



Ad hoc breeding and occasional disorders – a case study on current season needle necrosis in Nordmann fir (*Abies nordmanniana* (Steven) Spach)

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Abstract

Ad hoc breeding of forest trees, noted for its flexibility, has been predicted to be useful for addressing pests, diseases, and disorders under specific conditions. This study aimed to demonstrate the use of ad hoc breeding to address the occasional disorder current season needle necrosis (CSNN) in Nordmann fir. First, 1545 random reference trees from two plantation stands in Denmark (689 from Tørring and 856 from Mariager) with a known parental population (clonal seed orchard FP.266 Skibelund) were used. Second, to specifically screen for CSNN, 495 moderately to severely damaged trees (Sel-495) were selected from the most affected area in the Mariager stand. Clonal parents from FP.266 were ranked based on either the prevalence of CSNN in their offspring (Sel-495) or their breeding value following SNP-based parentage and quantitative genetic analysis (reference trees). In the Sel-495 dataset, 55.6% of the affected offspring originated from 20% of the parents, while 36% of the clonal parents had moderately or severely affected offspring in the reference trees. Moderate overlap was observed between clonal parents' ranking based on breeding values and the ranking based on the over-frequency of their offspring in Sel-495 trees. Overall, ad hoc breeding and parentage analysis facilitated backward selection against CSNN by enabling the estimation of genetic parameters ($h^2=0.08\pm0.04$) using reference trees and identifying high-risk parents. A combination of breeding value rankings and ranking based on CSNN prevalence in their offspring was used as a parental selection approach. The findings demonstrate that ad hoc breeding is an effective, flexible strategy for managing and breeding against unpredictable disorders in forest trees under changing climate conditions.

Keywords Ad hoc breeding · Current season needle necrosis · Conifers · Parentage · Single nucleotide polymorphisms

Introduction

The shifting climate profoundly alters the distribution and dynamics of forest pests and pathogens, along with their interactions with trees, ultimately affecting the resilience of forest ecosystems to withstand these challenges (Linnakoski et al. 2019). Moreover, biotic and abiotic stressors, such as insect pests, diseases, and wildfires, are expected to escalate, posing significant challenges for forest management

(Keenan 2012). An evident way of responding to newly emerging biotic and abiotic stress in forestry is breeding for resistance to new stress factors. Although targeted resistance breeding has shown promise in enhancing the health of certain commercially valuable tree species, progress has been slow, limited to a few species, and often requires decades (Yanchuk and Allard 2009; Sniezko and Koch 2017). The prolonged nature of tree breeding using traditional trials, coupled with substantial work and spatial investments, underscores the need for complementary techniques. Field trials offer insights into adaptation to climate change, but pests, diseases, and disorders can have an occasional/infrequent occurrence that can be difficult to predict and impossible to impose on conventional field trials already established with traditional methods.

The ad hoc breeding of trees is distinguished by its flexibility and non-dependence on continuity. Its flexibility

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comes from the fact that breeders can establish new genetic field trials (quasi-field trials) via DNA markers and pedigree reconstruction in ordinary production stands where and when the trials are needed (Hansen and McKinney 2010; Xu et al. 2018). In ad hoc breeding, DNA markers are used to reconstruct additive relationships between individuals in traditional field trials with no family information (e.g., provenance trials) or in production stands, thereby enabling assessment of the genetics behind the traits of interest. This approach can be especially beneficial in relation to pests, diseases, and disorders, which may only arise under certain circumstances (biotic or abiotic related), which therefore may only be observed in a limited time or spatial range. Thus, when a pest, disease, or disorder occurs in a forest production stand composed of offspring from a well-defined seed source with a bounded parent population, the establishment of quasi-field trials allows breeding in relation to either the parental seed source (backwards selection) or the offspring (forward selection).

