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High morphological differentiation in crown architecture is weakly reflected in the population genetic structure of German Norway spruce stands

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Abstract: High elevation sites in the low mountain ranges in Germany are naturally covered by Norway spruce (*Picea abies* [Karst.] L.) stands. Historically, large scale anthropogenic range expansion starting in the mid to late 18th century had a huge impact on the forest composition throughout Germany. Utilisation and exploitation often led to artificial regeneration, mostly carried out using seeds from allochthonous provenances. Usually autochthonous high elevation Norway spruce trees have narrow crown phenotypes, whereas lowland trees have broader crowns. Narrow crown phenotypes are likely due to the adaptation to heavy snow loads combined with high wind speeds. In the present study, neighbouring stand pairs of putative autochthonous and allochthonous origin with contrasting phenotypes in high elevation sites were investigated with 200 samples each. These stands are located in the Ore Mountains, the Thuringian Forest, and the Harz Mountains. Additionally, a relict population with the typical narrow high elevation phenotypes was sampled in Thuringia, known as "Schlossbergfichte". The objective of the study was to quantify supposedly adaptive phenotypic differences in crown architecture and the genetic differentiation at eleven putatively neutral nuclear microsatellite markers or simple sequence repeats (nSSRs). The high differentiation at morphological traits (P_{ST} = 0.952-0.989) between the neighbouring autochthonous and allochthonous stands of similar age contrasts with the very low neutral genetic differentiation (F_{ST} = 0.002-0.007; G''_{ST} = 0.002-0.030), suggesting that directional selection at adaptive gene loci was involved in phenotypic differentiation. Comparing the regions, a small isolation by distance effect for the Harz mountains was detected, suggesting landscape resistance restricting gene flow. Finally, the differentiation of the very old autochthonous (up to 250 years) stand "Schlossbergfichte" with typical high elevation phenotypes could cohere with the sampling of a relict genepool.

Keywords: *Picea abies*, microsatellites, crown architecture, morphology, phenotypic adaptation, genetic differentiation

1. Introduction

Genetic variation at neutral genetic markers across the distribution range of Norway Spruce (*Picea abies* [Karst.] L.) is very high, but genetic differentiation is usually relatively low [1,2] and contrasts with the high phenotypic differentiation of crown architecture between low and high elevation varieties [3–5]. Autochthonous high elevation stands are characterised by narrow crowned individuals as a result of potential adaptation to heavy snow loads, while in allochthonous stands of the same region broad crown shapes are prevailing. High differentiation at phenotypic traits between neighbouring stands in the same environment but low differentiation at randomly selected selectively neutral markers would suggest directional selection on genes related to these phenotypic traits [6], which are crown architecture traits in our study.

Norway spruce is one of the economically most important tree species in Germany and has been widely planted since the late 18th century [7]. However, some autochthonous stands are still present in the low mountain ranges at higher elevations. One of these rare stands is located in Thuringia known as "Schlossbergfichte". This stand is characterised by very old native trees (up to 250 years) with typical narrow crown phenotypes, whereas trees of neighbouring allochthonous stands mostly show the broad low elevation phenotypes.

Before extensive translocation of seeding material occurred [8], variation in crown phenotypes and typical narrow crown phenotypes were described in natural spruce stands from northern latitudes [9,10]. Crown characteristics are crucial for the resistance to snow breakage and high wind speeds [11]. Hence, trees with a narrow crown shape are considered to be more adapted to climatic conditions in high elevations or regions with high snow loads [7,12–14]. This assumption is also supported by the finding of higher frequencies of narrow crowned trees in areas with high snow break hazard [15]. Additionally, crown architecture is associated with temperature, altitude and precipitation [13], and a relation to temperature and elevation gradient had previously been reported [4,7]. Finally, trees with high elevation phenotypes showed a higher frost hardiness than low elevation phenotypes, while both "morphotypes" had sufficient frost tolerance to prevent late frost damage [16].

Sylvén [17] was one of the first who suggested that variation in crown shapes is heritable. The occurrence of neighbouring autochthonous and allochthonous stands with different crown shapes is a further indication of heritability of crown shapes. However, heritability estimates are not available from experimental trials. Kiellander [18] refers to a crossing experiment in Sweden 1942 and points to the heritability of crown architecture. In a formal way, inheritance of crown architecture was only assessed for the pendulous variety of Norway spruce (*Picea abies f. pendula*). Segregation ratios in open pollinated progenies suggested the control by a single or by a few dominant genes [19] and linkage to a RAPD (random amplified polymorphic DNA) marker [20].

Even though genetic differentiation across the species range is low, historical migration patterns can be reconstructed using neutral genetic markers and other evidence such as pollen data. Pollen data suggest that the first larger populations in the Holocene occurred within present day Germany in the Alps around 9-8 Ka BP [21]. Macrofossils and pollen give evidence for several potential refugia during the last glacial maximum, some of which had no contribution to the recolonization history, such as the Massif Central (France) or the Moldavian lowlands refugia [22]. Concurrently, different refugia are proposed as source of re-immigration, as *P. abies* can be divided in two main (Baltico Nordic and Alpine Central Europe) and one minor (Carpathian) domain [2,23–27]. Combined analysis from mtDNA (mitochondrial DNA) and pollen data reveal a more detailed view that suggest at least seven refugia from which recolonization occurred [28]. Despite this distinction even on larger geographical scales of several hundred kilometres the level of inter-population differentiation at nuclear markers remains relatively low, which is likely caused by high rates of pollen mediated gene flow [29–32].

Genetic and morphological analyses of neighbouring Norway Spruce populations which are morphologically differentiated in their crown architecture at the same altitudinal level are rare. The study by Greger [33] is the only example for an investigation of a possible link between crown morphology and genetic variation. More common are studies comparing stands or individuals along

altitudinal transects, characterising them, if at all, only on the stand level. Results from different sources showed no consistent pattern of genetic variation between low and high elevation stands. For example, Maghuly et al. [34] found a higher genetic diversity in high elevation type populations than in allochthonous middle and low elevation populations at nuclear microsatellites and mitochondrial markers. The authors further found higher expected heterozygosity in older stands than in younger stands at the nuclear simple sequence repeats (nSSR). However, other studies indicate no relationship between altitude and diversity [30] or the highest diversity in populations from intermediate elevations [35].

For the first time we identified neighboring populations with different crown architectures in the same high elevation environment located in three regions of the low mountain range in Germany.

Our objectives were to quantify phenotypic differences in crown architecture and genetic differentiation at nSSR markers between these neighbouring populations.

We addressed the following hypotheses:

1. High phenotypic differentiation (P_{ST}) between neighbouring autochthonous and allochthonous stands contrasts with low genetic differentiation at random neutral markers as signature of local adaptation.

2. The genetic structure of the stands is correlated to their phenotypic variation. This could only be expected if adaptive processes shaped the genetic structures significantly, and our neutral genetic markers are by chance linked to adaptive loci.

