 1996).

Forest tree genomics provides a rich variety of tools for the study of adaptation. However, in spite of explosively growing genomic resources and general awareness of the need of adaptive genetic markers (Gonzalez-Martinez et al. 2006; Grattapaglia et al. 2009; Neale & Ingvarsson 2008; Neale & Kremer 2011), empirical studies directly linking singlelocus and multilocus genotypes with environmental gradients have become appearing quite recently and are still scarce (Eckert et al. 2009,

2010; Namroud et al. 2008; Wachowiak et al. 2009).

Forestry research contributed to the study of adaptation by provenance trials, a type of common-garden experiments specific for this field of science. The term “provenance” denotes a local subpopulation of a defined origin. The aim of common gardens is elimination of the differences among different populations caused by large-scale environmental effects (macroclimate, parent rock) by growing plant material of different origins under equal conditions, and identification of the phenotypic response of these populations to transfer (Clausen et al. 1939; Turesson 1925). Most international provenance trials were organized by several working parties of the International Union of Forestry Research Organizations (IUFRO), and focused mainly on the trends of variation of commercially important traits (Giertych & Oleksyn 1992; Krutzsch 1992). Typically, provenances of widespread conifers of the Northern hemisphere originating from higher altitudes or northern latitudes showed slower growth, earlier budburst and growth termination during the vegetation season, a higher late-frost susceptibility, but also a higher resistance to deep frosts in the winter compared to southern

* Corresponding author

or low-elevation provenances, when planted together on the same site (Wright 1976).

Nevertheless, conifers do not represent ideal experimental systems for the study of local adaptation. First, their long generation time causes that they are exposed to varying environmental conditions during their lifespan. Consequently, phenotypic plasticity may be a more efficient strategy to cope with spatial and temporal heterogeneity of environment (Vitasse et al. 2009), however, hampering the evolutionary response (Gimeno et al. 2008; Mimura & Aitken 2010). Moreover, considering the fact that most populations have occupied their present locations quite recently during the Holocene, the evolutionary time (typically tens of generations) may have been too short to develop differentiation. Second, most conifers are wind-pollinated and with very efficient pollen dispersal over large distances (Lindgren et al. 1995). High levels of gene flow may prevent differentiation (Savolainen et al. 2007).

Norway spruce may serve as an example of a forest tree species with a large and distribution range comprising climatically and edaphically heterogeneous environments of the temperate and boreal zones of Europe. A considerable differentiation was observed in spruce both in gene-marker studies (Collignon et al. 2002; Heuertz et al. 2006; Lagercrantz & Ryman 1990; Nowakowska 2009; Sperisen et al. 2001; Vendramin et al. 2000) and provenance tests (Giertych 1976; Krutzsch 1974). However, at the rangewide scale, the effects of adaptation by natural selection on phenotypic traits and their underlying genetic structures can hardly be distinguished from those of neutral processes like migration, gene flow

and genetic drift. Therefore, our study focuses on local adaptation at a small scale. In Slovakia, Norway spruce is a montane species with a climatically very heterogeneous but small (180 × 80 km) distribution range. We examined two provenances from climatically contrasting sites for which additional phenotypic information was available by sequencing a set of adaptive EST-derived genetic markers, which proved to be highly polymorphic in spruce in an earlier study (Lamothe et al. 2006). The effects of demography on genetic structures are expected to be small in such material.

The objective of our study was identification of single nucleotide polymorphisms differentially represented in contrasting provenances (and thus potentially associated with adaptation to climate) and those associated with fitness-related physiological and growth traits.

Material and methods

Material of Norway spruce provenances tested within this study was collected in a spin-off of the IUFRO 1964/68 Inventory Provenance Experiment with Norway spruce (Krutzsch 1974). Selected foreign provenances represented in the experiment were completed by 11 Slovak provenances and planted in a series of 5 plots distributed along an altitudinal gradient from 450 to 1,250 m a.s.l. in 1968, in a randomized complete block design with 3 blocks and 49 plants per provenance and block (2 × 2 m spacing).

Measurements of common phenotypic traits (tree height, breast-height diameter) were done during the spring season of 2009. Moreover, phenological stage of budburst was recorded using a 7-point scale (0 – buds closed, 1 – buds swollen and somewhat elongated, 2 – buds elongating,

Table 1. Characteristics of provenances and trial sites included in the study and overview of the measured phenotypic traits.