Current season needle necrosis (CSNN) is a distinct needle disorder known in conifer Christmas tree production among several species from the genus *Abies* (Chastagner and Benson 2000; Çakar et al. 2023). It initiates during the development of shoots on the current year's growth, but its exact cause remains uncertain (Chastagner et al. 1990). Symptoms manifest as yellowish bands on needles, later turning reddish-brown, with potential necrosis of entire needles leading to shedding. Various hypotheses regarding its causal origin exist in the literature—for example, high temperature, calcium deficiency (Chastagner et al. 2012), nutrient disorder caused by heavy fertilization (Chastagner and Landgren 1998; Nielsen et al. 2019), or potential impact of fungi (Talgå et al. 2010; Çakar et al. 2023). The CSNN disorder is generally recognized to be more prevalent in plantations at lower elevations, and site conditions also severely impact damage levels. For example, plantings on Danish former farmland had more damage compared to the same plant material established on forest land (Nielsen and Christensen 1997). One of the things that make Christmas tree production special compared to normal forestry is the low tolerance to visible damage caused by pests or disorders, such as CSNN. Regardless of the cause of CSNN, reports indicate genetic variation in susceptibility to the disorder (Nielsen and Christensen 1997; Thompson 2005; Chastagner et al. 2012), providing an opportunity for selective improvement. Differences in susceptibility among trees are attributed to the unique genetic makeup of individual fir trees, as they are propagated from seed (Talgå et al. 2010). Additionally, significant differences in CSNN susceptibility have been observed among seed sources, with various populations showing varying levels of resistance in noble fir (Chastagner et al. 2008).

Christmas tree production in Europe was previously a side production of traditional forestry, exploiting the thinning trees from forest stands of, e.g., Norway spruce (*Picea abies*). However, in the last 50 years, it has gradually become a highly specialized production, mostly taking place on agricultural lands (Christensen 2024 pers. comm.). In Europe as well as North America, many *Abies* species are used in Christmas tree production due to factors such as nice appearance, soft needles, and good post-harvest needle retention (Nielsen et al. 2020). The most used Christmas tree in Europe today is Nordmann fir (*Abies nordmanniana*), with 40 million trees of 75 million consumed trees annually. Approximately 10 million of these trees are grown in Denmark of which a majority are exported (Christensen 2024 pers. comm.). In Denmark, severe CSNN damage have been observed in 13 out of 32 years during the period 1980 to 2012 - or approximately every 3rd year (Thomsen and Talgå 2013), but substantial geographic variation in the damage level have been observed (Hansen et al. 2012). In 1992, a Danish Nordmann fir Christmas tree breeding program was initiated (Nielsen 1994, 2000). It includes several gene pools, each with presumably different origins in the natural distribution area of the species, which grows naturally on the eastern side of the Black Sea, i.e., the Caucasus of Russia, Georgia, and northeastern Turkey (Liu 1971).

For the so-called Ambrolauri gene pool of the breeding program (see Ousmael et al. 2023 for details), two clonal seed orchards (CSOs) were established in 1995, grafted with scions from phenotypically selected (best-appearing) Christmas trees. These orchards produced seeds in mercantile amounts for the first time in 2009, which were used to establish many commercial Christmas tree stands. To select the best trees for the next generation through ad hoc breeding, commercial Christmas tree stands established with seeds from one of the CSOs were tracked to two sites—Mariager and Tørring. From these two stands, the best-appearing trees (plus trees) were selected. To enable genetic parameter estimation, two neighboring trees were included as reference trees for each plus tree (Xu et al. *in prep.*). Additionally, on one of the sites, 495 trees severely affected by CSNN (Sel-495) were selected to aid in parent tree selection based on the prevalence of CSNN in their offspring. This study used parentage analysis, by means of single nucleotide polymorphism (SNP) markers to select against occasional disorders in ordinary production stands, using CSNN and the two above-described commercial Christmas tree stands as a case study. Consequently, the specific aim of the study was to demonstrate how ad hoc breeding can be utilized as a versatile tool to breed against pests, diseases, and disorders based on opportunity and need. Using a combination of Sel-495 trees and randomly sampled reference trees, we tested simple investigation of parents for over-frequency

of their offspring in the affected population (Sel-495), as well as estimation of breeding values based on quantitative genetics within ordinary stands via ad hoc breeding. This study differs from previous studies using similar approaches (e.g., Xu et al. 2018) by targeting a specific and occasional disorder, rather than general improvement for multiple traits. It applies a dual-ranking strategy—based on both the frequency of affected offspring and estimated breeding values—to identify and select against high-risk parents. As such, it demonstrates a more diagnostic and responsive use of ad hoc breeding compared to broader gain-oriented strategies.

Material and methods

Origin of plant material – clonal seed orchard FP.266

In two Danish Christmas tree stands (F.808 Ny Saltbjerg and F.824 Tveden) with genetic material originating from the Ambrolauri Tlugi region in Georgia, 200 plus trees were phenotypically selected for superior Christmas tree quality in the early 1990's (tree ages 12 and 14 years). Of the 200 initially selected trees, 17 were discarded due to early flushing. Scions from the remaining 183 selected trees were grafted in 1995 in the CSO named FP.266 Skibelund (55.436208, 9.643111 DD) (Danish Nature Agency 2013). The orchard has later been thinned based on direct assessment of the grafted trees' own performance – mainly removing genotypes having poor postharvest needle retention. At the time of the first commercial seed harvest in 2009, which was a bumper crop year in Denmark, 140 clones were present in the CSO.