3. The genetic structure of the stands is correlated to their geographic location. This could be expected, if genetic structures are mainly affected by natural or artificial distribution of genetic information in space. Naturally, for example, such genetic structures can result from isolation by distance (IBD). Artificially, such pattern can result, if material of regionally different seed sources was planted.

Our results will be discussed with regard to the human influence on genetic structures and the phenotypic variation of *P. abies*.

2. Materials and Methods

Study sites and sampling

A total of 1325 adult trees were sampled in seven stands growing in altitudes from 770 to 1060 m a.s.l. Selected stands are located in the low mountain ranges of the Thuringian Forest, the Ore Mountains (Saxony) and the Harz Mountains (Lower-Saxony/Saxony-Anhalt), all being part of the Central German Uplands (Figure S1, a-d). In each region, two neighbouring stands were sampled, which represent two groups with different morphological characteristics of Norway spruce. The first group consists of narrow crowned mountain spruce phenotypes (high elevation type, HE) and the second group comprises lowland phenotypes with typical broad crowns (low elevation type, LE). In each stand terminal branches from 200 individual adult trees were collected between June and August 2016. We sampled all upper layer trees, starting from one edge of the stand, until 200 samples were collected. In addition, as a typical high elevation narrow crowned spruce stand, the relict population "Schlossbergfichte" near Oberhof, Thuringia was included with 75 individuals representing nearly the complete stock of old adult trees in this stand. All stands are growing under similar climatic conditions, namely mean temperature, length of the vegetation period, snow cover days and wind speed (Table 1.) Stands will be abbreviated as LE (low elevation type stand) or HE (high elevation type) for the major phenotypic groups followed with an underscore and the location Thy (Thuringian Forest), S (Ore mountains/ Saxony) or H (Harz).

Table 1: Climatological and stand specific data

Stand	N	Age of upper story trees	Mean multi annual air temp 1981-2010 (°C)	Mean vegetation period 1992-2015 (days)	Mean snow cover days 1981-2010 (days)	annual mean precipitation 1981-2010 (mm)	Mean wind in 20m a.g.l. m/s 1981-2000	Elevation range of sampled individuals (m a.s.l)
LE_Harz	200	180	4.7	181.0	141.5	1668.5	56	889-915
HE_Harz	250	300*	3.9	177.0	158.0	1793.0	79	1036-1065
LE_Sa	200	162	5.2	184.0	127.0	1210.0	55.5	972-1009
HE_Sa	200	142	4.9	182.0	136.0	1255.0	58	988-1014
LE_Thy	200	90	5.7	190.0	122.0	1331.0	57	899-912
HE_Thy	200	151	6.3	195.5	100.5	1211.0	39	761-776
Schlossbergfichte	75	250*	5.8	191.0	116.0	1331.0	50	818-840
Mean			5.2	185.8	128.7	1399.9	56.4	921

Le: Low elevation type; HE: high elevation type; Sa: Ore Mountains, Saxony; Thy: Thuringia. Values for climatological parameters were taken from extrapolated 1x1 km grid maps of the long-term average measurements [36–41]. For individuals falling in different grids the mean of these grid values is given and used for calculation of the total mean. The mean vegetation period is calculated from the grid values of the mean end-date minus the mean starting-date of the vegetation period (both are presented in days from New Year). Elevation data were taken from the GPS data and stand age according to information given by the forestry officials. *The age of the "Schlossbergfichte" population is based on the oldest trees and the age of the HE_Harz population is based on the oldest trees of equivalent stands at Mt. Broken [42].

Phenotypic assessment

During the collection of needle material, visual assessment of each tree for crown architectural characteristics was carried out. The spectrum of possible trait expressions was subdivided in three categories, representing the high elevation phenotype, an intermediate phenotype and the low elevation phenotype (Table 2) following Gruber [5]. Additional crown breakage and the occurrence of forking was noted. Breakage of the main stem was noted irrespective of the number of breakage points. Forking was diagnosed when more than one secondary stem replaced the lost apical shoot.

Table 2: The assessed phenotypic traits and phenotypes associated with elevation.

Trait	Mountainous phenotype	Intermediate phenotype	Lowland phenotype
overall crown architecture; structural appearance	narrow shaped crown	equivocal / intermediate shape	broad shape
angle of the first order branches	clearly downwards facing branches, stem and branches	no clear branch orientation up- or downwards	straight and upwards facing branches, all angles between stem and branches right or obtuse
branching pattern of the second order branches	plate or plate brush like	brush like	comb or comb brush like

The traits were scored in three categories which were summarizations of the categories used by various authors [4,7,12,13].

Marker analysis

All individuals were genotyped at 7 genomic SSRs and 4 expressed sequenced tag SSRs or EST-SSRs (Table 3). Additional information on potential gene functions of the EST-SSRs is provided in Table 4. A total of 46 unlabelled primer pairs were tested for amplification, including SSRs developed for *Picea abies* and SSRs adapted from other *Picea* species [43–50]. Further requirements for marker selection were the absence of null alleles in earlier studies [51,52] and the location in different linkage groups of *P. abies* [23,53,54]. A total of 24 primer pairs generated distinct amplification products in the expected size range and were used for PCR with fluorescent labelled forward primers. Eleven primer pairs that generated single-locus and polymorphic products were finally selected for the population analysis.

Table 3: Details on the SSR markers used for genotyping

SSR	Size min (bp)	Size max (bp)	Dye label and the PCR primer sequences (5`-3`)	Repeat motif	Primer reference
EATC1B2 [a]	197	219	F: FAM-TGGCATGAGATTTATGTGGTT R: GTGTGCCACTCAACCTCAC	$(ATC)_7(AT)_3$	[44]
EATC1D2 [a]	180	236	F: FAM-TTGTCATCGTCGTCATTGTC R: TTTAGCCTCTGTTTTCTAGCG	$(ATC)_3AT(ATC)_6$	[44]
EATC1E03 [a]	130	175	F: FAM-CCCCTTATTCCTAACGTCAAA R: TACCAGTGGTGACAACGATG	$(CAT)_4CGT(CAT)_8CGT$- $(CAT)_4CGT(CT)_4CGT(CAT)_4$	[44]
EATC2G05 [a]	193	254	F: HEX-TGGAGCATGGGTAAATCG R: TACCTCACACCCGTGAGAAT	$(AAT)_5(CAT)_{16}CAA(CAT)_4$	[44]
PaGB3 [b]	109	150	F: FAM-AGTGATTAAACTCCTGACCAC R: CACTGAATACACCCATTATCC	$(AT)_{11}$	[50]
PaGB8 [b]	95	203	F: FAM-AGCATGTACAAAATGAAGATTCTC R: CCCTTTAGTGTTTTCTCTTTCTAC	$(AC)_{12}$	[50]
SpAG2 [a]	88	122	F: FAM-GCTCTTCACGTGTACTTGATC R: TTCGAAGATCCTCCAAGATAC	$(TC)_{16}$	[47]
SpAGC1 [a]	71	121	F: HEX-TTCACCTTAGCCGAGAACC R: CACTGGAGATCTTCGTTCTGA	$(TC)_5TT(TC)_{10}$	[47]
SpAGG3 [a]	109	149	F: HEX-AGCATGTTGTCCCATATAGACC R: CTCCAACATTCCCATGTAGC	$(GA)_{24}$	[47]
WS00016.O09 [b]	386	402	F: HEX-CTTTGGGGGCTAGCAAGTTT R: ATTCGGGCTTCATAGCACAA	$(AT)_9$	[45]
WS00111.K13 [b]	212	272	F: HEX-GACTGAAGATGCCGATATGC R: GGCCATATCATCTCAAAATAAAGAA	$(AT)_9$	[45]

[a] gSSRs, [b] EST-SSRs.