	Nr.	Longitude /Latitude	Altitude (m a.s.l.)	Soil	T^1 (°C)	P^2 (mm)
Provenance						
Beňuš	P1	19°53'/49°50'	700	Dystric Cambisol	5.7	852
Habovka 49b	P12	19°41'/49°15'	1000	Dystric Cambisol	3.7	1199
TANAP	P49	20°15'/49°11'	1450	Haplic Podsol	2.9	1154
Trial site						
Veľký Lom	T1	19°21'/49°20'	450	Eutric Cambisol	7.9	727
Mútne-Zákamenné	T5	19°17'/49°32'	1250	Dystric Cambisol	3.1	1336
Trait	Description*					
height	tree height at the age of 45 years					
diameter	breast-height diameter at the age of 45 years					
stem volume	stem volume calculated from the height and BHD (Pajtik and Petras 1991)					
phenology	mean budburst stage calculated from 2 scoring dates					
I_{20}	index of frost injury at the freezing temperature of –20 °C (Flint et al. 1967)					
I_{80}	index of frost injury at the freezing temperature of –80 °C (Flint et al. 1967)					
F_0	basic fluorescence; the yield fluorescence in the absence of photosynthetic light, when all reaction centres of photosystem II are open					
F_m	maximum fluorescence					
F_v	variable fluorescence; $F_m - F_0$					
F_v/F_m	the quantum efficiency of open photosystem II centres					
T_m	time required for the increase of fluorescence from F_0 to F_m					
$Area$	the area above the induction curve between the basic and maximum fluorescence					

¹Mean annual temperature, ² Mean annual precipitations

* for details, see Gömöry et al. (2010) and Maxwell & Johnson (2000)

Table 2. Proportions of polymorphic sites and nucleotide diversities for individual markers.

Provenance		M002	M007B2	M007C2	M007D1	M007G1	M024	Sum/Mean
P1	<i>nP</i>	31	18	17	19	32	35	152
	<i>PP</i>	0.052	0.041	0.033	0.029	0.049	0.038	0.040
	π	0.215	0.087	0.145	0.280	0.154	0.099	0.163
	<i>nHW</i>	0	0	0	1	1	2	4
P12	<i>nP</i>	24	20	17	17	24	22	124
	<i>PP</i>	0.041	0.045	0.033	0.026	0.037	0.024	0.033
	π	0.195	0.129	0.151	0.265	0.154	0.073	0.161
	<i>nHW</i>	0	4	0	1	0	0	5
P49	<i>nP</i>	29	15	17	17	25	30	133
	<i>PP</i>	0.050	0.034	0.033	0.026	0.039	0.033	0.035
	π	0.204	0.098	0.174	0.258	0.135	0.111	0.163
	<i>nHW</i>	0	0	1	0	0	0	1
Total	<i>nP</i>	34	28	23	21	39	45	190
Sequence length (bp)		585	444	507	653	649	921	3759

nP – number of polymorphic sites, *PP* – proportion of polymorphic sites, π – nucleotide diversity, *nHW* – number of sites showing significant deviation from Hardy-Weinberg equilibrium (without Bonferroni correction)

fully broken, needles not protruded, scale cup on the apex, 3 – new green foliage visible and protruding, shoots soft and short (up to 6 cm long), 4 – protruded needles light green, shoots extending (approx. 7–12 cm long) but still soft, 5 – needles dark green, shoot elongation terminated, shoots straight, 6 – fully matured shoots with dark foliage).

For the evaluation of genetic variation, we chose three provenances originating from contrasting environments planted in the lowermost and uppermost plots of the series (Table 1). Climbers collected branches from the isolated upper part of the crown from 10 trees per provenance on October 27 and 28, 2009 in Velký Lom and Mútne-Zákamenné, respectively. Frost-resistance index based on electrolyte leakage after artificial freezing and the parameters of the rapid phase of chlorophyll *a* fluorescence giving information of the efficiency of photosystem II were measured in one-year-old needles as described in Gömöry et al. (2010).

Total genomic DNA was isolated from all individuals of the populations Beňuš (P1; 19 trees) and TANAP (P49; 20 trees) and in a subset of individuals of the provenance Habovka 49b (P12; 11 trees) using modified CTAB protocol following Doyle & Doyle (1987). For the isolation, 10 mg of silica dried needles was used. DNA concentration was measured spectrophotometrically.

Six highly polymorphic EST-derived markers according to Lamothe et al. (2006) were sequenced (M002, M007B2, M007C2, M007D1, M007G1, M024). These markers represent candidate genes for cold tolerance or embryogenesis, they are thus expected to be responsive to climatic contrasts. Primer sequences and thermal cycling profile for PCR followed Lamothe et al. (2006). The PCR mixtures for all markers were done in volume 20 μ L consisting of 1 $\times$ PCR buffer, 2 mM MgCl₂, 0.2 μ M of primer, 0.3 μ M dNTP, 0.5 U *Taq* DNA polymerase (GeneCraft), 0.8 μ g/ μ L 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. Sequences were reduced to sites exhibiting single nucleotide polymorphisms (SNPs). For statistical evaluations, each polymorphic site was treated as separate locus. Only the most contrasting populations P1 and P49 were considered in most analyses to ensure sufficient sample size for

statistical tests. First, differences in allelic frequencies between P1 and P49 were tested using exact probability test, whereby the resulting significances of differences were corrected using sequential Bonferroni procedure. Subsequently, Hardy-Weinberg and linkage disequilibria were tested for the loci showing significant inter-population differentiation. Nucleotide diversity for individual markers was calculated according to Tajima (1983). A two-level locus-by-locus analysis of molecular variance was used to assess the among-population variance components for polymorphic sites. Calculations were done using Genepop 3.1 (Raymond & Rousset 1995) and Arlequin 2.000 (Schneider et al. 2000).

