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Molecular Genetic Identification Explains Differences in Bud Burst Timing among Progenies of Selected Trees of the Swedish Douglas Fir Breeding Programme

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Abstract: Douglas fir is expected to play an increasingly important role in Swedish forestry under a changing climate. Thus far, seed orchards with clones of phenotypically selected trees (plus trees) have been established to supply the market with highly qualitative reproductive material. Given the high genetic variation of the species, its growth properties are significantly affected by the provenance. Here, we applied microsatellite markers to identify the origin of clones selected within the Swedish breeding programme. Moreover, we analysed the timing of bud burst in open-pollinated families of these clones. In particular, we aimed to explain the provenance effect on phenology by using molecular identification as a proxy. A Bayesian clustering analysis with microsatellite data enabled the assignment of the clones to one of the two varieties and also resolved within-variety origins. The phenological observations indicated an earlier bud burst of the interior variety. Within the coastal variety, the northern provenances exhibited a later bud burst. We found a significant effect of the identified origin on bud burst timing. The results of this study will be used to support further breeding efforts.

Keywords: *Pseudotsuga menziesii*; non-native forest tree; forest tree breeding; microsatellites; phenology; origin identification

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1. Introduction

Tree breeding is an important tool to increase forest growth and much of the forest reproductive material (FRM) has great potential for genetic improvement. Such improvement of the FRM provides forest owners with a robust cropping option to meet the climate challenges of the future. Improved trees can very effectively, and without negative side effects, increase forest growth and contribute to mitigate global warming. Understanding the basic genetic relationships and how they interact with environmental conditions for different traits is important to optimise the tree breeding system.

In western North America, Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) grows naturally over a large land area across several climatic gradients with generally dry summers [1]. Within this range, it displays a high genetic variation, both quantitative and molecular [2–4]. Douglas fir has a long tradition as a non-native in European forestry, which dates back to the 19th century [5]. In Scandinavian countries, it has been identified as one of the most promising non-native forest trees with a high potential in a changing climate. It is highly relevant in the south of Sweden as it is expected that Douglas fir will benefit from increasing temperature due to climate change [6–8]. In addition, this tree species has several desirable wood properties well suited for future wood and bio-refinery

products. Thus, there is a strong interest to analyse and investigate the effects of different growing materials for the forest conditions in Sweden.

European provenance research shows that there is much to be gained from using the correct provenance for one's Douglas fir growing stock [8]. In central Europe, the coastal variety (*P. menziesii* var. *menziesii*) is preferred, while the inland variety (*P. menziesii* var. *glauca*) is suggested for higher northern latitudes [1]. As the natural range of Douglas fir covers a large area, differences within and between provenances of the two varieties have been identified based on common garden trials [2,8–10]. Important differences between the two varieties are often linked to their inherent frost hardiness and growth since both are strongly dependent on their geographical origin [8]. In particular, the interior provenance is known to cope better with early autumn and winter frosts (especially provenances from British Columbia, Canada). However, it is more susceptible to late frost in spring, which is attributed to phenological differences; coastal provenances are usually characterised by a late bud burst in spring and late bud set in autumn [11].

Swedish experience with Douglas fir is limited. Only a small number of stands have been established since its introduction in the beginning of the 1900s. All of these stands grow in southern Sweden [1,6]. The introduction of Douglas fir in Swedish forestry was soon followed by the establishment of provenance trials. However, only limited conclusions were possible from these oldest trials due to their low survival, which was most probably caused by non-hardy seed sources from a too southern origin in the native distribution range, and severe browsing impacts (cf. [12]). Further provenance research in Sweden led to the establishment of a combined provenance and progeny trial series on six sites in south and central Sweden in 1990–1991, with the purpose to test seed sources from more northern latitudes, of which the two most southern trials were S2265 Bruzaholm and S2266 Tönnersjöheden [13,14]. In 2009 and 2010, two new provenance trials were established at 12 sites to study interior and coastal provenances in southern Sweden [12]. An early evaluation, based on six-year-old data of this field material, showed that the interior provenances exhibited a higher seedling survival in most parts of southern Sweden, with exceptions in the very south of Sweden and in coastal areas [15].

Today in Sweden, a low-input tree breeding programme for the next generation of Douglas fir is operated by Skogforsk (the Forestry Research Institute of Sweden) [16]. The objective of the Swedish Douglas fir breeding programme is to secure an adapted seed source of Douglas fir cultivation for southern Sweden. The programme is currently advancing its efforts, as new trials with progenies from a second batch of selected genotypes (plus trees) will be planted in 2022. The first plus tree selection was made in 1995, when 65 plus trees were selected in well-growing stands in south Sweden. The provenance of the stands was unknown. Open pollination seeds were obtained from 35 trees and progeny tests were planted at six sites in 2000. Another progeny trial series consisting of 103 open-pollinated families from Danish seed orchards were planted in 1998 on four sites. The two progeny test series were evaluated and forward selection of 31 genotypes from the best families in the two respective trial series was carried out in 2010. Selections were made in four of the trial sites; F1307 Trolleholm-Bäringelund, F1308 Tönnersjö-Älvasjön, F1335 Knutstorp-Teglaröd and F1336 Tönnersjö-Åsamöllevägen. In 2011, 81 well-growing trees in the provenance trials S2265 Bruzaholm and S2266 Tönnersjöheden were additionally identified and chosen for selection to broaden the breeding population. A total of 108 trees were finally grafted into two seed orchards, FP-200 Gåtebo (56.84° N, 16.77° E) and FP-201 Slogstorp (55.75° N, 13.45° E), and to a clonal archive at the Ekebo field station (55.95° N, 13.11° E). These comprise the breeding population for future deployment of FRM [16].

