GENETIC VARIATION OF NORWAY SPRUCE (*PICEA ABIES* [L.] KARST.) POPULATIONS IN AUSTRIA

II. MICROSPATIAL PATTERNS DERIVED FROM NUCLEAR SEQUENCE TAGGED MICROSATELLITE SITES

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ABSTRACT

In three putatively autochthonous, high elevated Norway spruce populations, microspatial genetic pattern was studied by using three nuclear sequenced tagged microsatellite sites (STMS). In total, 273 trees were genotyped in the Austrian populations. Different measures such as number of alleles per locus (range: 28–38), effective allele number or gene pool diversity v (range: 7.6–9.7) (GREGORIUS 1978) and total population differentiation δ_T (range: .88–.90) (GREGORIUS 1987) indicated a high amount of genetic variation. Expectedly genetic distances among populations were also high. Population differentiation δ reached .33. Measures were several folds higher than measures based on isozyme data. Spatial patterns of STMS variation were studied by spatial autocorrelation using *Autocorrelation Indices for DNA Analysis* (*AIDA*) (BERTORELLE & BARBUJANI 1995). Genetic variants were mainly randomly distributed in space. At a certain STMS in one population significant positive *II*-values in short distance classes and negative *II*-values in long-distance classes were obtained. This pattern was interpreted as the likely result of selection rather than due to limited gene flow.

Key words: *Picea abies*, isozymes, SSR, STR, STMS, microsatellite, differentiation, AIDA, spatial autocorrelation.

INTRODUCTION

Norway spruce (*Picea abies*) is one of the most important conifers in Eurasia. Its natural distribution ranges from northern Scandinavia to southern areas of the Balkan Peninsula or from eastern France to far beyond the Ural Mountains (SCHMIDT-VOGT 1977, *l.c.* pp.164 –242). In *Picea abies* much genetic data have been accumulated based on field trials (*e.g.*, KRUTZSCH 1992) or were derived from experiments performed under controlled conditions (*e.g.*, KOSKI 1994).

Morphological markers of *Picea abies* have been used in the early fifties (LANGNER 1953) and roughly two decades later allozymes were for the first time introduced to forest genetics by studying the genetic control of multiple forms of esterase in this conifer (BARTELS 1971). Meanwhile an exhaustive number of allozyme papers in Norway spruce are available (see KRUTOVSKII & BERGMANN 1995 for review). For instance data on mating system and gene flow (PAULE *et al.* 1993), disgenic disequilibrium (GEBUREK 1998), microspatial (LEONARDI *et al.*1996) and macrospatial pattern (LAGERCRANTZ & RYMAN 1990) or selection processes (RADDI *et al.* 1994) have been published.

The using of restriction enzymes (BOTSTEIN *et al.* 1980) and the introducing of polymerase chain reaction (PCR, MULLIS *et al.* 1986) have provided a variety of different DNA markers for plant analysis (KARP *et al.* 1997). One of the most promising marker types in plant genetics are sequence tagged mircosatellite sites (STMS). Microsatellites are simple sequence repeats (SSR), sometimes also called short tandem repeats (STR).They are arrays of very short, 1–5 bp long repetitive units and reach normally a total length smaller than 200 bp and it is likely that they are well distributed in the eukaryotic genome. Microsatellites supposedly originate from DNA slippage. Number of repeats is highly variable and can be detected by PCR-amplification of the DNA stretch including the microsatellite using primers specific to the flanking regions.