Differences among genotypes in phenotypic traits (growth, phenology, photosynthetic parameters, frost resistance) were tested using a two-way analysis of variance with fixed effect of test location (Velký Lom, Mútne-Zákamenné) and random effect of genotype (procedure GLM, SAS 2010). Again, probabilities associated with the *F*-tests were corrected using sequential Bonferroni correction, and Hardy-Weinberg and linkage disequilibria were tested for the loci showing significant associations with phenotypic traits.

Results

Although the six studied ESTP markers were developed on the basis *Picea glauca* cDNAs, all primers proved to be useful also in *P. abies* (as already suggested by Lamothe et al. 2006) and yielded readable sequences. Overall, 5.1% of sites were polymorphic in the studied material, although the within-population proportion of polymorphic sites was slightly lower (3–4%, see Table 2) and varied among the examined markers. This is quite much, considering the fact that expressed (and thus potentially selected) sequences were studied.

The differences in variation levels among populations were quite small. Numbers or proportions of polymorphic sites can be directly compared only for provenances represented by identical sample sizes. They are higher in P1 compared to P49, and this difference is quite consistent over markers. On the other hand there are no differences in nucleotide diversity among provenances (Table 2).

Table 3. Analysis of molecular variance based on polymorphic sites for provenances P1 and P49.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Between populations	1	16.462	0.30898	6.53***
Within populations	76	335.922	4.42003	93.47

Table 4. Polymorphic sites exhibiting significant differences between provenances P1 and P49: allelic frequencies, significance tests and between-population variance components (AMOVA).

Marker	Site	P^1	P^2	Nucleotide	Genomic region /effect ³	Provenance		Variance component ² (%)
						P1	P49	
M002	104	**	*	C	intron	0.31	0.05	17.66*
				T		0.69	0.95	
	217	**	**	G	intron	0.53	0.18	20.78*
M007D1	098	**	*	T	Y↔S	0.47	0.82	17.48***
				A		0.00	0.20	
				C		1.00	0.80	
M024	882	**	**	A	3'UTR	0.71	0.97	19.45*
				T		0.29	0.03	
M002	121	*		A	intron	0.31	0.08	13.09
				T		0.69	0.92	
	129	*		C	intron	0.72	0.95	14.75
				T		0.28	0.05	
	165	*		G	intron	0.31	0.11	9.23
				C		0.69	0.90	
	306	*		G	V↔F	1.00	0.82	15.75**
				T		0.00	0.18	
M007C2	256	*		C	intron	0.40	0.18	8.91
				T		0.61	0.83	
M024	191	*		A	A↔T	0.03	0.18	9.54
				G		0.97	0.82	
	318	*		A	intron	0.00	0.12	9.86
				C		1.00	0.88	
	496	*		A	A↔T	0.00	0.15	13.01
				G		1.00	0.85	
	848	*		G	3'UTR	0.76	0.97	14.10
				C		0.24	0.03	

P – significance of the exact probability test of differences in allelic frequencies: ¹significance without Bonferroni correction, ²significance after Bonferroni correction, ³symbols and abbreviations: Y – tyrosine, S – serine, F – phenylalanine, V – valine, A – alanine, T – threonine

Significance labels: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Although the overall between-population variance component assessed by the AMOVA based on both extreme populations P1 and P49 was quite low (6.53%), the differentiation was highly significant (Table 3). However, quite few polymorphic sites contributed to the differentiation. Thirteen loci exhibited significant differences between populations, but after Bonferroni correction of significances, only 4 loci remained. As this study should only be regarded as a pilot one, we presented all 13 sites in Table 4, because they may be prospective for further testing in more detailed studies. The results of locus-by-locus AMOVA and exact probability tests are in a good accord with one exception of the site M002/306, exhibiting a rather high and significant between-population variance component in AMOVA but already excluded in Bonferroni correction of exact probability tests. The relative between-population variance components for the four most differentiated loci range between 17 and 21%, and exceed 8% in the remaining 9 polymorphic sites (Table 4).