An important part of Douglas fir breeding is the characterisation of the genetic origin of the plus trees. Molecular markers have been successfully used to describe the genetic structure of this species in its native range [3,17] and also to assess the origin and genetic diversity of introduced stands in Europe [18–20]. The use of highly variable microsatellite markers in the last two decades [9,20] has improved the resolution of such analyses in comparison to earlier studies based on isoenzymes [18,19]. Many stands in southern and

central Sweden were derived from direct seed imports from southern British Columbia in Canada and northern Washington in the USA or from Danish stands [1,21].

The knowledge of the population genetic constitution of the plus trees in the Swedish breeding programme is currently poor and this study aims to fill some important knowledge gaps. An in-depth population genetic analysis of the plus trees presented in this paper offers valuable knowledge to design further breeding and genetic improvements of Douglas fir in Sweden. This may provide a direct benefit to the use of seed orchards and may increase their profitability and also provide valuable knowledge to design the further breeding of Douglas fir in Sweden.

In this study, we used molecular markers and phenological observations to assess the effect of the native origin of bud burst phenology on the plant nursery seedlings of the breeding population of Douglas fir in Sweden. We are specifically interested in (i) assessing the origin of 108 clones comprising the breeding population of Douglas fir in Sweden utilising molecular markers, (ii) comparing the genetic diversity between subsamples of the selection population and native reference samples of the same origin, (iii) assessing differences in bud burst timing among the open-pollinated progenies from 76 of these clones and (iv) using molecular genetic identification as a proxy for explaining phenological differences among the progenies.

2. Materials and Methods

In September 2019, the genetic material for the study was sampled from plus trees comprising the breeding population of Douglas fir in Sweden. 106 genotypes were sampled in the seed orchard FP-200 Gåtebo, and two additional clones were sampled at the clonal archive in Ekebo. A total of 77 of the 108 clones sampled for genotyping originated from the provenance trials S2265 Bruzaholm and S2266 Tönnersjöheden, while the remaining 31 were selected in the open-pollinated progeny test of Danish clones. In this paper, we refer to these 108 samples collectively as the Swedish Douglas fir clones or genotypes. The origin of the sampled clones is shown in Table 1. The origin of the 77 clones from the provenance trial plots (Bruzaholm and Tönnersjöheden) is native, as the respective genotypes arose through random mating within the natural distribution range in North America and were then planted in the Swedish provenance test. On the contrary, we assigned a non-native origin to the 31 clones of the progeny test of Danish clones, as the mating events giving rise to these genotypes took place in Europe. For genotyping, a few young current-year shoots were collected, put in a labelled paper bag and dried in a ventilated storage facility at the Ekebo field station for a couple of days before they were shipped to the molecular genetics laboratory of the Institute of Silviculture, BOKU (Vienna, Austria).

Cones from 89 of the 108 genotypes sampled for DNA analysis were collected. Open-pollinated cones for each genotype were collected at the same time as the tissue (shoot) collection individually in separate net bags, either as fully mature brown cones or as immature green cones; all cone bags were put for after-ripening in a cold storage room at the Ekebo field station for some weeks before seed extraction. The open-pollinated seeds were used to establish new progeny trials in the Swedish tree breeding programme of Douglas fir. Part of the seeds were sown in late May 2020 for container seedling production with a standard protocol in the plant nursery at the Ekebo field station. A HIKO V-150 multi-cell tray growing system with, in total, 24 cells was used for the plant production. A complementary batch of seeds was sown early in spring 2021 to ensure an adequate number of seedlings from all 89 genotypes were available for the new progeny trial. The trial plots will be set up in 2022 with both one- and two-year-old seedlings.

For genotyping, genomic DNA was extracted from all tissue samples using a commercial extraction kit (DNEasy Plant Minikit, Qiagen) in the laboratory of the Institute of Silviculture (BOKU, Vienna). The quality and quantity of the extracted DNA was assessed by running a 1% agarose gel and by measuring the extracts with a nanophotometer (Implen).

Table 1. Provenances included in the study, origin and number of individuals (clones) genotyped per provenance. Native origin = genotypes which arose from mating within the native range. Non-native origin = genotypes arising from mating in Europe, after introduction. BC = British Columbia, Canada; MT = Montana, USA, OR = Oregon, USA; WA = Washington, USA.

Provenance	Origin	Number of Individuals (Clones)
Bella Coola	Native	6 [1,2]
Blue River	Native	19 [1,2]
Boston Bar	Native	12 [1,2]
Campbell River	Native	5 [1,2]
Darrington	Native	5 [2]
Hoh Valley	Native	4 [2]
Horsefly	Native	3 [1,2]
Prince George	Native	5 [2]
Quesnel	Native	7 [1,2]
Sicamous	Native	11 [1,2]
Stendal 136	Non-native	12 [3]
Other	Non-native	19 [3,*]
Coastal OR (R05-Dufur)	Native (reference)	20 [4]
Coastal WA (R12-Darrington)	Native (reference)	20 [4]
Coastal BC (R38-Alger Creek)	Native (reference)	20 [4]
Interior BC (R39-Tercer)	Native (reference)	20 [4]
Interior MT (R33-Lewis & Clark N.F)	Native (reference)	18 [4]

[1] Genotypes selected in the provenance trial plot S2265 Bruzaholm. [2] Genotypes selected in the provenance trial plot S2266 Tönnersjöheden. [3] Genotypes selected in the progeny test of Danish clones. [4] Origin of individuals described in [4]. * Native seed origin partly known.