Considering tree species, STMS have been identified for instance in *Citrus spp.* (KIJAS *et al.* 1995), *Dryobalanops lanceolata* (TERAUCHI 1994), *Gliricidia sepium* (DAWSON *et al.* 1997), *Larix spp.* (VOLKAERT 1995), *Picea abies* (MORGANTE *et al.* 1996, PFEIFFER *et al.* 1997), *Pinus radiata* (SMITH & DEVEY 1994), *Pinus strobus* (ECHT *et al.*, 1996), *P. sylvestris* (KOSTIA *et al.* 1995, SORANZO *et al.* 1998), *Pithecellobium*

elegans (CHASE *et al.* 1996), *Quercus macrocarpa* (DOW *et al.* 1995), and *Q. petrea* (STEINKELLNER *et al.* 1997). Despite their high potential usefulness, these markers have still not often been used in population or ecological genetics. KARHU *et al.* (1996) compared the differentiation pattern in *Pinus sylvestris* by using different marker types and CHASE *et al.* (1996) tested the STMS they developed in two tropical tree (*Pithecellobium elegans*) populations.

In this paper, we studied three autochthonous Norway spruce (*Picea abies*) populations by using three nuclear STMS. Results on genetic differentiation among and within populations are presented and were discussed *inter alia* in comparison with isozyme data.

MATERIAL AND METHODS

A detailed description of the three Austrian *Picea abies* populations which are called POP–1, –2, –3 was already given in a previous paper (GEBUREK 1998). In total, 51 (POP–1), 129 (POP–2), and 93 trees (POP–3) were used for this study.

DNA was extracted from megagametophytes according to HEINZE *et al.* (1996). Genomic DNA was pooled from megagametophytes collected from single trees. For roughly 90% of the specimens 10 megagametophytes were pooled while for the remaining 10% of trees a smaller number was used. However, DNA from at least 6 megagametophytes was analysed and amplified by PCR. Three out of the 7 STMS representing single polymorphic markers in Norway spruce (PFEIFFER *et al.* 1997) proved to provide interpretable fragment patterns in our material. The following PCR-primer sequences were used to amplify STMS markers:

SpAGC1: 5'TTCACCTTAGCCGAGAACC3'/
5'CACTGGAGATCTTCGTTCTGA3'
SpAGC2: 5'TACCATTCAACGCAAGGG3'/
5'GTGTATGGTTTTCTTTTCGCA3'
SpAGG3: 5'CTCCAACATTCCCATGTAGC3'/
5'AGCATGTTGTCCCATATAGACC3'.

A 12.5 µl PCR reaction contained: 0.5 units of DNA polymerase (DynaZyme II from Finnzymes Oy), 200 µM of each dNTP (Pharmacia), 2.5 mM $MgCl_2$, 0.2 µM per each primer, 1–10 ng of genomic DNA. Samples were overlaid with one droplet of mineral oil (Perkin Elmer). Amplifications were performed in MJ Research PTC 100 thermocycler using 96 well plates (Thermowell, Costair) with the following amplification profile: 5 min 95 °C, 4 min 80 °C, 37 cycles (45 s 94 °C, 45 s 57 °C, 45 s 72 °C), 10 min 72 °C. Three µl of a USB denaturing stop solution (95 % formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05 % xylene cyanol) and 3 µl of internal standard (50 and 230 bp long DNA fragments) were added to 4 µl of the amplified samples and denatured at 80 °C for 5 minutes just before loading. The polyacrylamide gels were prepared on film carriers (GelBond PAG, FMC) containing 20 ml of Long Ranger 50% (w/v) (FMC 50610), 12 ml of 10× TBE (Tris-borate, EDTA), 0.7 M urea, 0.5 ml 10% (w/v) ammonium persulphate (APS), 50 µl N,N,N', N'-tetramethyletylenediamine (TEMED) in 100 ml gel solution. The amplified samples were separated electrophoretically on a preheated (40 °C) DUAL SLAB Gel Unit, model DSG-250 (CBS), gel size 14 × 26 cm, at 1000 V for 1.5 hours.

After electrophoresis the gels were washed with H_2O (three times) to remove carbamide, and were fixed with 10% ethanol and 0.5% glacial acetic acid for 30 min. Gels were silver stained in a 5.89 mM $AgNO_3$ solution for 45 to 60 min, washed twice in H_2O, incubated for 15 min in a solution of 0.375M NaOH, 2.6 mM $NaBH_4$, 0.5 mM HCHO, and then treated in 0.071 M Na_2CO_3 for 10 min.