If 10% is taken as a lower allele-frequency limit,

major polymorphisms are evenly distributed in both populations over the loci showing significant differentiation. Among the 13 SNPs, four completely monomorphic sites were found only in the low-elevation provenance P1 (Table 4).

Numerous loci showed significant association with physiological and growth traits. Again, all of them are presented in Table 5 because they might potentially be interesting for further studies. However, after Bonferroni correction, only four sites remained. The M007G1 locus showed an association with biomass production (sites 498 and 538; breast-height diameter and stem volume), whereas the sites of the M024 marker were associated with photosynthesis parameters (sites 232 and 496 with T_m and F_0 , respectively).

We have no information from the source paper (Lamothe et al. 2006) about linkage of the markers used. Linkage disequilibria seem, however, to be generally restricted to the within-marker level, and the absence of significant disequilibria for all sites within a marker is probably only due to small sample sizes (Ta-

Table 5. Polymorphic sites exhibiting significant associations with phenotypic traits.

Trait	Locus	Site	Alleles	Genomic region /effect ³	Partial R^2	DF		F -test	P^1	P^2
						nom	den			
diameter	M007G1	498	A, C	syn.	0.150	1	43	7.91	**	*
diameter		538	A, C	M↔L	0.238	1	43	14.11	***	***
stem volume	M024	538	A, C	M↔L	0.216	1	42	11.75	**	**
T_m		232	C, T	intron	0.143	1	43	7.46	**	*
F_0		496	A, G	3'UTR	0.153	1	43	7.86	**	*
F_v	M002	254	A, G	syn.	0.068	1	44	4.16	*	
T_m		306	G, T	V↔F	0.107	1	44	5.45	*	
I_{20}	M007B2	306	G, T	V↔F	0.094	1	44	5.35	*	
height		413	C, T	syn.	0.048	1	40	4.19	*	
phenology		510	A, G	3'UTR	0.097	1	41	5.65	*	
Area	M007B2	068	C, T	A↔V	0.089	1	46	4.59	*	
Area		206	A, G	3'UTR	0.082	1	46	4.22	*	
Area		252	A, G	3'UTR	0.157	2	45	4.3	*	
I_{80}	M007B2	252	A, G	3'UTR	0.115	2	45	3.68	*	
F_v / F_m		302	A, G	3'UTR	0.099	2	45	3.22	*	
T_m		326	A, G	3'UTR	0.093	1	46	4.9	*	
phenology	M007C2	207	C, T	intron	0.112	2	43	3.44	*	
phenology		256	C, T	intron	0.109	2	43	3.36	*	
phenology		258	A, T	intron	0.087	1	44	5.31	*	
phenology	M007D1	269	A, T	intron	0.112	2	43	3.44	*	
phenology		308	A, G	intron	0.079	1	44	4.74	*	
T_m		333	A, G	N↔S	0.128	2	46	3.37	*	
I_{20}	M007D1	098	A, C	Y↔S	0.095	1	47	5.69	*	
phenology		132	C, T	syn.	0.079	1	44	4.74	*	
Area		371	G, C	D↔E	0.088	1	47	4.65	*	
phenology	M007G1	463	A, G	R↔K	0.079	1	44	4.74	*	
F_v / F_m		219	A, C	intron	0.072	1	46	4.6	*	
I_{80}		219	A, C	intron	0.078	1	46	4.85	*	
F_v / F_m	M007G1	242	G, T	intron	0.065	1	46	4.14	*	
diameter		284	A, C	intron	0.109	1	43	5.49	*	
stem volume		284	A, C	intron	0.101	1	42	4.76	*	
I_{20}	M007G1	450	G, C	syn.	0.131	2	45	4.04	*	
phenology		450	G, C	syn.	0.131	2	42	4.11	*	
F_0		498	A, C	syn.	0.092	1	46	4.74	*	
stem volume	M007G1	498	A, C	syn.	0.138	1	42	6.8	*	
F_0		538	A, C	M↔L	0.093	1	46	4.81	*	
height		538	A, C	M↔L	0.051	1	42	4.83	*	
diameter	M007G1	601	C, T	H↔Y	0.135	1	43	7.01	*	
stem volume		601	C, T	H↔Y	0.095	1	42	4.48	*	
T_m	M024	069	G, T	S↔I	0.106	1	43	5.28	*	
T_m		135	C, T	L↔P	0.128	1	43	6.58	*	
T_m	M024	191	A, G	A↔T	0.113	1	43	5.69	*	
I_{80}		194	A, C	K↔Q	0.124	2	42	3.7	*	
phenology		194	A, C	K↔Q	0.141	2	39	4.19	*	
I_{80}	M024	202	C, T	syn.	0.124	2	42	3.71	*	
phenology		202	C, T	syn.	0.142	2	39	4.24	*	
I_{80}		211	C, T	syn.	0.124	2	42	3.7	*	
phenology	M024	211	C, T	syn.	0.141	2	39	4.19	*	
F_0		318	A, C	intron	0.087	1	43	4.13	*	
F_v		318	A, C	intron	0.092	1	43	5.24	*	
F_m	M024	318	A, C	intron	0.091	1	43	5.08	*	
F_v		496	A, G	A↔T	0.077	1	43	4.33	*	
F_m		496	A, G	A↔T	0.087	1	43	4.83	*	
T_m	M024	496	A, G	A↔T	0.136	1	43	7.04	*	