176 Table 4: Annotation of the four EST-sequences with SSRs (EST-SSRs) used in this study [45,50]

EST-SSR	GenBank accession no.	Annotation	Location of SSR in the EST
PaGB3	AJ133748	*P. abies* mRNA for major intrinsic protein (aquaporin)	3′ UTR
PaGB8	AF100429	*P. abies* clone PA12H2 repetitive DNA sequence	
*WS00016.O09**	CN480894	NP 197764—expressed protein (*A. thaliana*)	3′UTR
*WS00111.K13**	CN480897	BAB86071—putative beta-glucosidase (*O. sativa* [japonica cultivar-group])	3′UTR

177 *Based on BLAST [55] search.

178 *DNA extraction*

179 From each sample tree approximately 50 mg fresh needle tissue was cut into small pieces, frozen
180 in liquid nitrogen and ground in a MM300 ball mill (Retsch, Haan, Germany) for 2 min at 30 Hz. For
181 the extraction of total DNA we used the DNeasy ™ 96 Plant Kit (Qiagen, Hilden, Germany) according
182 to the manufacturer`s protocol with the minor modification of adding 5 ml of 26 %
183 polyphenylpyrovat solution to the 90.5 ml lysis buffer. Initial DNA concentration was measured
184 using a NanoDrop ™ 2000 spectrophotometer (Thermo Fisher Scientific, Madison, USA.).

185 All PCR reactions were performed in 14 µl total volume, containing 1 µl of 1:10 diluted DNA
186 (ca. 20ng). The PCR mix contained 1X reaction buffer B (Solis BioDyne, Tartu, Estonia), 2.68 mM
187 $MgCl_2$, 178.57 µM for each dNTP and one unit of HOT FIREPol® (Solis BioDyne, Tartu, Estonia) *Taq*
188 polymerase. Each forward and reverse primer was added in a concentration given in Table S1.

189 The reaction started with an initial incubation for 15 min at 95°C, 10 touch-down cycles of
190 denaturation at 94°C followed by annealing at 60°C (Δ-1°C) and extension at 72°C each for 1 min, 25
191 cycles at 94°C, 50°C and 72°C with each temperature level kept for 1 min, final extension at 72°C for
192 20 min. All reactions were run in a Biometra TProfessional Basic thermocycler (Analytic Jena AG,
193 Jena, Germany). Microsatellite fragments were separated on an ABI™ 3130xl Genetic Analyzer
194 (Applied Biosystems, Foster City, USA) with the size standard GeneScan™500 ROX™ as reference
195 (Applied Biosystems, Foster City, USA). Peak calling was done in the GeneMapper™ v4.1 software
196 (Applied Biosystems, Foster City, USA).

197 *Statistical analysis*

198 To assess differentiation and structuring at phenotypic traits and to compare the results with the
199 genetic data we used comparable multivariate analyses.

200 Phenotypic variation

201 The commonly used genetic differentiation measure G_{ST} has its analogy in Q_{ST} to characterize
202 the among population genetic variance exhibited by quantitative traits [56]. In case the among
203 population additive genetic component of variance is unknown, Q_{ST} can be estimated based on the
204 phenotypic data. This estimate of phenotypic differentiation among populations (P_{ST}) is reliant on the
205 scaling parameters c (the proportion of total variance explained by the additive effect) and h^2 (narrow
206 sense heritability) [57,58]. Usually, no prior knowledge of c/h^2 exists, and since this ratio can also vary
207 across population pairs, robustness of the estimated P_{ST} on this ratio should be evaluated [58]. We
208 calculated P_{ST} on the phenotypic data and evaluated its robustness with the Pstat-package [59] in R
209 [60].

Additionally, we used discriminant analysis (DA) of principal components (PCs) to analyse the phenotypic dataset. In contrast to principal component analysis (PCA), where the PCs are optimised for the maximal represented total variance, DAPC (Discriminate Analysis of Principal Components) maximises the between group variance while minimizing within group variance [61]. To choose the number of retained PCs we ran a stratified cross-validation step with 5000 replications, and the number of used discriminant functions (DFs) was fixed to 2, as these always had much higher eigenvalues (EVs) than the remaining DFs. Two PCs were chosen as these had the lowest mean squared error, the highest number of correctly assigned individuals of the subsample and explained 58.1% of the variance. To test for significance of group separation a multivariate analysis of variance (MANOVA) on the retained PCs was performed, where the independent variables, contrary to DA, are the population groups. Finally, a classical PCA and a spatial principal component analysis (sPCA), as described below, were calculated.

Genetic variation - Microsatellite analyses

Allele binning was done using a custom R [60] script. Histograms of raw peak size data per locus were plotted with fine scale breakpoints and the binning limits of each allele per locus were manually defined. Then, we double checked the allele sizing further, and, if necessary, corrected allele sizes to make them consistent and reliable.

The Hardy-Weinberg equilibrium (HWE) was tested using the exact test proposed by Engels [62] and implemented in the R package "HWxtest" [63].

Linkage disequilibrium (LD) was assessed as the standardized index of association $\bar{r}_d$ [64] in a pairwise loci comparison for each stand. Occurrence of significance of the LD values in each stand was evaluated by 10000 permutations using the "poppr" program [65] in the R package.

Null allele frequencies were estimated by the expectation maximisation (EM) algorithm [66] in the joint maximum likelihood (ML) estimation implementation in genepop [67]. Additionally, the ML method in ML-Nullfreq [68] was used. Averaging these results should improve the estimate, and applying a frequency threshold of ≥ 5% for reporting can further reduce false positives [69]. Nevertheless, occurrence of null alleles does not hinder usage of affected markers, as conclusions drawn from assignment analyses or F_{ST} based estimates are unlikely to be influenced by the presence of null alleles [70].

Expected (H_e) and observed (H_o) heterozygosity, fixation index (F_{IS}) and its p-values were calculated using Arlequin 3.5.2.2 [71]. Pairwise F_{ST}, G''_{ST} and corresponding p-values based on 10000 permutations were calculated using GenAlEx 6.5 [72]. For mean number of alleles per locus (A), mean allelic richness after rarefaction (A_r) and the number of private allelic states within populations were calculated using the "hierfstat" [73] and "poppr" [65] R-packages. In order to test for differences in diversity between stands, regions and between high and low elevation stands linear mixed models accounting for differences in diversity between loci based on the refracted allelic richness were calculated with the R-package "lme4" [74].