Electronic images of the gels were taken by an UVP GDS 7500 camera system. The size of the DNA fragments were determined by the RFLP scan software (Scanalytics) using the internal DNA length standards (50 and 230 bp long DNA fragments included in each lane).

Allozyme data of 16 gene loci obtained for the same trees from the same three populations were used for comparisons. Details can be found in the previous paper (GEBUREK 1998).

Population analysis

The following measures of genetic variation within populations were employed: gene pool diversity v, which measures the mean effective number of alleles (GREGORIUS 1978), total population differentiation δ_T (GREGORIUS 1987), and gene pool distance (arithmetic mean over single locus distances) (GREGORIUS 1984). As measures of genetic variation among the populations, population differentiation D and δ were used (GREGORIUS & ROBERDS 1986). The calculations were done by using the GSED software (GILLET 1994). Since certain measures are sensitive to different sample sizes, a reduced data set was additionally used. This set consisted of equal sample sizes (N = 51) for all populations, disregarding of the marker type (STMS, isozymes) used.

The microspatial pattern was analyzed using BERTORELLE & BARBUJANI's (1995) approach for DNA data. Each locus was represented by 2 haplotypes binarically coded. For each populations 10 distance classes (0 – <10 m, 10 – <20 m, ... >90 m) were chosen based on individually spatial coordinates (see GEBUREK

$$II = \frac{n \sum_{i=1}^{n-1} \sum_{j>i}^{n} w_{ij} \sum_{k=1}^{S} (p_{ik}-\bar{p}_k)(p_{jk}-\bar{p}_k)}{W \sum_{i=1}^{n} \sum_{k=1}^{S} (p_{ik}-\bar{p}_k)^2}$$

1998) for each distance class *AIDA II* was calculated as in which n is the sample size, W is the number of pairwise comparisons in the distance class of interest, p_{ik} and p_{jk} are the binaric genotypes of the i^{th} and j^{th} tree, respectively, at the k^{th} site, $\bar{p}_k$ is the k^{th} element of the average vector (BERTORELLE & BARBUJANI 1995). If the trees i and j fall in the distance class of interest, then the weight w_{ij} is 1 and 0 otherwise. Summation is over S polymorphic sites, and for all n trees in the sample. *II* value ranges from ≥−1 to ≤ +1 for large samples sizes (n) and E(*II*) equals to $-1/(n-1)$. A permutation analysis was done to evaluate significance. Binaric genotypes were randomly distributed to sampled localities and AIDA-values were computed for all distance classes. This procedure was redone 1000 times to construct confidence limits for each distance class. Genetic similarity among trees within the population is shown by positive values and *vice versa* genetic dissimilarity is shown by negative *II*-values. Further details are found in BERTORELLE & BARBUJANI (1995).

RESULTS AND DISCUSSION

A very high number of different alleles were detected at the STMS. For all populations 28 alleles were found in total at SpAGC1, 38 at SpAGC2, and 34 at SpAGG3, respectively. Many alleles occurred at very low frequencies and were specifically found in certain populations. This is also indicated by the mean average effective number of alleles v, which is much smaller (range from 7.6 to 9.7) than the observed number (Table 1). Interestingly, the same order of rank of the populations were observed disregarding whether STMS or isozymes were considered. Thus POP–3 was characterized by the greatest estimates of genetic variation, while POP–1 and POP–2 were similar and less variable than POP–3 (Table 1). This holds true disregarding whether all trees in the populations were considered or a reduced data set (identical sample size for all populations) was used. The number of alleles at STMS varies strongly. For instance in the tropical tree species, *Pithecellobium elegans,* 5 STMS were studied in 52 specimens originating from two localities and number of alleles found varied from 1 to 12 (CHASE *et al.* 1996) and in *Quercus petrea* in 17 STMS, allele number varied from 6 to 13 (STEINKELLNER *et al.* 1997). Expectedly, absolute estimates of gene pool diversity and total population differentiation in our study was much higher for STMS than for isozymes even though for the latter group of markers exclusively polymorphic loci were used (Table 1). Recalling the high number of alleles in the three populations, many alleles were found only in single trees. Hence, the allelic structures were characterized by a high proportion of alleles with even frequencies.