The following thirteen microsatellite loci were amplified by means of PCR: Pm_OSU_1C3, Pm_OSU_1F9, Pm_OSU_2C2, Pm_OSU_2D4, Pm_OSU_2D6, Pm_OSU_2D9, Pm_OSU_2G12, Pm_OSU_3B2, Pm_OSU_3B9, Pm_OSU_3F1, Pm_OSU_3D5, Pm_OSU_4A7 and Pm_OSU_5A8 [22]. The applied PCR conditions are described in [4]. The PCR success was checked by means of agarose gel electrophoresis (1.5% gel).

For allele scoring, capillary electrophoresis was carried out in a genetic analyser (SeqStudio, Thermo Fischer, Waltham, Massachusetts, USA). The allele scoring was performed by applying a fragment length analysis using the software GeneMapper v.6.0 (Thermofischer). Allele binning was performed manually. The genotype lists were exported for use in population genetic analyses.

In order to identify the origin of the 108 clones, 98 additional individuals from five reference native populations were used (Table 1, Figure 1). These five populations were subsamples of the native populations published in [4,20]. Bayesian clustering analysis was performed with the whole dataset of 206 individuals (i) in order to identify a predefined number of **K** genetic clusters (in which the Hardy–Weinberg Equilibrium is nearly fulfilled, according to the method) and (ii) to assign the analysed individuals a membership proportion to each one of the K clusters.

For the analysis, the STRUCTURE software [23,24] was used. Twenty independent runs were performed for each of **K** = 1 . . . 10 assumed clusters. The STRUCTURE settings were the same as in [20], namely, 50,000 burnin replications followed by 100,000 MCMC iterations applied at each run, assuming the admixture model and correlated allele frequencies. After the analysis, the data were processed with the programmes (i) Structure Harvester [25] to calculate the statistic Δ**K** and infer the most likely number of **K** clusters according to [26], as well as (ii) the on-line platform CLUMPAK [27], which implements the CLUMPP algorithm [28] for cluster alignment and averaging among runs, as well as for detecting multimodality (different clustering solutions). The criteria for selecting the most likely numbers of **K** clusters were: (i) maximisation of the statistic Δ**K** and (ii) consistency among the clustering solutions (a high degree of unimodality, i.e., similarity of the clustering pattern across runs, as revealed by CLUMPAK).

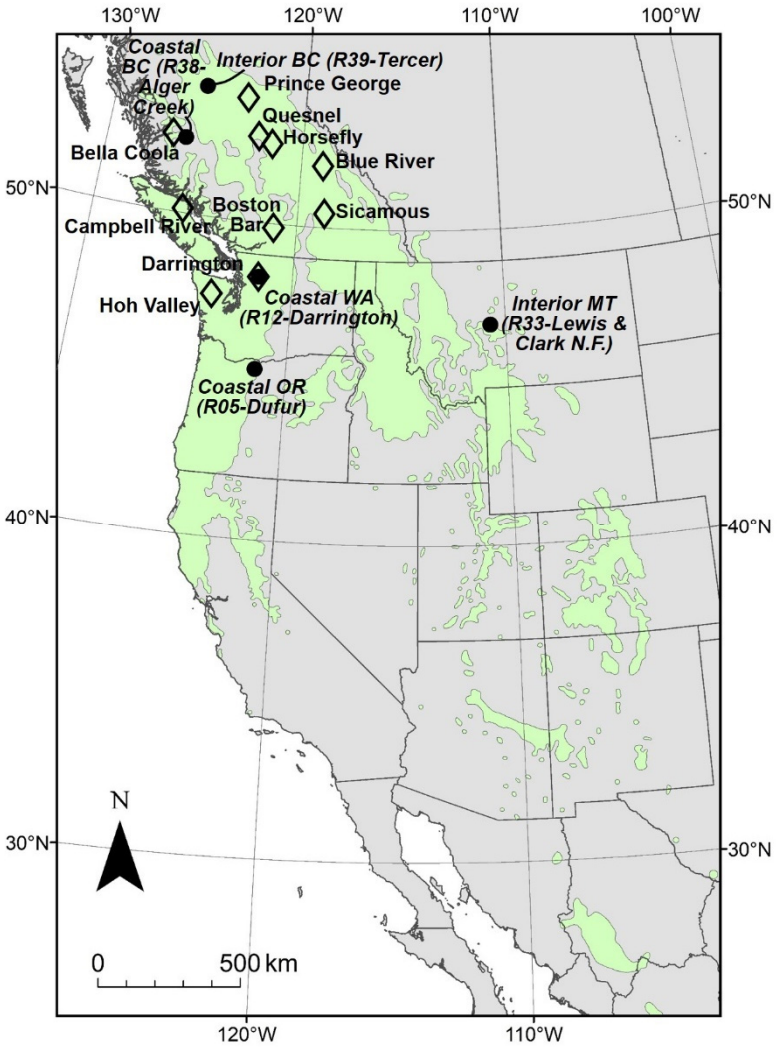


Figure 1. Provenances with a documented native origin. Documented native provenances of the genotyped Swedish clones are marked with empty diamonds, reference provenances used for molecular genetic assignment are marked with black filled circles and labels in italics. The distribution area of Douglas fir is shown with light green colour.