We assumed that all SSR loci are selectively neutral and checked this assumption with Lositan [75], Arlequin 3.5.2.2 [71] and BayeScan 2.1 [76]. We used the recommended workflow in Lositan by first estimating the neutral F_{ST} value (0.003674) using the random nuclear SSRs, and then running the analysis including the EST-SSRs with the stepwise mutation model and 50,000 replications. In Arlequin 50,000 simulations of 100 demes per group were run with the finite island model also based on F_{ST}. The default parameters were used to run the Markov chain Monte Carlo simulations implemented in BayeScan 2.1.

Hierarchical Analysis of Molecular Variance (AMOVA) [77] was performed in Arlequin 3.5.2.2 [71] to partition variance between regions, stands and stand types. The hierarchical structure of the

dataset was described as the region in which the stands grow, the population/stand itself and the single individuals. Alternatively, the stand classification as HE or LE stand was used as highest hierarchical level, to compare if this grouping explains more variation than the grouping by regions. The within and between group variance was tested for significance using 5000 permutations.

To study relatedness of the populations in the light of hypothetical gene flow we applied the approach of Sundqvist et al. [78] that projects the populations on a relative migration network. This method estimates direction and rate of migration based on either G_{ST} [79], *D* [80,81] or Nm_{Alcala} [82]. We used G_{ST}, as it was shown to perform best in most scenarios [78], and further tested for significant directional migration by 10000 permutations in the "diveRsity"- R-package [83].

To study structuring of individuals at the SSR markers accounting for spatial autocorrelation we used sPCA [84] that optimizes the product of the variance and the spatial autocorrelation measured by Moran's *I* [85,86]. Global or local structures with positive or negative autocorrelation are indicated by positive or negative EVs. To test whether global or local structures are significantly different from the null hypothesis of alleles being randomly distributed across space, the estimated distribution of the EVs via Monte Carlo (MC) sampling was used. This test has higher statistical power than the previously suggested method based on R^2 [84,87]. We calculated the sPCA and the test procedure as implemented in the R-package "adegenet" [88,89]. Spatial distribution was described by spatial weights that are directly proportional to the inverse of the geographic distance matrix. The EV-MC test for local and global structure was run 10^3 times. As with the phenotypic traits we calculated the alternatives PCA and DAPC.

Finally, to determine if the sampled individuals can be grouped into different genetic clusters (*K*) the program STRUCTURE [90] was used. Admixture proportions were estimated for K = 1 to 12 clusters with 15 replicated iterations for each cluster run with 10000 burn-in and 50000 following iterations. The ancestry model considering admixed individuals was chosen. Sample location information was considered as prior known for the model and both the degree of admixture alpha and the parameter lambda for the distribution of allele frequencies were set to be estimated from the data. Further, the model considers allele frequencies between populations to be correlated, as the result may give more accurate estimates of admixture, produces more detailed clustering results and gives the same results as independent models in the absence of correlation [91,92]. Averaging different runs for the same *K* was done in CLUMPAK [93] with the default settings. The runs for each *K* were summarised according to a similarity score. If different runs pass a certain threshold value in the comparison, they are included in the major model, which is a summary of the majority of the runs, or in one or more minor models depending on their similarity. Methods based on DeltaK [94] and on the log-likelihood probability of *K* [90]were used to help choosing the most likely number of clusters (*K*).

3. Results

Phenotypic differentiation between low and high elevation types

A clear difference in the phenotypic traits was found between the selected high and low elevation type stands. The overall crown shape, angle and the branching pattern are strongly correlated (Pearson correlation 0.64-0.76), due to the dependence of the crown appearance on the angle and pattern of the branches. The relative frequencies of the traits in each stand are presented in Figure 1 (values are also provided in Table S2). In the relict stand "Schlossbergfichte" from Thuringia all trees expressed the high elevation type according to the assessed traits, and no crown damage and forking were detected. High elevation characteristics were also found in more than half of the observed trees (~55%) in the potential autochthonous HE-Thy stand, and no damage and forking occurred. The neighbouring low elevation type stand was predominantly comprised of lowland crown shapes (96.5%) with all trees being damaged. In the Harz Mountains the high elevation stand was comprised of 37% narrow crowned individuals, but most trees showed intermediate (62.6%)

crown shape. Varying branching patterns were also observed there, and all trees were damaged. In the neighbouring low elevation type stand most trees had broad (68%) or intermediate (31.5%) crowns, and most of them showed crown breakage (94%). In Saxony the trees in the high elevation type stand were of narrow crowned (56.5%) or intermediate (37.5%) character with few trees showing sights of breakage (8.5%). Intermediate crown type (73%) and branching pattern (66%) were typical for the low elevation type stand in Saxony, where also only few trees were damaged (4.5%). Accordingly, the divergence for phenotypic traits (P_{ST}) showed high between group variance compared to within group variance, as the crown shape traits as well as the damage traits had P_{ST} value of 0.952 to 0.989 for $c/h^2 = 1$. Only a weak effect of varying ratios of c/h^2 could be noted. Even for low $c/h^2 \geq 0.1$ ratios the P_{ST} value was above or near 0.95 (forking occurrence and branching pattern with P_{ST} values of 0.945 and 0.936 for $c/h^2 = 0.1$, Figure S2).