Our findings are similar to those in other conifers. SMITH & DEVEY (1994) studied the variation at 2 STMS in 40 *Pinus radiata* specimens derived from 4 localities. Expected heterozygosity (H_e) amounted to roughly .70 in their study. Comparable estimates (H_e = .77) were calculated from two *P. sylvestris* samples (N_{total} = 50) based on 2 STMS in a Finnish study (KARHU *et al.* 1996). These estimates for the two *Pinus* species are both much higher than typical isozyme based values ranging up to .2 or .3. Also in 2 populations of *Pithecellobium elegans,* H_e averaged .65, while estimates based on isozyme data were less than half (CHASE *et al.* 1996). Our δ_T values for single markers varied from .734 to .934 in single populations and were thus slightly higher than the H_e values in above-mentioned studies using STMS markers. The higher estimates of this study may be due to bigger sample sizes (up to 129 trees were used). Accordingly, the gene pool distances among the three populations based on STMS were very high and reached up to .44 (.518 reduced data set), whilst isozyme based values of identical trees did not exceed .026 (.037 reduced data set) (Table 2). As far as STMS variation among populations is concerned, gene pool differentiation δ reached .332 (.398

Table 1. Gene pool diversity υ and total population differentiation δ_T of the gene pool in three *Picea abies* populations (POP-1, POP-2, POP-3) based on three STMS and 16 isozyme markers. Values in brackets refer to adjusted, i.e., equal sample sizes (reduced data set).

Parameter	Marker	POP–1	POP–2	POP–3
υ	STMS	7.60 (7.60)	7.60 (6.30)	9.67 (8.83)
	Isozymes	1.20 (1.19)	1.20 (1.18)	1.21 (1.23)
δ_T	STMS	.877 (.877)	.872 (.850)	.902 (.896)
	Isozymes	.165 (.159)	.165 (.153)	.175 (.187)

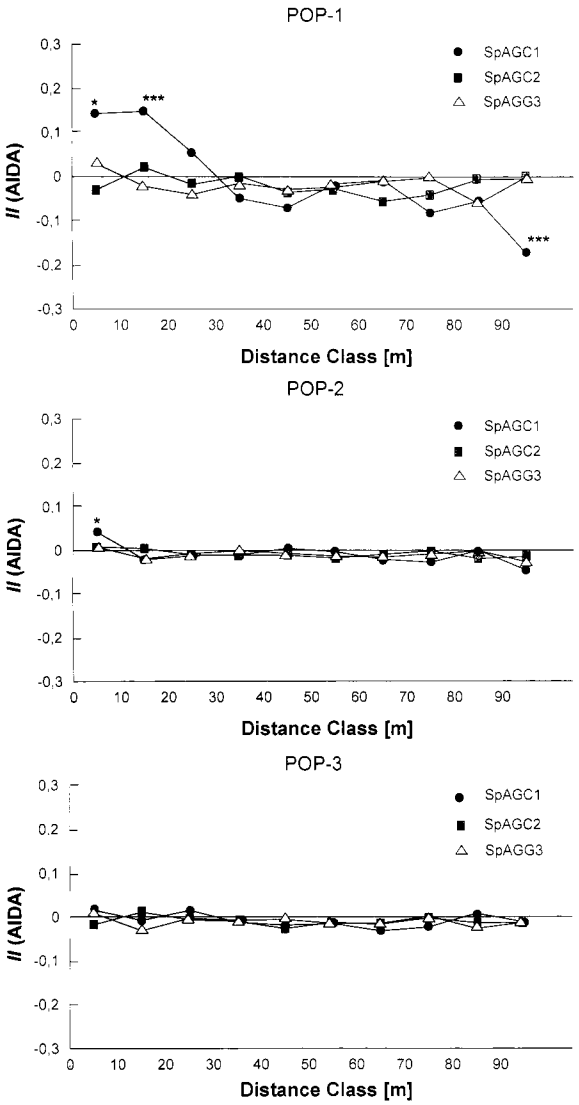

Figure 1. Correlograms for three *Picea abies* populations (POP–1, POP2, POP–3) based on three STMS markers. Autocorrelation indices *II* (AIDA) (BERTORELLE & BARBUJANI 1995) were plotted at the upper limits of distance classes (* 5% significance level, *** 0.5% significance level).