For further inspecting the relationships between the clusters, pairwise F_{ST}s [29] were calculated with the FSTAT software [30] for all clustering solutions above **K** = 2, which were selected according to the aforementioned criteria. The significance of the pairwise F_{ST} values was tested by applying the maximum possible number of permutations of genotypes between the populations (i.e., not assuming Hardy–Weinberg equilibrium within the clusters) in each case.

Analyses of genetic diversity were performed within the genetic clusters inferred by Bayesian clustering analysis for different values of **K**. First, the software GenAlEx [31,32] was used to compute different measures of genetic diversity. As several measures are strongly influenced by population size, allelic richness was also calculated using the technique of rarefaction to account for sample size differences [33]. The rarefaction size of six alleles (three fully genotyped individuals) was defined based on the smallest number of genotyped alleles at a given marker across all clustering solutions. Calculations of allelic richness with rarefaction were performed using the software package ADZE [34]. Moreover, we performed Wilcoxon signed-rank tests across loci to compare the allelic richness and the inbreeding coefficients between the Swedish clones and the reference samples of a given genetic cluster. The other diversity parameters calculated were not used for comparison because they are strongly affected by sample size differences [33].

An investigation of the correspondence of phenology and information of the genetic background was based on the open family seedlings produced for the new progeny trials. The assessment of the phenology data was made in the plant nursery on all plants in one randomly selected tray from each family from the first seedling batch. One-year-old seedlings from 76 families could then be scored for bud burst on two occasions in 2021, May 17th (Phen1) and 24th (Phen2), with a six-stage key based on photographs following [11]. Statistical analyses were carried out in several steps using ASReml [35]. Preliminary univariate analyses of the phenology traits (Phen1, Phen2, Hast = Phen2 − Phen1) were obtained to acquire an overview of the trait, test additional explaining variables and to obtain starting values for the bivariate analyses. The final mixed-model equation used for the analyses of the individual observations was:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z}_1\mathbf{m} + \mathbf{Z}_2\mathbf{s} + \mathbf{e} \quad (1)$$

with $\mathbf{y} = \left[\mathbf{y}'_1 \ldots \mathbf{y}'_n\right]'$, $\mathbf{b} = \left[\mathbf{b}'_1 \ldots \mathbf{b}'_n\right]'$, $\mathbf{m} = \left[\mathbf{m}'_1 \ldots \mathbf{m}'_n\right]'$ and $\mathbf{s} = \left[\mathbf{s}'_1 \ldots \mathbf{s}'_n\right]'$, where the vector **y** holds the phenotypic observations of trait 1 to n (n = 1 for univariate and 2 for bivariate); **b**, **m**, **s** and **e** are the vectors of the fixed effects, the random mother effect (the general combining ability, GCA), the random effect of the interaction between the STRUCTURE assignments for the **K** = 4 assumed cluster configuration (CL4GRPS discrete groups) and provenance (see Table 1) (CL4GRPS × provenance) data and the residual deviations, respectively; **X**, $\mathbf{Z}_1$ and $\mathbf{Z}_2$ are the corresponding incidence matrices. For the discrete variable CL4GRPS, the progeny of a given clone was assigned to the given provenance and to a STRUCTURE cluster based on the membership proportion (q value) of the mother tree to a certain cluster derived by the STRUCTURE analysis for **K** = 4. The random terms m, **s** and **e** were assumed to be independent and normally distributed with zero means and (co)variances as:

$$\mathbf{Var}\begin{bmatrix}\mathbf{m}\\ \mathbf{s}\\ \mathbf{e}\end{bmatrix} = \begin{bmatrix}\mathbf{M}\otimes\mathbf{I} & \mathbf{0} & \mathbf{0}\\ \mathbf{0} & \mathbf{K}\otimes\mathbf{I} & \mathbf{0}\\ \mathbf{0} & \mathbf{0} & \mathbf{R}\otimes\mathbf{I}\end{bmatrix} \quad (2)$$

where **0** is a null matrix; **I** is an identity matrix; and $\mathbf{M} = \{\sigma_{\mathbf{m}_i\mathbf{m}_j}\}$, $\mathbf{K} = \{\sigma_{\mathbf{s}_i\mathbf{s}_j}\}$ and $\mathbf{R} = \{\sigma_{\mathbf{e}_i\mathbf{e}_j}\}$ are the symmetric genetic mother, $\mathbf{K}_4$ × provenance and residual (co)variance matrices, and $\otimes$ is the direct product (i, j = 1 to trait n, denoting variance when i = j, e.g., $\sigma_{\mathbf{m}_i\mathbf{m}_j} = \sigma^2_{\mathbf{m}_i}$). Models with alternative sets of fixed effects were compared using the conditional F test (type III) in ASReml. Estimated variance components were assumed non-zero when they exceeded twice their approximate standard error. Otherwise, the likelihood-ratio test (LRT) [35] was used to justify the acceptance or rejection of a non-zero estimate.

Using the estimated components of variance, the phenotypic variance (σ^2_P) and narrow-sense heritabilities $\left(h^2\right)$ were estimated as: $\sigma^2_P = \sigma^2_A + \sigma^2_E$ and $h^2 = \sigma^2_A/\sigma^2_P$ with the additive genetic variance as $\sigma^2_A = 3\sigma^2_{\mathbf{m}}$ (i.e., assuming a mixed crossing pattern of full- and half-sibs). Additive genetic correlations between traits 1 and 2 ($r_{A_{12}}$) were calculated from bivariate analysis variance components according to Falconer and Mackay (1996) as $r_{A_{12}} = \sigma_{A_{12}}/\sigma_{A_1}\sigma_{A_2}$. The standard errors of the derived parameter estimates were approximated by Taylor series expansion, based on the ASReml results.