Significance labels: see Table 4

³symbols and abbreviations: K – lysine, Q – glutamine, L – leucine, P – proline, I – isoleucine, H – histidine, M – methionine, R – arginine, D – aspartic acid, E – glutamic acid; otherwise see table 4

ble 6). Nevertheless, this has consequences for the interpretation of the association tests. The loci are not completely independent, the M002 sites 104 and 217 show significant linkage disequilibrium in both populations. The same applies (although in a smaller extent) to the associations with fitness-related traits: pairs of

sites in both M007G1 and M024 show significant linkage disequilibrium in the high-elevation provenance P49 (Table 5).

The effects of the mutations at polymorphic sites are derived from the protein sequences as given in the GenBank database (accession numbers DQ120079,

Table 6. Groups of loci associated with altitude and/or phenotypic traits, showing significant linkage disequilibria.

Provenance P1													
Marker	M002									M007C2		M007D1	
Site	104	121	129	165	217	254	306	413	510	308	132	463	
Marker	M007C2												
Site	207	256	258	269	333								
Marker	M007G1												
Site	219		194	202	211								
Marker	M024												
Site	69	135	191'	232									
Marker	M024												
Site	848	882											
Provenance P49													
Marker	M002									M007B2			
Site	104	121	129	165	217	254	306	413	510	302'			
Marker	M007B2												
Site	206'	252	302'										
Marker	M007C2												
Site	207	256	258	269									
Marker	M007G1												
Site	284'	498'	538	69	135	191	194	202	211	232	318	496	848 882

Dashed sites: loci showing LD with >50% of the remaining sites within the linkage group

DQ120081, DQ120083, DQ120084, DQ120089, DQ120092; Lamothe et al. 2006). As no sequencing of the produced proteins was done directly in *Picea abies*, the location of polymorphisms in genomic regions as well as their effects on aminoacid substitutions must be regarded as putative.

Discussion

Trees are often claimed to be a good model for adaptation studies, as local adaptation is an essential component of their life strategies (Petit & Hampe 2006). However, our experience based on the same material as that used in this study shows different. We analyzed the patterns of the responses to transfer of 11 domestic Norway spruce provenances (see Materials and methods) measured by fitness-related traits (height, diameter and volume growth, survival). The outcomes indicate that all provenances are adapted to the same optimum and no clear signs of local adaptation were identified (Gömöry et al. 2011). A study of physiological traits of the most contrasting provenances from this set (Gömöry et al. 2010) also revealed that frost resistance and photosystem II efficiency differ more in relation to the plantation site than the site of origin, indicating that the differences are caused by physiological acclimation rather than climate-driven natural selection. We were nevertheless curious whether any signs of adaptation can be identified in sequence that proved to have adaptive significance, although in a different but related species.

Several approaches are available for the studies of functionally important genetic variation (Gonzalez-Martinez et al. 2006; Neale & Ingvarsson 2008; Vasemägi & Primmer 2005). As our problem was detecting loci underlying adaptive phenotypic responses to climate (altitude was used as a proxy for temperature and

precipitation variation) and/or associated with fitness-related phenotypic traits such as growth and vegetative phenology, the first option was the association analysis, looking for non-random associations between genetic variation and climatic variables or phenotypic traits. This is based on the coincidence of clines or contrasts of allelic frequencies with environmental clines or contrasts.

Temperature and precipitation are the main climatic drivers of phenotypic variation patterns for forest trees. The genetic basis of adaptive phenotypes related to climate has been proved in common-garden experiments for many tree species (König 2005). Majority of the signals of adaptation among forest tree populations is thus expected to occur along climatic gradients which are usually identical with geographical clines. Most commonly, latitude has been taken as a surrogate for climate, as it correlates with many biologically relevant environmental gradients including photoperiod (Vasemägi & Primmer 2005). However, a plenty of studies (including those in forest trees) has demonstrated that neutral processes such as colonization, isolation by distance and genetic drift can also lead to the formation of clines or sharp contrasts in genetic variation (Ballian et al. 2006; Comps et al. 2001). Neutral mechanisms affect the whole genome including genes with adaptive significance, although their effects may vary from locus to locus. Taking a significant correlation between a geographical coordinate or environmental variable and allele or haplotype frequency as a proof of adaptation is therefore problematic, mainly in large-scale studies, because commonly there is no population-specific null expectation regarding to what extent neutral polymorphisms also show clinal pattern. Nevertheless, these problems become less important in small-scale studies such as ours. As stated by Vasemägi & Primmer (2005), scale is not directly related to