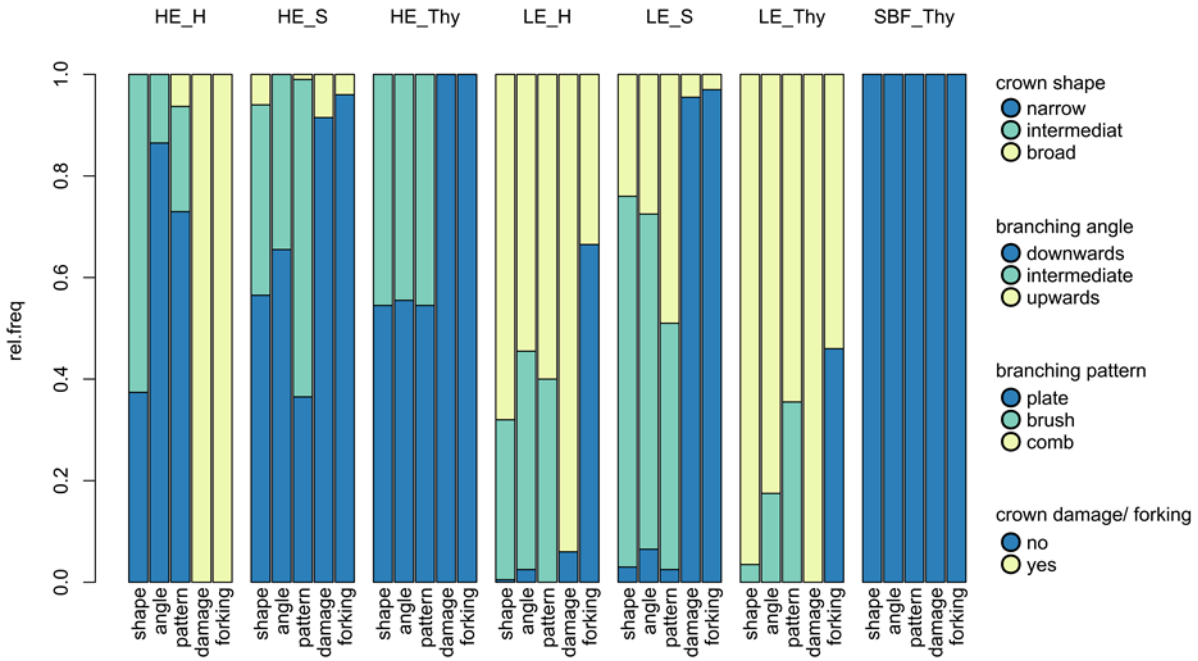

Figure 1: Relative frequencies of the assessed phenotypic traits (overall crown shape, angel of the first order branches, second order branching pattern, occurrence of crown damage and occurrence of forking) in the studied stands. Categories for the traits are given in the right-hand legend.

The DAPC results demonstrated that the between group structure of the phenotypic data was mainly explained by the first two DFs as reflected in the corresponding high EVs. The low elevation stands were separated from the high elevation stands with the LE_S stand as a transition, as its 95% distribution density on the first DF overlaps with all but the SBF-Thy and HE_H distributions. The second DF mostly separates the HE_H and LE_S stands from the other stands (Figure 2). Further testing the prior group membership of the individuals by MANOVA yielded a high significance ($P \approx 0$).

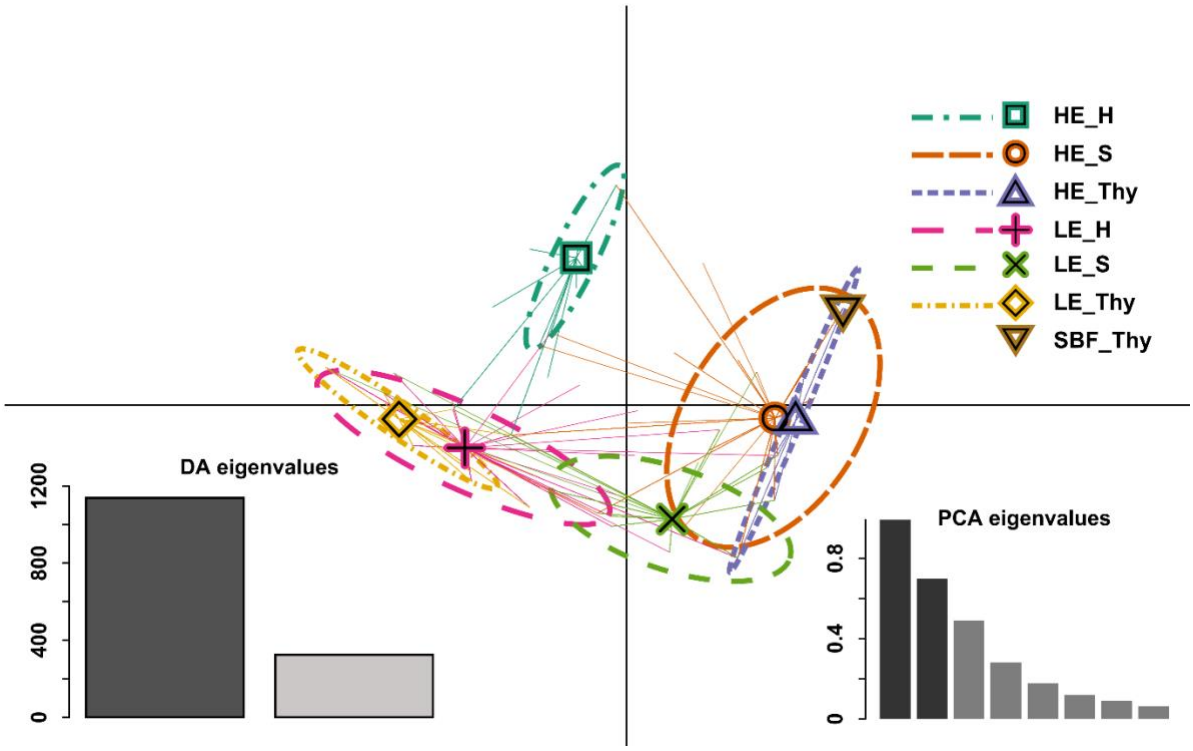

Figure 2: Discriminant analysis of principal component (DAPC) [61] on multivariate phenotypic data. Data points are displayed on the first and second discriminate axes (x- and y-axes, respectively) of the DAPC indicated by the stars originating from the corresponding distribution centre. Groups are depicted by 95% inertia ellipses with corresponding barycentre as symbol. Note that, for the stand SBF_Thy no ellipse is displayed as all data points are identical. In the bottom left corner, the DA eigenvalues are displayed, where the first and second DA are highlighted in dark grey and grey, respectively. The PCA eigenvalues are displayed in the lower right corner, where the retained PC's eigenvalues are highlighted in dark grey.

PCA gave a similar data distribution on the first two axes, nevertheless separation of the single stands was weaker compared to DAPC, as the DA step is not performed. Centres for each stand distribution were more separated in DAPC as well as the 95% inertia ellipses. In the sPCA strong and highly significant global structure (positive autocorrelation), attributed to inter- and intra-regional separation of the stands with contrasting phenotypes was observed. No local structure (negative autocorrelation) could be detected, which reflects the absence of clustering of similar types within the stands.

Summarizing these results, it is evident that a high phenotypic differentiation between high and low elevation stands was observed at the assessed traits.

Genetic variation and differentiation

No significant differences in diversity between populations, regions and stand-types could be detected based on linear mixed effect models accounting for between loci diversity divergences. In total 9 (*EATC1E03* & *WS00016-O09*) to 41 (*PaGB8(a)*) alleles per locus were observed and only small differences in H_o, H_e and A_r values between the stands were found. The H_o values ranged from 0.630 to 0.702, with the highest values found in the HE_Thy (0.702) and SBF_Thy (0.692) stands and lowest in the LE_H (0.640) and HE_S (0.630) stands. Values for H_e were similar among stands (0.730-0.757). The rarefied allelic richness of the relict stand SBF_Thy had a value (16.79) comparable to the other stands (16.13-16.83). HE_H (16.51) had a slightly reduced A_r compared to other HE stands (16.51-16.83). All but one (LE_H: 0.041) F_{IS} value were significant, ranging from 0.079 in SBF_Thy to 0.142 in HE_H (Table 5).

Table 5: Summary of basic population genetic parameters.