reduced data set) in the three populations, while for isozyme data .021 (.029 reduced data set) was calculated (Table 3). Conversely, in *Pinus sylvestris* isozyme variation among populations was higher than estimates based on 2 STMS, however number of populations studied using isozyme loci was unequal to those studied using STMS markers (KARHU *et al.* 1996). Non coding parts of the genome harbor much more mutations and thus carry much more alleles at STMS than structural genes, such as isozymes, do. Allozymes may be selectively constrained in ways that non coding region are not (see CLEGG 1989 for review). Balancing selection acting on isozymes loci can result in an overestimation of genetic similarity among populations compared to

Table 2. Gene pool distance between three *Picea abies* populations (POP–1, POP2, POP–3) based on three STMS (below diagonal) and 16 isozyme markers (above diagonal). Values in brackets refer to adjusted, *i.e.*, equal sample sizes (reduced data set).

	POP–1	POP–2	POP–3
POP–1	–	.021 (.026)	.025 (.035)
POP–2	.441 (.518)	–	.026 (.037)
POP–3	.357 (.363)	.355 (.459)	–

strictly neutral loci (ALTUKHOV 1991). On the other hand, heterozygote instability will inflate STMS diversity more than the allozymic ones (AMOS & HARWOOD 1998). Hence estimates of absolute and relative genetic variation are expected to be different when different genetic markers are used.

The vast majority of autocorrelation indices showed that genetic variation was randomly distributed in space. Most *AIDA II*-values were not significantly different from random expectations (Fig. 1). Although this is true with respect to the total number of indices times the number of distance classes times the number of loci times the number of populations, this may be misleading in terms of the number of loci times the number of populations. From nine cases of locus/population combinations significant deviations from random distribution were found in two of these. Locus SpAGC1 indicated a non-random spatial pattern both in POP–1 and POP–2. Thus, in POP–1 significant positive *II*-values in short distance classes and a significant negative *II*-value in a long distance classes were obtained [$II_{0\text{–}10m} = .144$ ($p < .05$), $II_{10\text{–}20\,m} = .149$ ($p < .0005$), $II_{>90\,m} = -.172$, ($p < .0005$)] and in POP–3 a significant case was found in the distance class 0–10 m [$II_{0\text{–}10m} = .044$ ($p < .05$)] (see Fig.1). However, it should be mentioned here, that $II_{0\text{–}10m}$ in POP–1 was based on only 39 pairs. Because of edge effects correlograms should be truncated when the number of pairs is less than 30 in a distance class (D. WARTENBERG, personell comm., cited in WASER & MITCHELL 1990). In all other distances classes with significant indices number of pairs exceeded 100. Although significant deviations from random spatial pattern based on that locus were only found in four out of 30 distance clases (3 populations times 10 distance classes each), or approximately 10%, three of four cases were found only in close distance clases of the most closely located trees with significant and positive *AIDA II* indices showing non-random positive association of similar genotypes among close neighbors, where one may expect the highest positive association of similar genotypes, whilst the forth case was for a long distance

Table 3. Measures of genetic differentiation *D* and *δ* among three *Picea abies* populations (POP–1, POP–2, POP–3) based on three STMS and 16 isozyme markers, respectively. Values in brackets refer to adjusted, *i.e.*, equal sample sizes (reduced data set).