Experimental sites, selected trees, and setup

As part of an ad hoc breeding effort for Christmas tree quality in the Ambrolauri gene pool, progeny in commercial Christmas tree plantings from the 2009 harvest of the Danish Nature Agency 2013 were tracked to two locations: Mariager (56.681262, 10.095587 DD) and Tørring (55.899949, 9.504696 DD). The two sites were planted in spring 2016 (Mariager) and 2015 (Tørring), and the selected areas in these two sites comprise 21,500 and 17,600 trees, respectively. In each commercial plantation, a research design was laid out. Mariager comprises six blocks, each containing 18 sub-blocks with approximately 200 trees per sub-block. Tørring comprises eight blocks, each containing between 9 and 14 sub-blocks, with approximately 200 trees per sub-block. The spacing between trees is 1.2 m × 1.0 m. In each sub-block, four plus trees were selected. Selection criteria were Christmas

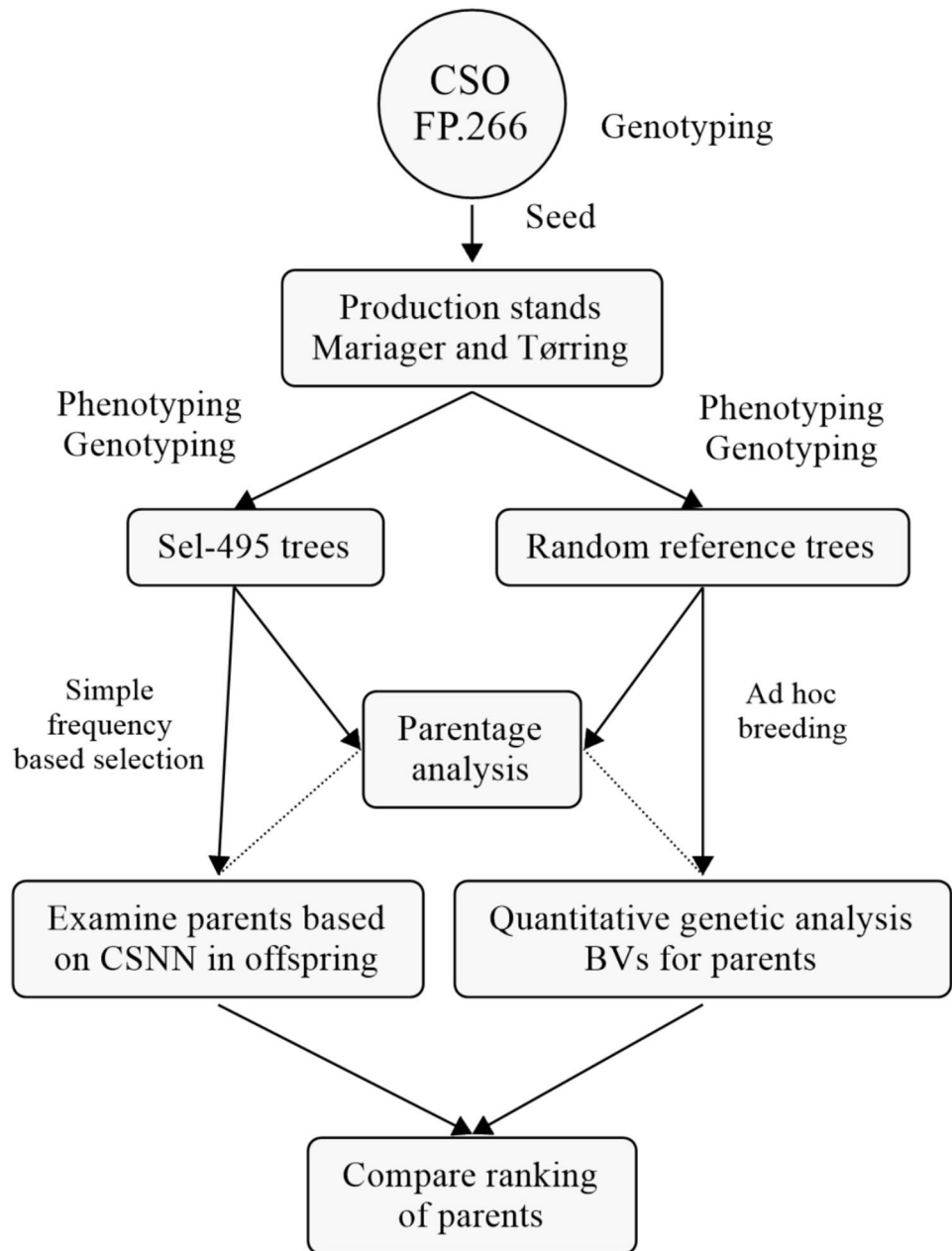
tree quality traits, including high branch density, upright branch angle, and no visible CSNN. This led to the selection of 784 plus trees. For each of the 784 selected plus trees, two neighboring reference trees planted next to the plus tree in the same row were included (systematically south and north of the selected trees). The role of the reference trees was to allow estimation of the response to selection as well as genetic parameters, such as genetic variances and heritability. In this specific study of and breeding for CSNN, we utilized 856 reference trees at Mariager and 689 reference trees at Tørring, which gives a random sample of 1545 offspring from the FP.266 CSO in relation to CSNN—referred to as reference trees. In contrast, the plus trees selected for good Christmas tree traits had a bias against CSNN, as this was a reason to deselect trees as plus trees, and data from the plus trees was consequently not used in this study.