Stand	H_o	H_e	A	A_r	Private alleles	F_{IS}	P
HE_H	0.655	0.733	17.09	16.51	0	0.142	0.000
HE_S	0.640	0.751	16.90	16.83	2	0.124	0.000
HE_Thy	0.702	0.757	17.00	16.67	4	0.129	0.006
LE_H	0.630	0.730	16.46	16.13	4	0.041	0.099
LE_S	0.673	0.746	16.64	16.64	0	0.108	0.000
LE_Thy	0.668	0.734	16.73	16.46	5	0.137	0.000
SBF_Thy	0.692	0.749	14.55	16.79	0	0.079	0.007

H_o and H_e - expected and observed heterozygosity, respectively; A - mean number of alleles per locus; A_r - allelic richness adjusted for different sample size by rarefaction; F_{IS} – index of fixation with corresponding P-values based on permutation. Parameters were calculated using Arlequin 3.5.2.2 [71], except A, A_r and private alleles that were calculated using the "hierfstat" [73] and "poppr" [65] R-packages.

Deviation from HWE was found for five loci in several stands. Locus *WS0016-O09* deviated in all stands from HWE, *PaGB3* in all stands but SBF_Thy, *EATCG05* in all stands but LE_Thy and SBF_Thy. Furthermore, in four stands significant deviation from HWE was found for the loci *SpAGC1* and *SpAGG3* and in one or two stands for *EATC1B2*, *EATC1E03* and *SpAG2*, *WS0011-K13*, respectively (Figure S3). The estimated null allele frequencies per locus were mostly similar in the stands, thus underestimation of genetic diversities as result of null alleles would have affected all stands equally. Loci surpassing null allele frequencies of 5% were mostly in the more complex trinucleotide repeats, namely the *EATC*-loci. The dinucleotide repeats expressed much lower values, which narrowly exceeded 5% (Figure S4). Insignificant LD values ($\bar{r}_d$) were found for all locus pairs based on population wise permutation test (Figure S5). In the applied F_{ST} outlier test none of the loci exceeded the lower or upper 95% confidence intervals of the simulation run in Lositan (Figure S6). In accordance, no directional selection was found by the Bayesian method used as well.

The high phenotypic differences between high and low elevation type populations were not reflected in genetic variation patterns at the screened supposedly selectively neutral SSRs. Overall, very low genetic differentiation was observed among populations and regions based on the 11 supposedly neutral polymorphic SSR markers. Partitioning the molecular variance indicated that variation was mostly attributed to variability of the individuals, as 90.21 % of the variance is contained within the individuals, only 0.16 % of the variance was attributed to among population variation within regions, and 0.24 % was distributed between geographical regions. The remaining 9.39 % were attributed to the variance of individuals within the populations (Table S3). When the phenotypic stand characteristics (high or low elevation type stand) were used as the highest hierarchical level no variance was partitioned between these two groups (the actual value of the percentage of variation among groups was -0.09 %; negative values may arise due to the geometric distance calculation and can be considered as approximately zero).

In the pairwise comparison low and non-significant F_{ST} values of 0.002 were observed between low elevation stands in Saxony and Thuringia and in both stands in the Harz Mountains. Slightly higher values of 0.004-0.007 were detected between the relict stand "Schlossbergfichte" and all but the HE_Thy stand (Table S4). Comparison based on G''_{ST} yield a similar picture. Further, most of the highest G''_{ST} values (0.016-0.030) were found between the Harz Mountains and both the regions Thuringia and Saxony (Table S5). Compared to the estimated P_{ST} values, even for low c/h^2 ratios, all F_{ST} and G''_{ST} values were much lower. Estimated rates of gene flow between stands of the same region were relatively high (0.58-1), but lower between stand SBF_Thy and the other Thuringian stands. For this stand a high gene flow rate (0.5) was only estimated towards the HE_Thy stand. Between Saxon and Thuringian stands (also excluding SBF) high bidirectional gene flow rates were estimated. As a general pattern across all regions a stronger gene flow from the low to the high elevation stand was detected. Nevertheless, no significant asymmetric migration was found at $\alpha = 0.05$ (Figure S7).

We applied sPCA, which also incorporates spatial relationships among stands, to detect potential genetic structuring correlated with the geographic distances. The results reveal weak isolation by distance between the Harz Mountains and the two other regions. In Figure 3 the distribution of data on the first two sPCAs shows separation of the Harz population on the first axis, whereas the second axis slightly separates the HE_Thy and SBF_Thy populations from the LE_Thy population. The MC-EV test showed the absence of local structuring but revealed highly significant global structuring ($P < 0.001$), which is attributed to the first four PCs. When only the between group variation is used, the DAPC analysis yields similar separation on the first DF, but a slightly stronger difference for SBF_Thy and HE_S stands. Both the regional and population differentiation were supported by MANOVA ($P < 0.001$). Weak geographical differentiation between Harz populations and the others was also confirmed by STRUCTURE. Evaluation of the cluster number K suggested two distinct clusters supported both by delta K and the log-likelihood probability of K (Figure S8). Summarizing all runs for $K = 2$ presented in Figure 4, individuals of the Harz populations formed one cluster, and individuals of all other populations were assigned to the second cluster, with a considerable amount of estimated admixture in all individuals. The analysis of the 15 runs in CLUMPAK gave one major and one minor models, which are very similar and consistent in their interpretation.