the physical distances among populations as such but rather depends on the spatial scale of dispersal and steepness of the environmental gradient. In our case, the number of populations is too small to speak about a cline or gradient. However, all available knowledge about the reach of gene flow by pollen in spruces indicates that it by far exceeds the distances between the studied provenances (Burczyk et al. 2004; O'Connell et al. 2007; Tollefsrud et al. 2009). We also do not dispose of information letting us suspect that bottlenecks, founder events or similar phenomena might have affected genetic structures in our material. Differentiation of spruce in Slovakia at neutral allozyme loci was reported to be very small (genetic distance among natural stands not exceeding 0.02; Gömöry 1992). Differentiation for some polymorphic sites in this study was considerably higher (see Table 4). Although we did not conduct a parallel analysis of differentiation at neutral loci, we suppose that selection is the only plausible explanation for such high differentiation levels.

During the last years, substantial advances in plant genomics have been achieved, mainly in model organisms and agricultural crops (*Arabidopsis*, maize, rice etc.). In spite of this rapid development, understanding of plant adaptation at the molecular level remains incomplete (Eckert et al. 2010). This is even more so in forest trees, where a long lifespan and organism size represent additional complications in the study of the genetic basis of phenotypic variation. In many cases, the function of proteins coded by genes containing potentially adaptive SNPs is unknown. This is actually the case of the markers used in this study. Quite many mutations putatively occurred in non-coding regions or are synonymous, but their locations and effects in translation are only derived from the comparison to homologous sequences in *Arabidopsis*. Lamothe et al. (2006) give information about the homology of marker sequences to those found in plant genomic databases, but it is questionable whether the proteins coded by these sequences fulfill the same function in spruce as in *Arabidopsis*. Quesada et al. (2008) who compared transcriptomes of *Populus* and *Arabidopsis* showed that except a small group of genes associated with essential functions, expression patterns diverged substantially. Considering this, we even cannot dare speculations what might be the physiological basis of the selective processes at loci that we suspect to be subjected to selection or what might be the mechanisms by which the identified loci affect phenotypic traits.

The presented results should be considered only a pilot study, regarding sample size in terms of the number of populations, trees per population and the number of markers, and needs extension. Both neutral marker studies and traditional approaches based on common-garden tests evaluation already reached their limits and hardly can fundamentally contribute to the understanding of adaptation. In forest trees, such understanding is desperately needed at least for two reasons: regulation of the transfer of forest reproductive material (which is still based on prejudices rather than knowledge of

the genetic and physiological basis and mechanisms of adaptation processes) and mitigation of climate change, already having serious consequences for the health condition of forests (see a large-scale dieback of spruce forests in Slovakia even in the montane and subalpine zones, i.e. in the core of the natural range; Ministry of Agriculture 2010). Any practical measures require knowledge about which genes are the most relevant for climatic adaptation, what are the physiological mechanisms of their phenotypic expression, and how their allelic variation (or variation of expression) is distributed over the range. This study should be considered one of the very first steps in this direction.

Acknowledgements

The study was supported by a research grant of the Slovak Agency for Research and Development APVV-0441-07. Data analysis was also supported by a grant of the Slovak Grant Agency for Science VEGA 1/0218/11.