3. Results

3.1. Identification of the Clone Origin Using Bayesian Cluster Analysis

According to the posterior probability and the modes of the STRUCTURE runs, as well the values of the Δ*K* statistic, the clustering solutions for **K** = 2, 4 and 6 were kept for further investigation. For these values, the Δ**K** statistic peaked (Figure S1), whereas the clustering solution was rather consistent among runs (results of cluster modes and posterior probability of the data in Table S1).

As expected, the solution for the **K** = 2 cluster configuration corresponded to the two varieties—coastal (*Pseudotsuga menziesii* var. *menziesii*) and interior (*Pseudotsuga menziesii* var. *glauca*). All Swedish samples from S2265 Bruzaholm and/or S2266 Tönnersjöheden with a documented origin from Horsefly, Prince George, Quesnel and Sicamous (all from the Rocky Mts. in British Columbia, Canada) were assigned to the cluster of the interior variety (Figure 1). All Swedish Douglas fir clones with a declared origin from Bella Coola (British Columbia, Canada) and Darrington (Washington, DC, USA) clustered together with the coastal variety. Most Swedish individuals from Blue River (Rocky Mts., British Columbia, Canada) showed a higher membership proportion to the cluster of the interior variety. Oppositely, most individuals from Boston Bar showed an affinity to the coastal variety. Finally, the 31 individuals selected among open-pollinated progenies of Danish clones (Table 1) were mostly identified as coastal Douglas fir (Figure 2).

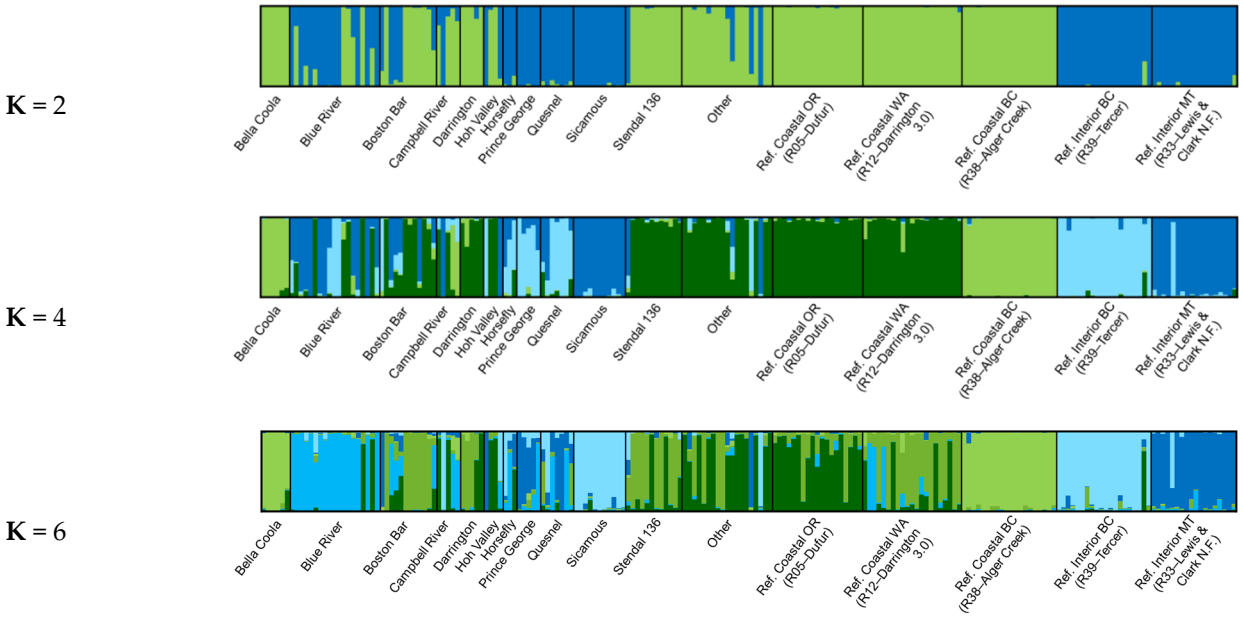


Figure 2. Membership proportion of individuals (slim bars) to clusters identified by the STRUCTURE (Bayesian clustering) analysis (green = coastal, blue = interior) for **K** = 2, 4 and 6 modelled clusters. Individuals belonging to the same reference population (as listed in Table 1) are marked by black boarder lines. The first 12 boxes from left to right (Bella Coola to Other) include the 108 Swedish clones, while the last five boxes include the five native reference populations. BC = British Columbia, Canada; MT = Montana, USA; OR = Oregon, USA; WA = Washington, USA.

At **K** = 4, splits could be observed within both varieties, separating northern from southern origins. Within the coastal variety, the southern reference populations Dufur (Oregon, OR, USA) and Darrington 3.0 (Washington, WA, USA) belonged to a different cluster than the northern Alger Creek (British Columbia, Canada) (Figure 2). Henceforth, we refer to these two clusters as the southern and northern coastal cluster, respectively. Within the interior variety, Tercer (British Columbia, Canada) on the one hand and Montana Lewis and Clark National Forest (Montana, MT, USA) on the other, formed different clusters (Figure 2), hereinafter named the northern and southern interior clusters, respectively.