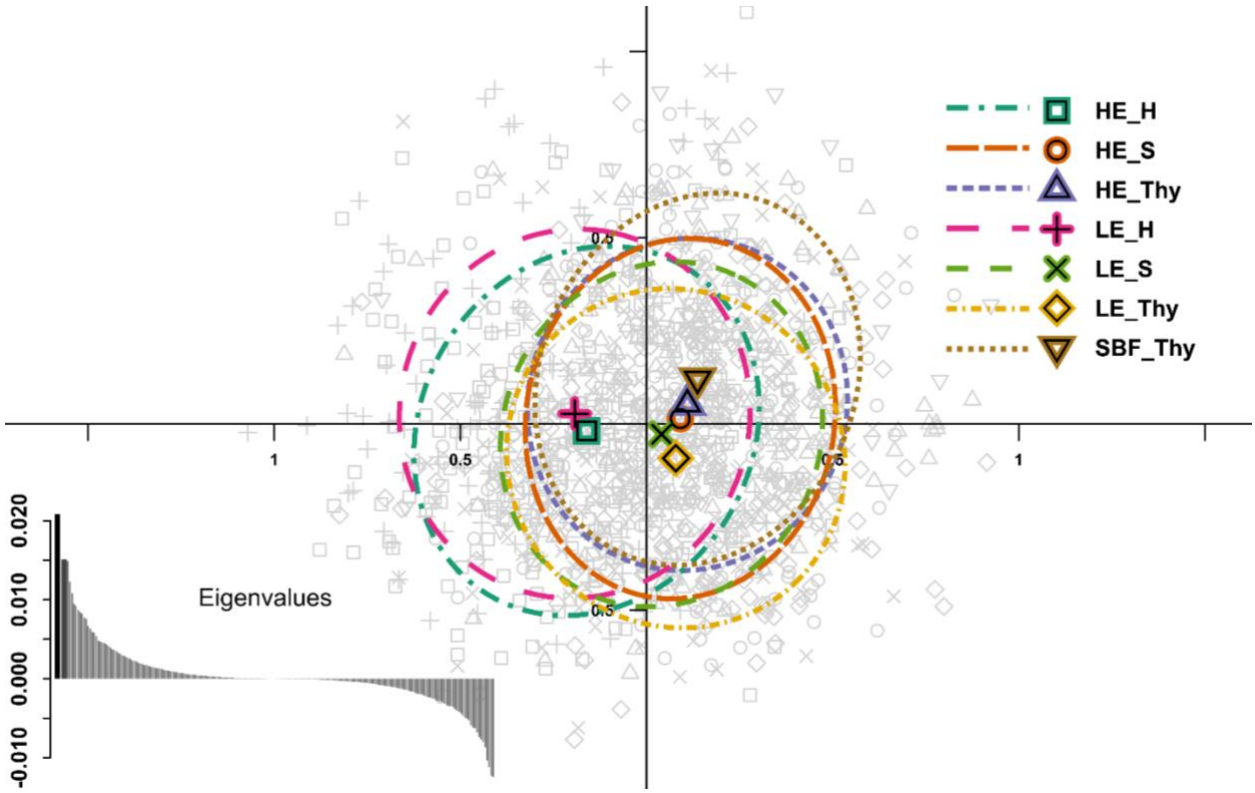

Figure 3: Spatial principal component analysis [84] based on the allele data, reflecting low spatial genetic differentiation for the SSR loci. Data points (grey) are displayed on the first and second principal components (x- and y-axes, respectively), where the symbols represent the different stands in the upper right legend. Groups are depicted by 95% inertia ellipses with corresponding barycentre as symbol. In the bottom left corner, the eigenvalues are displayed, were the first and second eigenvalues are displayed with wider bars and highlights in darker grey

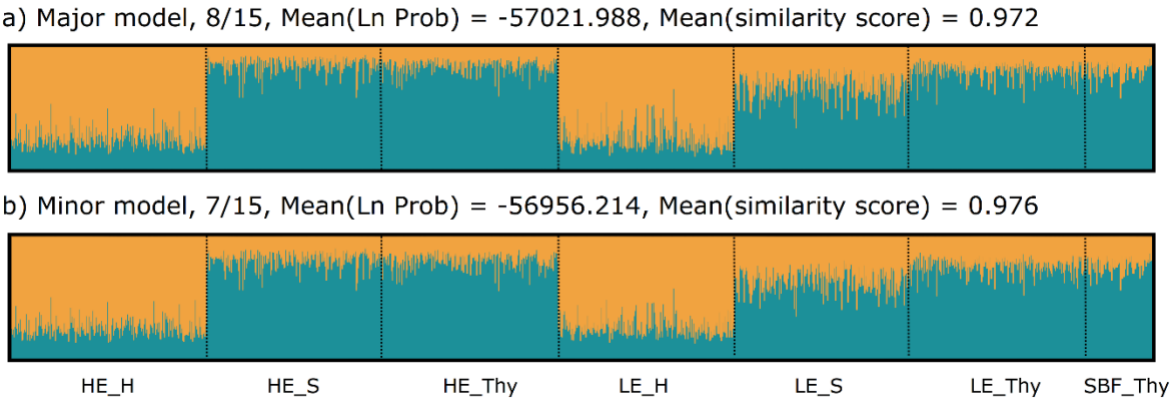

Figure 4: Clustering result from STRUCTURE (Pritchard et al. 2000), displayed as average over the 15 runs obtained from CLUMPAK [93]. In the major model (a) 8 of the 15 runs were summarized and an additional minor model of 7 runs was obtained. The average logarithmic probability of the data for the given mode and the average similarity of the runs summarized is also given.

4. Discussion

Norway spruce is still one of the economically most important tree species for forestry in Europe, but the species' range is assumed to decline due to the predicted climate change [95]. Forestry in Germany also heavily relies on Norway spruce [7], but the projected future area will be more limited to the low mountain ranges [96]. Thus, the sampled locations represent important regions of todays and future spruce stands, also in terms of genetic variation of this species. With a complete inventory of stands we tried to minimise random sampling effects.

Phenotypic differentiation

Our comparison of stand pairs of potentially autochthonous and allochthonous spruce stands, respectively, showed a high phenotypic differentiation between stands of natural (autochthonous) and artificial (allochthonous) origin. The phenotypic differentiation can further be interpreted as an adaptational mechanism that is genetically controlled, but not reflected by the supposedly neutral markers used in this study. The differentiation was revealed by both the high P_{ST} values (> 0.9) and the stand separation in the DAPC analysis with further confirmation by the significant MANOVA results. Comparison between the estimated P_{ST} and F_{ST} values at neutral loci can point to the control of the phenotypical differences. As in the present case with $P_{ST} >> F_{ST}$ directional selection is likely involved in the divergence at the trait level between different populations [6]. Also, comparing G''_{ST} to P_{ST} this observation holds true. While the discussion on which measure to use is extensive [e.g. 72,90,91], it is in the reported case likely irrelevant which common measure of genetic differentiation (X) is used, as it will supposedly not change the observation $X >> F_{ST}$. Additionally, as our estimated P_{ST} values remained high (> 0.85) on the complete range of the c/h^2 ratios examined, and $P_{ST} >> F_{ST}$ holds, the draw conclusions can be considered as very robust [58].

Morphological differences could also be attributed to phenotypic plasticity. For instance, Gruber [5] assumed that the low elevation phenotype has a relative high plasticity and could resemble the high elevation phenotype under certain conditions. In the past, however, allochthonous Norway stands were planted with about 10000 plants/ha, and after decades the artificial stands, especially in Thuringia and the Harz Mountains still show a strong phenotypic differentiation from the natural stands. The expected lower frequencies or absence of crown damage in narrow crowned trees were confirmed in the Thuringian and Saxony stands, but not in the high elevation stand of the Harz Mountains. This stand is located just below the natural tree-line at Mount Broken with around 1100 m a.s.l. [99] and subjected to the highest average wind speeds and the most average snow cover days in this study, which may explain that even the narrow crowned trees may not withstand these conditions.

Hence, our results support the assumption that Norway spruce trees of natural origin in high elevation stands have morphologically adapted to extreme weather conditions by alteration of crown architecture [12,13,100]. Moreover, these results are in agreement with the hypothesis that crown morphology is at least partially genetically controlled [12] as all stands or stand pairs were subjected to similar events of snow and wind (Table 1) but still showed considerable phenotypical differences. In stands with both high and low elevation types, trees with different crown types were randomly distributed suggesting that micro-environmental differences within the stand have a minor effect on crown architecture.