Marker	*D*			*δ*
	POP–1	POP–2	POP–3	
STMS	.375	.361	.260	.332
	(.400)	(.473)	(.320)	(.398)
Isozymes	.019	.020	.024	.021
	(.025)	(.026)	(.035)	(.029)

class with a significant and negative *AIDA II* index showing non-random positive association of nonsimilar (unlike) genotypes. However, absolute significant *AIDA II* values were small. This suggests a weak non-random spatial pattern for locus SpAGC1. Theoretically several population genetic processes can produce deviation from spatial randomness as discussed earlier in the preceeding paper of this series (GEBUREK 1998). Generally geneticists who analyze gene-frequency surfaces based on a set of population specific allele frequencies would like to determine which correlograms indicate gene flow, reflect selection, and which are a corollary of isolation-by-distance. Unfortunately, spatial correlograms are affected by several sources of variation as pointed out by SLATKIN & ARTER (1991): (1) sampling variation, (2) stochastic variation – sometimes also called realization variance – caused by unpredictable events in the history of each allele, (3) parametric variation caused by differences among processes governing allele frequencies at different loci, and (4) variation caused by differences in initial conditions and it is difficult to differentiate among evolutionary causes. Another problem is, that currently no tests are available to determine significant differences among different population and marker specific correlograms. When – as in the present study – non-random spatial pattern is weak, conclusions become even more difficult to draw. Having these limitations in mind, it is proposed that the overall pattern is not due to restricted gene flow, *i.e.*, a patchy structure of genetically (over all loci) similar specimens due to limited pollen and/or gene flow (cf. WRIGHT 1943) and SpAGC1 pattern in POP–1 may be due to microselection.

Few studies on the genetic neighborhood have been done in *Picea abies*. While BRUNEL & RODOLPHE (1985) detected a slight but significant correlation between genetic relationship and topographic distance, spatial autocorrelation based on isozymes and DNA polymorphism resulted in no or in a weak non-random structure. In an Italian allozyme study, in most cases a random distribution of genotypes were found and less than 11 % of genotype pairs showed positive association in the short distance class (up to 10 m). On a small spatial scale, certain genotypes appear in clumps (LEONARDI *et al.* 1996). Based on 20 mapped random amplified polymorphic DNA marker, the microspatial pattern in Norway spruce was analyzed by BUCCI & MENOZZI (1995). Positive associations in short distance classes were found for certain markers, however most genotypes were randomly distributed. Outcrossing rate in *Picea abies* is high (*e.g.*, PAULE *et al.* 1993) and pollen and seed dispersal are easily distributed over distances of 20–30 m. Clumping of genetically similar specimens are therefore not to be expected unless microselection has acted. In a previous paper (GEBUREK 1998) the three Austrian spruce populations have been already investigated by means of isozyme and spatial autocorrelation analyses. While a higher number of significant standard normal variates were detected than could be expected on a 5 % level, the overall picture indicated spatial randomness. Comparisons between STMS and isozyme based data is complicated by the fact that it is not possible to use a common identical spatial analysis. Theoretically STMS alleles can be also analysed by MORAN's (1950) index, which was used in GEBUREK's (1998) earlier paper. However, due to the very high number of alleles found at STMS loci and the much smaller sample size, single allele frequency were extremely small which unavoidably would exclude most alleles from the analysis. Hence, comparisons between the two studies must remain limited.

CONCLUSIONS

1. The high number of alleles found at the three STMS makes this type of marker very effective to describe and differentiate Norway spruce populations. The markers can also be effectively used for studying the reproductive pattern.

2. Intrinsic difference of STMS compared to isozyme data causes different results regarding the genetic variation.

3. A single STMS showed that a patchy genetic structure was present within populations. This finding contrasts the random distribution of genes found in the same populations at other STMS and isozyme markers. Though it cannot be excluded that this result is due to statistical artefacts, it is not unlikely that STMS can detect a more subtle spatial variation pattern than isozymes. It may be too early to recommend minimum distances among seed bearing trees that are to be

harvested but unless contradictory results have been found it appears wise to regard 30 m as the minimum distance especially when only a small number of trees in a single stand are harvested.

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