Within the four interior provenances represented among the Swedish samples, Horsefly, Prince George and Quesnel were assigned to the northern, whereas Sicamous was assigned to the southern cluster of the interior variety. This is in line with their geographic distribution (Figure 1). Within the Swedish samples of coastal Douglas fir, clones from Bella Coola showed membership to the northern coastal cluster, which also fits to their geographic origin (Figure 1). Accordingly, the clones from Darrington were assigned to the southern coastal cluster. All samples of the non-native clones (i.e., the genotypes selected from the progeny test of Danish clones; Stendal 136 and other in Table 1) displayed affinity

to the southern coastal cluster. Douglas fir clones of the provenance test from Blue River, Boston Bar, Campbell River and Hoh Valley showed a mixed ancestry.

At **K** = 6, a further distinction was detectable within the southern cluster of the coastal variety. Most individuals from Dufur (Oregon, OR, USA), but also half of the 31 Douglas fir genotypes from the progeny test of Danish clones (labelled as "other" in Table 1 and Figure 2) exhibited a high membership proportion to a "southernmost" cluster of the coastal variety (Figure 2). Interestingly, the Swedish Douglas fir clones from Blue River, which had shown a mixed ancestry under **K** = 2 and 4, formed a separate cluster (Figure 2). This seems to reflect their intermediate position between the northern and the southern reference populations of the interior Douglas fir.

Pairwise F_{ST} values quantified the genetic differentiation among the STRUCTURE clusters. Both at **K** = 4 and **K** = 6, pairwise comparisons between the northern coastal and any of the other clusters displayed the highest F_{ST}s, all of them being statistically significant (Tables 2 and 3). Interestingly, comparisons across varieties did not necessarily result in higher F_{ST}s than at the within-variety level. For instance, the pairwise F_{ST} values between the northern interior cluster and any of the coastal clusters at **K** = 6 ranged between 0.035 and 0.090 and were in all cases significant (Table 3). Within the interior variety, the F_{ST} between the northern and the southern interior cluster was also within this range, but not significant at **K** = 6 (Table 3). Finally, the 'Blue River' cluster did not clearly show a higher affinity to the one or to the other variety (Table 3).

Table 2. Pairwise F_{ST} values (below diagonal) between clusters for the **K** = 4 clustering solution (according to the STRUCTURE analysis) and probability of random F_{ST} value being higher than the observed (above diagonal). The indicative adjusted nominal level (5%) for multiple comparisons is 0.008.

Cluster	Coastal South	Coastal North	Interior North	Interior South
Coastal south		0.008	0.008	0.008
Coastal north	0.046		0.008	0.008
Interior north	0.040	0.080		0.008
Interior south	0.028	0.072	0.036	

Table 3. Pairwise F_{ST} values (below diagonal) between clusters for the **K** = 6 clustering solution (according to the STRUCTURE analysis) and probability of random F_{ST} value being higher than the observed (above diagonal). The indicative adjusted nominal level (5%) for multiple comparisons is 0.003.

Cluster	Coastal South	Coastal Centre	Coastal North	Blue River	Interior North	Interior South
Coastal south		0.003	0.003	0.003	0.003	0.003
Coastal centre	0.022		0.003	0.003	0.003	0.003
Coastal north	0.061	0.059		0.003	0.003	0.003
Blue River	0.037	0.039	0.075		0.003	0.003
Interior north	0.035	0.047	0.090	0.054		0.010
Interior south	0.055	0.054	0.094	0.059	0.037	

3.2. Comparing the Genetic Diversity of Swedish Douglas Fir Clones vs. the Reference Populations

The coastal Douglas fir clones from Sweden exhibited slightly higher values of mean and effective allele number per locus, allelic richness with rarefaction and expected heterozygosity versus our reference samples from the native range (the STRUCTURE clustering solution for **K** = 2, see Table 4). For all these parameters, the values for the Swedish versus the reference populations were within the standard error of each other. On the other hand, the observed heterozygosity was lower among the Swedish samples of the coastal cluster which resulted in a significantly higher inbreeding coefficient compared to the native range ($p < 0.05$, Wilcoxon signed rank test). A comparison between the reference and Swedish Douglas firs assigned to the interior variety gives a similar picture, whereas the inbreeding coefficient did not differ significantly. The diversity values for the Swedish samples

were higher, but within the standard error range of the reference estimates (Table 4). The Wilcoxon signed rank test also showed that the differences in the allelic richness between the Swedish and the reference samples of the interior cluster were not significant.

Table 4. Average ± standard error of various diversity measures for Swedish and native Douglas fir (reference populations) assigned to one of the two varieties by STRUCTURE for **K** = 2. A/L = number of alleles per locus, A_e = effective number of alleles, AR_6 = allelic richness; rarefaction size = 6 alleles, H_o = observed heterozygosity, H_e = expected heterozygosity, F = fixation index.