References

- Ballian D., Longauer R., Mikić T., Paule L., Kajba D. & Gömöry D. 2006. Genetic structure of a rare European conifer, Serbian spruce (*Picea omorika* (Panc.) Purk.). *Plant. Syst. Evol.* **260**: 53–63.
- Burczyk J., Lewandowski A. & Chalupka W. 2004. Local pollen dispersal and distant gene flow in Norway spruce (*Picea abies* [L.] Karst.) *For. Ecol. Manage.* **197**: 39–48.
- Clausen J., Keck D.D. & Heisey W.M. 1939. The concept of species based on experiment. *Am. J. Bot.* **26**: 103–106.
- Collignon A.H., van de Sype H. & Favre J.M. 2002. Geographical variation in random amplified polymorphic DNA and quantitative traits in Norway spruce. *Can. J. For. Res.* **32**: 266–282.
- Comps B., Gömöry D., Letouzey J., Thiébaud B. & Petit R.J. 2001. Diverging trends between heterozygosity and allelic richness during postglacial colonization in the European beech. *Genetics* **157**: 389–397.
- Doyle J.J. & Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19**: 11–15.
- Eckert A.J., Bower A.D., Gonzalez-Martinez S.C., Wegrzyn J.L., Coop G. & Neale D.B. 2010. Back to nature: ecological genomics of loblolly pine (*Pinus taeda*, Pinaceae). *Mol. Ecol.* **19**: 3789–3805.
- Eckert A.J., Wegrzyn J.L., Pande B., Jermstad K.D., Lee J.M., Liechty J.D., Tearse B.R., Krutovsky K.V. & Neale D.B. 2009. Multilocus patterns of nucleotide diversity and divergence reveal positive selection at candidate genes related to cold hardiness in coastal Douglas fir (*Pseudotsuga menziesii* var. *menziesii*). *Genetics* **183**: 289–298.
- Flint H.L., Boyce B.R. & Beattie D.J. 1967. Index of injury – a useful expression of freezing injury to plant tissues as determined by the electrolytic method. *Can. J. Plant Sci.* **47**: 229–230.
- Geburek T. 2000. Effects of environmental pollution on the genetics of forest trees, pp 135–158. In: Young A., Boshier D. & Boyle T. (eds), *Forest Conservation Genetics, Principles and Practice*, CSIRO Publishing, Colingwood and CABI Publishing, Oxon.
- Giertych M. 1976. Summary results of the IUFRO 1938 Norway spruce (*Picea abies* (L.) Karst.) provenance experiment. Height growth. *Silvae Genet.* **25**: 154–164.
- Giertych M. & Oleksyn J. 1992. Studies on genetic variation in Scots pine (*Pinus sylvestris* L.) coordinated by IUFRO. *Silvae Genet.* **41**: 133–143.
- Gimeno T.E., Pias B., Lemos J.P. & Valladares F. 2009. Plasticity and stress tolerance override local adaptation in the responses