Genetic differentiation

The relatively weak genetic structure and differentiation were in contrast to the morphological differentiation. However, both Harz populations, as well as the "Schlossbergfichte" population, were slightly differentiated from all other populations. As the genotyped SSR loci showed no sign of directional selection, these markers likely reflect neutral genetic variation patterns. Our estimates of F_{ST} between the populations ranged from 0.002 to 0.007 (G''_{ST} = 0.002 – 0.030) and are comparable to the overall very low differentiation found for nSSRs on similar or even greater geographical scales, such as for Austrian (F_{ST} = 0.0004–0.0035 [101]), Swedish (F_{ST} = 0.000–0.0206 [31]), Italian Alpine (F_{ST} = 0.00–0.04 [102]) and Scandinavian, Baltic and Russian populations (F_{ST} = 0.087 [1]). These results of low differentiation between stands were mostly attributed to the high gene flow through pollen. Even between populations from putative different refugial lineages, and thus different gene pools, estimates remain low (F_{ST} = 0.12 based on isozymes [103]; F_{ST} = 0.0585 based on EST-SSRs [104]).

However, detailed assessment based on the pairwise F_{ST}, G''_{ST} and sPCA showed a differentiation of the Harz region and the "Schlossbergfichte" population from the other populations, even though differences were small and accounted for a very small fraction of the overall variation based on the AMOVA results. From the perspective of recolonization history the regions in focus should belong to the same lineage from the Carpathian refugium, covering both the Harz and the Bohemian Massif [28]. Nevertheless, a more recent recolonization history of the Harz Mountains than for the Bohemian Massif is presumed from pollen records [22]. This could suggest a still noticeable founder effect in the Harz typical for the found pattern. Historically the Harz Mountains were a centre of forest reproductive material export [8]. Plantations within the region are more likely to be of local origin, and thus may have retained the broad spatial structure and the genetic similarity at neutral markers between LE and HE stands. In the Bayesian clustering analysis, we found one cluster to be comprised of the Harz stands and the second cluster comprising all stands from Saxony and Thuringia. The small IBD found here is also in accordance to previous studies between Austrian montane and subalpine [105] and high elevation populations [101], such as it is expected for more geographically scattered populations [104].

The relict population "Schlossbergfichte" comprised > 250 year old and younger adult trees (approximately 180), which originated from natural regeneration. As seed dispersal in spruce is limited compared to pollen dispersal [106], at least the maternal part is retained within the stand. Thus, the initial genetic composition of the typical Thuringian Forest high elevation provenance may have been retained in this remnant population through the oldest trees, and a temporal isolation effect could be the reason for the found small genetic divergence to the other stands. This genetic divergence was also reflected in the migration network, as the rates towards the "Schlossbergfichte" population were overall lower than between other stands, and only higher migration from this stand towards the HE_Thy population was found.

From the investigations of Meyer et al. [42] the age of single individuals in the HE stand at Mount Broken, Harz, was estimated with up to 300 years, whereas the stand is supposedly highly age structured. In comparison to the population "Schlossbergfichte" the relict character of this stand is represented by the oldest trees, as with decreasing age the influence from the allochthonous gene pool rises, as discussed below.

Regarding the differentiation between low and high elevation type stands, we could not detect any differences in allelic richness or diversity between high and low elevation type stands or stands

in general. Such differences in diversity between natural and artificial stands were suggested by Gömöry [35] and Finkeldey and Ziehe [107]. Comparing artificial stands with high altitude stands [34] or with stands characterised as mountainous ecotypes [108] of Norway spruce confirmed the occurrence of these differences. Recently, Máchová et al. [109] characterised eight Czech populations and nine subpopulations of a gene conservation unit at eight EST-SSRs and one nSSR. These stands were classified as either alpine, mountainous or lowland ecotypes, and the authors found the sampling units of identical ecotypes clustered together based on Nei's genetic distance. In contrast to our results, no separation among geographic regions was observed. Also, Jeandroz et al. [110] compared autochthonous mountainous spruce stands from the Vogues mountains to allochthonous stands and distinguished these groups based on RAPD markers. Nevertheless, no detailed assessment of crown types was reported in either study. In the upper Harz mountains, Greger [33] studied the relationship between morphological traits and the isozyme locus *PGI* (phosphoglucose isomerase) and identified a higher proportion of *PGI-B 22* genotypes in the autochthonous stands with high elevation crown types.

Our results exemplify the presence of pollen mediated gene flow in combination with human aided translocation of material that may have homogenized genetic structures and diluted adaptive genetic variation over generations. Human impact strongly altered the composition of forests in Central Europe especially in Germany [111,112], and may have had a strong impact on the neutral and adaptive genetic variation [107,113]. In Norway spruce translocation and planting of non-local material started in the 18th century throughout Europe, also with the organised expansion of its natural range. In general, anthropogenic gene flow through extensive translocation of regeneration material had major effects on the genetic diversity of forest tress [8].

In our study high rates of gene flow were estimated between HE and LE stands. Even though, no significant directional migration was found, a strong influence of pollen mediated gene flow from the majority of artificial to the few natural stands can be assumed. Over generations this process can lead to lower genetic differentiation in general and specifically to a dilution of adaptive genetic variation.

Hence, we suggest that the partially unsuited morphological characteristics in the younger autochthonous stands reflect the influence of gene flow from artificially introduced plant material. In our study the oldest stand SBF_Thy showed pure high elevation ecotypes, whereas the younger autochthonous HE stands showed higher morphological variability with a mixture of intermediate, sometimes even low, and high elevation ecotypes. Thus, our results are related to the stand age and history. The older the stands the smaller the anthropogenic influence should be, especially in terms of translocation of material and subsequent gene flow between artificial and autochthonous stands [8,33]. Accordingly, the > 250-year-old trees of the "Schlossberfichte" stand should be comprised of the original genepool of the local ecotype. The other potential autochthonous "younger" stands experienced gene flow from neighbouring stands. As such, the dilution of the original genepool by immigrant pollen and seeds could have altered the frequency of genes coding for the high elevation morphological characteristics.

5. Conclusions

1. High phenotypic variation contrasting with low genetic variation measured by the nSSR markers reflects different local adaptations. The occurrence of high elevation and low elevation crown types in neighbouring stands, and the random distribution of trees with different crown types in mixed stand suggested that crown architecture is under strong genetic control. Common garden experiments are needed to derive heritability estimates for this trait.
2. High and low elevation stands were not differentiated at neutral markers. Candidate gene approaches or genome scans could reveal adaptive genes associated with crown architecture.
3. A weak geographic structure was detected based on the nSSR markers. However, the Harz region and the relict population showed a weak, but significant differentiation from the remaining stands. Gene flow by pollen and seeds likely resulted in decreased phenotypic

differentiation in younger autochthonous stands, while all trees in the relict population "Schlossbergfichte" were characterized by typical high elevation crown types.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Document (pdf; 13pp) including Tables S1-S5 and Figures S1-S8.

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