Pop.	*N*	A/L	A_e	AR_6	H_o	H_e	F
Coastal Sweden	56	26.46 ± 2.87	13.93 ± 1.93	4.94 ± 0.18	0.580 ± 0.046	0.903 ± 0.018	0.362 ± 0.047
Coastal reference (native)	60	26.08 ± 2.49	12.74 ± 1.44	4.91 ± 0.17	0.673 ± 0.050	0.901 ± 0.018	0.255 ± 0.051
Interior Sweden	52	22.69 ± 2.09	12.75 ± 1.35	4.92 ± 0.16	0.548 ± 0.046	0.903 ± 0.016	0.397 ± 0.045
Interior reference	38	19.46 ± 2.04	10.48 ± 1.44	4.71 ± 0.20	0.509 ± 0.062	0.872 ± 0.023	0.423 ± 0.064

A further splitting to clusters within the varieties (STRUCTURE clustering solution for **K** = 4) revealed that the diversity differences between the Swedish and reference populations remained small and within the standard error range (Table 5). This was particularly true for the rarefied allelic richness (AR_6 in Table 5) which is less affected by differences in sample size [36]. A significantly higher inbreeding coefficient could be observed within the Swedish Douglas firs assigned to the southern coastal cluster compared to the reference genotypes of the same cluster ($p < 0.01$, Wilcoxon signed rank test). The lower genetic diversity of the Swedish Douglas firs assigned to the northern coastal cluster in terms of the mean and effective number of alleles and heterozygosity might have been due to the small sample size (eight individuals). The difference between the Swedish and reference samples assigned to this cluster was not statistically significant. The only significant difference in the rarefied allelic richness was found between the Swedish and reference genotypes of the interior northern cluster ($p < 0.05$, Wilcoxon signed rank test), with the latter being less diverse.

Table 5. Average ± standard error of various diversity measures for Swedish and native Douglas fir (reference populations) assigned to one of the two varieties by STRUCTURE for **K** = 4. A/L = number of alleles per locus, A_e = effective number of alleles, AR_6 = allelic richness; rarefaction size = 6 alleles, H_o = observed heterozygosity, H_e = expected heterozygosity, F = fixation index.

Pop.	*N*	A/L	A_e	AR_6	H_o	H_e	F
Coastal south Sweden	48	25.08 ± 2.62	13.68 ± 1.75	4.97 ± 0.17	0.581 ± 0.045	0.905 ± 0.017	0.361 ± 0.046
Coastal south reference	40	22.69 ± 2.09	12.69 ± 1.44	4.96 ± 0.17	0.695 ± 0.045	0.902 ± 0.018	0.231 ± 0.045
Coastal north Sweden	8	7.77 ± 0.70	6.14 ± 0.65	3.81 ± 0.25	0.572 ± 0.073	0.801 ± 0.032	0.308 ± 0.076
Coastal north reference	20	10.54 ± 0.86	5.70 ± 0.50	4.14 ± 0.16	0.623 ± 0.075	0.802 ± 0.023	0.222 ± 0.090
Interior north Sweden	18	13.38 ± 1.10	8.82 ± 0.77	4.51 ± 0.18	0.544 ± 0.056	0.869 ± 0.018	0.378 ± 0.059
Interior north reference	21	14.85 ± 1.88	10.23 ± 1.60	4.16 ± 0.18	0.486 ± 0.068	0.847 ± 0.035	0.442 ± 0.066
Interior south Sweden	34	19.69 ± 1.85	12.01 ± 1.26	4.80 ± 0.17	0.551 ± 0.047	0.899 ± 0.015	0.392 ± 0.047
Interior south reference	17	9.23 ± 0.80	5.81 ± 0.63	4.60 ± 0.29	0.537 ± 0.068	0.794 ± 0.027	0.322 ± 0.083

3.3. Differences of Bud Burst Timing among Progenies of Swedish Douglas Fir

The univariate analysis of the bud burst timing for the cluster groups of the **K** = 4 cluster configuration from the STRUCTURE analysis (i.e., the coastal north and south, the interior north and south) showed the following results (see Table 6): (i) The predicted phenological scores were higher for the interior variety, indicating an earlier bud burst versus the coastal Douglas fir. Differences could also be observed within the coastal variety. (ii) The progenies of the clones assigned to the southern cluster flushed earlier compared to northern provenances, as shown by their higher phenological score. The progress of bud burst seemed to be faster in the southern cluster (Table 6). (iii) Within the interior variety, the differences between the northern and southern provenances were within standard error, and the progress of bud burst was similarly fast between the northern versus the southern cluster.

Table 6. Predicted value for Phen1 (phenological score of bud burst on 17 May 2021), Phen2 (phenological score of bud burst on 24 May 2021) and Hast (difference between the two phenological scores as an indicator of bud burst speed).

CL4GRPS	Phen1	Phen2	Hast
K4_cl1 (coastal south)	2.23 ± 0.03	2.94 ± 0.04	0.71 ± 0.02
K4_cl2 (coastal north)	1.96 ± 0.07	2.54 ± 0.09	0.61 ± 0.05
K4_cl3 (interior north)	2.84 ± 0.05	3.75 ± 0.06	0.90 ± 0.04
K4_cl4 (interior south)	2.79 ± 0.04	3.69 ± 0.05	0.90 ± 0.03

The bivariate result of the genetic parameters is shown in Table 7. The inclusion of the STRUCTURE result in the provenance (CL4GRPS × provenance) in the model made it possible to better account for the provenance effects. A comparison of the cluster groups **K** = 2, 4 or 6 in the model (CL2GRPS, CL4GRPS, CL6GRPS) decreased the heritability but gave the best support to the inclusion of CL4GRPS when comparing the AIC (Akaike Information Criterion) values (data not shown).

Table 7. Estimates of parameters (±standard error) obtained from the bivariate analyses. Additive genetic correlation estimates above the diagonals and phenotypic correlations below the diagonals.