Furthermore, for specific screening and to complement the ad hoc breeding effort in relation to CSNN, 495 moderately to severely damaged trees—referred to as Sel-495 (damage score ≥ 4 , see below)—were additionally selected at Mariager in an area of the site where the disorder was most pronounced. Needles from reference trees as well as Sel-495 trees were collected for DNA extraction and subsequent genotyping and parentage analysis. Figure 1 provides an overview of the methods and approach used in the study and is further described in the following sections.

Assessment of current season needle necrosis

Current season needle necrosis occurs after or during shoot elongation. Red needles are seen on the current year's shoot. Red bands on few or many needles are the first signs of damage and are often seen in late June. The number of red needles can increase throughout the summer and into August, depending on the weather conditions. A damage score was developed grouping damage as follows: none (0), minor damage (1–3), moderate damage (4–6), and severe damage (7–9). Both moderately and severely damaged trees exhibit needle discoloration that can lead to commercial downgrading when trees for harvest are marked in early September and October. In general, many of the red needles will be shed during autumn, leaving moderately damaged trees with only a few missing—non-critical—needles by the time of harvest in November. However, severely damaged trees, due to extensive needle discoloration and immediate or delayed needle drop, will appear with unacceptably bare shoots, rendering many of them unsalable for the current season. For severely damaged trees, it takes at least two additional growing seasons to recover from needle loss through new growth. Severely damaged trees also tend to have a higher risk of developing further problems in

Fig. 1 Overview of the methods used for breeding against CSNN in the Mariager and Tørring production stands



subsequent years. Figure 2 shows pictures of moderate and severe levels of damage.