- of Mediterranean holm oak seedlings to drought and cold. *Tree Physiol.* **29**: 87–98.
- Gömöry D. 1992. Effect of stand origin on the genetic diversity of Norway spruce (*Picea abies* Karst.) populations. *For. Ecol. Manage.* **54**: 215–223.
- Gömöry D., Foffová E., Kmeť J., Longauer R. & Romšáková I. 2010. Norway spruce (*Picea abies* Karst.) provenance variation in autumn cold hardiness: adaptation or acclimation? *Acta Biol. Crac. Ser. Bot.* **52**: 42–49.
- Gömöry D., Longauer R., Hlásny T., Pacalaj M., Strmeň S. & Krajmerová K. 2011. Limited small-scale local adaptation in Norway spruce (*Picea abies* Karst.). *Eur. J. For. Res.* **131**: 401–411.
- Gonzalez-Martinez S.C., Krutovsky K.V. & Neale D.B. 2006. Forest-tree population genomics and adaptive evolution. *New Phytol.* **170**: 227–238.
- Grattapaglia D., Plomion C., Kirst M. & Sederoff R.R. 2009. Genomics of growth traits in forest trees. *Curr. Opin. Plant Biol.* **12**: 148–156.
- Heuertz M., De Paoli E., Kallman T., Larsson H., Jurman I., Morgante M., Lascoux M. & Gyllenstrand N. 2006. Multi-locus patterns of nucleotide diversity, linkage disequilibrium and demographic history of Norway spruce [*Picea abies* (L.) Karst.]. *Genetics* **174**: 2095–2105.
- Kawecki T.J. & Ebert D. 2004. Conceptual issues in local adaptation. *Ecol. Lett.* **7**: 225–234.
- König A. 2005. Provenance research: evaluation the spatial pattern of genetic variation, pp. 275–334. In: Geburek T. & Turok J. (eds), *Conservation and Management of Forest Genetic Resources in Europe*, Arbora Publishers, Zvolen and IPGRI, Rome.
- Krutzsch P. 1974. The IUFRO 1964/8 provenance test with Norway spruce (*Picea abies* [L.] Karst.). *Silvae Genet.* **23**: 58–62.
- Krutzsch P. 1992. IUFRO's role in coniferous tree improvement – Norway spruce (*Picea abies* (L.) Karst.). *Silvae Genet.* **41**: 143–150.
- Lagercrantz U. & Ryman N. 1990. Genetic structure of Norway spruce (*Picea abies*): concordance of morphological and allozymic variation. *Evolution* **44**: 38–53.
- Lamothe M., Meirmans P. & Isabel N. 2006. A set of polymorphic EST-derived markers for *Picea* species. *Mol. Ecol. Notes* **6**: 237–240.
- Lindgren D., Paule L., Shen X.H., Yazdani R., Segerstrom U., Wallin J.E. & Lejdebö M.L. 1995. Can viable pollen carry Scots pine genes over long distances? *Grana* **34**: 64–69.
- Linhart Y.B. & Grant M.C. 1996. Evolutionary significance of local genetic differentiation in plants. *Annu. Rev. Ecol. Syst.* **27**: 237–277.
- Mátyás C. 1996. Climatic adaptation of trees: rediscovering provenance tests. *Euphytica* **92**: 45–54.
- Mimura M. & Aitken S.N. 2010. Local adaptation at the range peripheries of Sitka spruce. *J. Evol. Biol.* **23**: 249–258.
- Ministry of Agriculture 2010. Report on the Status of Forestry in the Slovak Republic of 2009. Ministry of Agriculture and Rural Development of the Slovak Republic, Bratislava and National Forest Centre – Forest Research Institute Zvolen, 128 pp.
- Namroud M.C., Beaulieu J., Juge N., Laroche J. & Bousquet J. 2008. Scanning the genome for gene single nucleotide polymorphisms involved in adaptive population differentiation in white spruce. *Mol. Ecol.* **17**: 3599–3613.
- Neale D.B. & Ingvarsson P.K. 2008. Population, quantitative and comparative genomics of adaptation in forest trees. *Curr. Opin. Plant Biol.* **11**: 149–155.
- Neale D.B. & Kremer A. 2011. Forest tree genomics: growing resources and applications. *Nature Rev. Genet.* **12**: 111–122.
- Nowakowska J.A. 2009. Mitochondrial and nuclear DNA differentiation of *Picea abies* populations in Poland. *Dendrobiology* **61(suppl.)**: 119–129.
- O'Connell L.M., Mosseler A. & Rajora O.P. 2007. Extensive long-distance pollen dispersal in a fragmented landscape maintains genetic diversity in white spruce. *J. Hered.* **98**: 640–645.
- Petit R.J. & Hampe A. 2006. Some evolutionary consequences of being a tree. *Annu. Rev. Ecol. Syst.* **37**: 187–214.
- Petráš R. & Pajtík J. 1991. Sústava česko-slovenských objemových tabuliek drevín. *Lesn. čas.* **37**: 49–56.
- Quesada T., Li Z., Dervinis C., Bock P.N., Tuskan G.A., Casella G., Davis J.M. & Kirst M. 2008. Comparative analysis of the transcriptomes of *Populus trichocarpa* and *Arabidopsis thaliana* suggests extensive evolution of gene expression regulation in angiosperms. *New Phytol.* **180**: 408–420.
- Raymond M. & Rousset F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* **86**: 248–249.
- SAS 2010. SAS®STAT User's Guide. http://support.sas.com/documentation/onlinedoc/91pdf/sasdoc91/stat_ug_7313.pdf
- Savolainen O., Pyhäjärvi T. & Knurr T. 2007. Gene flow and local adaptation in trees. *Annu. Rev. Ecol. Evol. Syst.* **38**: 595–619.
- Schneider S.D., Roesli D. & Excoffier L. 2000. ARLEQUIN, version 2.0: a software for population genetic data analysis. University of Geneva, Geneva, 111 pp.
- Sperisen C., Buchler U., Gugerli F., Matyas G., Geburek T. & Vendramin G.G. 2001. Tandem repeats in plant mitochondrial genomes: application to the analysis of population differentiation in the conifer Norway spruce. *Mol. Ecol.* **10**: 257–263.
- Tajima F. 1983. Evolutionary relationship of DNA sequences in finite populations. *Genetics* **105**: 437–460.
- Tollefsrud M.M., Kissling R., Gugerli F., Johnsen Ø., Skrøppa T. et al. 2008. Genetic consequences of glacial survival and postglacial colonization in Norway spruce: combined analysis of mitochondrial DNA and fossil pollen. *Mol. Ecol.* **17**: 4134–4150.
- Turesson G. 1925. The plant species in relation to habitat and climate. *Hereditas* **6**: 147–236.
- Vasemägi A. & Primmer C.R. 2005. Challenges for identifying functionally important genetic variation: the promise of combining complementary research strategies. *Mol. Ecol.* **14**: 3623–3642.
- Vendramin G.G., Anzidei M., Madaghiele A., Sperisen C. & Bucci G. 2000. Chloroplast microsatellite analysis reveals the presence of population subdivision in Norway spruce (*Picea abies* K.). *Genome* **43**: 68–78.
- Vitasse Y., Delzon S., Bresson C.C., Michalet R. & Kremer A. 2009. Altitudinal differentiation in growth and phenology among populations of temperate-zone tree species growing in a common garden. *Can. J. For. Res.* **39**: 1259–1269.
- Wachowiak W., Balk P.A. & Savolainen O. 2009. Search for nucleotide diversity patterns of local adaptation in dehydrins and other cold-related candidate genes in Scots pine (*Pinus sylvestris* L.). *Tree Genet. Genomes* **5**: 117–132.
- Wright J.W. 1976. *Introduction to Forest Genetics*. Academic Press, London, 463 pp.

Received September 9, 2011

Accepted April 12, 2012