Trait	Phen1	Phen2	Hast	h^2
Phen1	-	0.91 ± 0.03	0.35 ± 0.15	0.62 ± 0.14
Phen2	0.86 ± 0.02	-	0.71 ± 0.08	0.71 ± 0.15
Hast	0.10 ± 0.05	0.60 ± 0.03	-	0.52 ± 0.11

4. Discussion

We observed a north–south differentiation within the Douglas fir varieties using a well-established set of markers. By increasing the number of assumed clusters in the STRUCTURE analysis, we could differentiate between the two varieties, but also resolve genetic clusters within the varieties. Interestingly, when assuming six genetic clusters (**K** = 6), we could identify a genetic cluster with genetic affinity to the coastal variety (see Table 3). This cluster was mainly represented by the Swedish clones selected in the provenance test (plots S2265 Bruzaholm and S2266 Tönnersjöheden) originating from Blue River, which lies within the range of the interior variety (see Figures 1 and 2). The genotypes of these clones showed a mixed membership to the coastal and interior variety when we opted for **K** = 4 assumed clusters in the STRUCTURE analysis. We interpret this as a result of genetic introgression. An introgression zone covers wide parts of the Rocky Mountains in a west–east direction at this latitude, as shown by studies based on plastid and nuclear DNA markers [4,9,37].

With few exceptions, the molecular genetic identification of the clones agreed with the documented provenance for most of the 77 clones of the provenance test. One of these exceptions is clone S21K1160092, which showed a genetic affinity to the southern cluster of

the interior Douglas fir although its documented origin is Hoh Valley, Olympic Peninsula, Washington, within the range of the coastal variety. Interestingly, the open-pollinated progeny, established with seeds collected from the same tree used for genotyping, exhibited an early bud burst which is typical for the interior variety (see also discussion below). This unexpected result could be a rootstock of interior origin outgrowing the scion, which is a typical cause of error involving incorrect graft assignment in seed orchards [38,39]. A similar observation was made for the clone S21K1160095, also from Hoh Valley (coastal) and again genetically assigned to the interior variety. In this case, the progeny was late flushing, suggesting a coastal origin. A labelling error could also account for such a result.

In contrast to the native clones originating from the provenance trial, the 31 genotypes from forward selection in the progeny test originating from Danish seed sources should be considered as non-native, as they belong at least to the second generation after introduction to Europe. With three exceptions, i.e., one intermediate and two interior genotypes, they were all assigned to the southern coastal cluster. This reveals an origin from the region between Washington and northern Oregon, USA (the origin of our reference populations representing the southern coastal cluster) for most Douglas fir plus trees included in the Danish seed orchard. Due to repeated seed imports from Washington, many of the Danish Douglas fir stands established in the early 20th century most likely originated from this state [1].

The molecular genetic analysis provided insights into the genetic diversity of introduced Douglas fir populations in Sweden. The diversity levels are comparable between the native reference populations and the clones of the Swedish breeding programme after assigning them to the correct cluster. The high inbreeding coefficient among Swedish clones of the southern coastal cluster is notable (see Table 5). Given that most of the clones (29 out of 48) originated from the progeny test of Danish seed sources, this effect could be due to mating patterns in the selected seed stand or seed orchards. In particular, mating between related genotypes or self-mating may have both increased the inbreeding coefficient over the generations [40,41]. A marker-based pedigree reconstruction involving two generations could address this effect [41,42].

In addition to the molecular genetic structure, the origin differences were mirrored in the phenological behaviour of the progenies of the Swedish Douglas fir clones. In particular, the phenology pattern for bud burst timing among the 76 open-pollinated families followed the expectation that coastal provenances respond later in spring than interior provenances [43,44]. In addition, differences were found within the coastal variety. The progenies of the clones assigned to the southern cluster flushed earlier, on average. Similar observations were made in common garden experiments in the native range [45]. The earlier bud burst of southern coastal provenances was interpreted as an adaptation to increasing summer drought towards the south [45]. An early flushing results in growth gains during spring before the onset of summer moisture deficit which takes place earlier and is more intense in the south [45].

5. Conclusions

Planting Douglas fir offers a forest management adaptation option against climate change impacts in southern Sweden. The strong relationship between bud burst and genetic background allows the selection of Douglas fir reforestation material for different growing conditions in Sweden. Molecular provenance identification (e.g., [20]) is a useful approach if the origin of introduced Douglas fir stands is unknown. Origin assessment of selected phenotypes provides an early indication about their adaptive characteristics and growth performance (see also [9]). This assists future selections for different regions (breeding zones) and supports the Swedish Douglas fir breeding programme because the applied set of markers provide insights into the genetic variation of introduced Douglas fir stands. The results of our work will guide further breeding efforts of Douglas fir in southern Sweden. The provenance analysis of the Swedish plus trees will help to optimise harvesting strategies of Swedish seed orchards. Knowledge of the geographical origin of

the different plus trees can be used for the selective harvest of clones to produce a more genetically uniform seed crop in seed orchards. The ability to match the right provenance with the growing location is important for forest management, since late flushing genotypes can be promoted for use in sites prone to late frosts in spring. Further developments of the breeding programme should focus on the establishment of separate selection populations and seed orchards for different breeding zones. A parallel step would be the establishment of new seed orchards by separating clones according to their origin and adaptive traits. This would prevent outbreeding depression caused by mating between parents with different genetic backgrounds [46].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13060895/s1, Figure S1: value of the statistic ΔK for assumed numbers of clusters ranging from 2 to 9, Table S1: different modes identified for STRUCTURE runs.

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