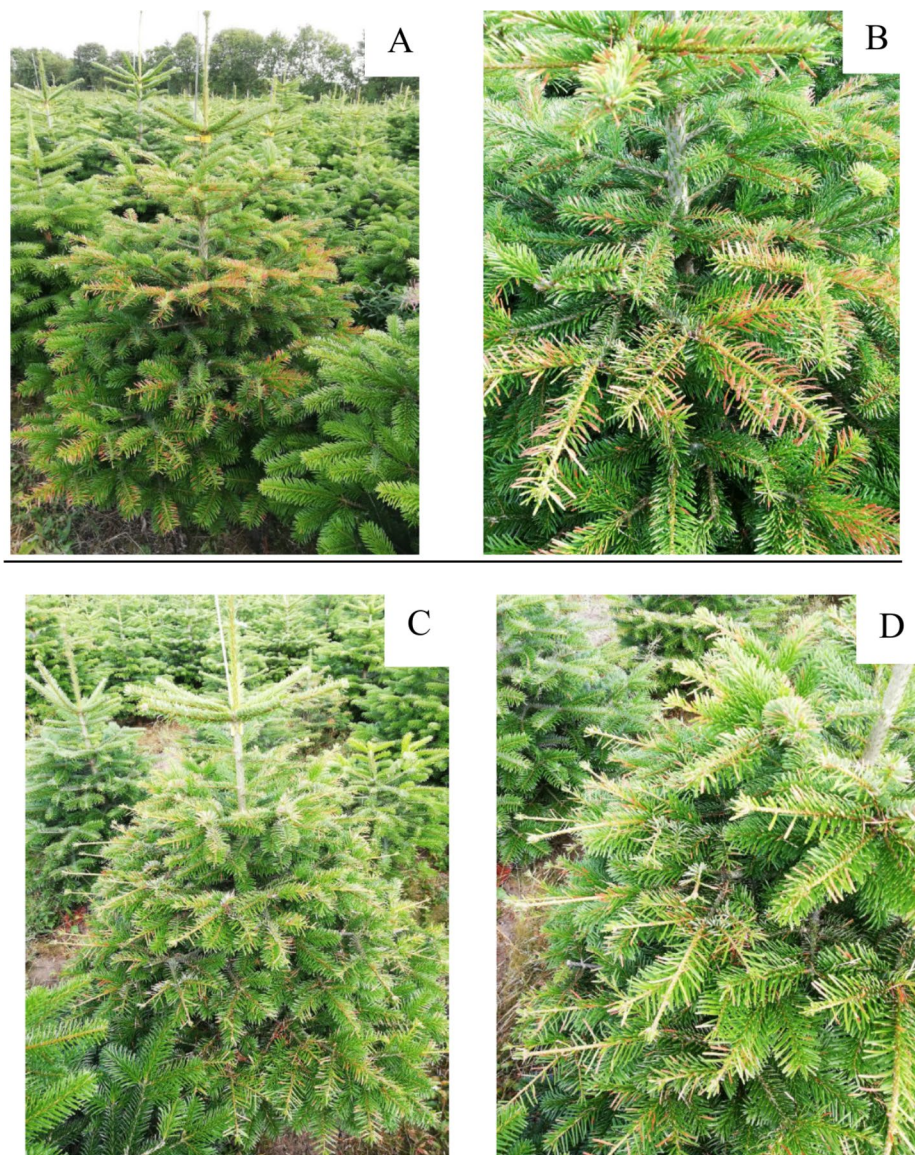
The 495 specifically selected damaged trees (Sel-495) at Mariager were assessed September 26–27, 2021. All reference trees at Mariager were scored for CSNN on August 10–26, 2021. In Tørring, reference trees were scored on August 20–24, 2021.

SNP genotyping

Targeted amplicon sequencing for 2180 (successfully genotyped) trees using 451 amplicon sequencing primers was accomplished by means of the Hi-Plex approach at Floodlight

Genomics LLC, Knoxville, Tennessee, USA (see Ousmael et al. (2023) for information on identification of the SNPs used in this study). The resulting amplicons were sequenced on an Illumina NovaSEQ 6000 (Admera Health LLC, Plainfield, NJ, USA) according to the manufacturer's instructions. The 2180 individuals included 856 reference trees from Mariager, 689 reference trees from Tørring, 495 CSNN selected (Sel-495) trees (also from Mariager), and 140 parent clones from the Danish Nature Agency 2013. In the case of the latter, three ramets of each clone were typically genotyped. We used ANGSD v. 0.935 (Korneliussen et al. 2014) for genotype calling. The filtering criteria in ANGSD included retaining reads with a mapping quality > 20, selecting only truly

Fig. 2 Photos **A** and **B** show moderate damage (CSNN score=4), while **C** and **D** illustrate severe damage (CSNN score=9) in Nordmann fir trees. The average tree height is 185 cm in Tørring and 133 cm in Mariager. Photos: Else Møller



variable SNPs (p values < 0.001), and retaining loci with a posterior genotype probability > 0.95 to ensure high-confidence SNP detection and genotyping.

Parentage analysis

Parentage analysis was conducted to identify parent pairs from FP.266 to the offspring trees in the two stands. The analysis was performed using the CERVUS 3.0.7 software (Marshall et al. 1998; Kalinowski et al. 2007) using the filtered SNPs. CERVUS employs a likelihood-based approach that incorporates allele frequency data to evaluate parent–offspring relationships. First, allele frequency analysis was performed site-wise, using a combination of the 140 clonal parent genotypes and individuals from each of the Mariager and Tørring sites separately. This was followed

by simulation analysis, creating 10,000 offspring by means of the allele frequency data generated in the previous step. The simulation of parentage analysis was used to evaluate the confidence in assignment of parentage to the most likely parents based on the observed allele frequencies. Parentage analysis was likewise performed separately for the Sel-495 trees from Mariager. Skewness in the distribution of parentage contribution was calculated in Microsoft Excel to assess the potential reproductive imbalance among parents.

Quantitative genetic analysis

To obtain breeding values for the parental clones in FP.266, the following linear mixed model was used for the CSNN score for 1371 reference trees with assigned parentage from Mariager and Tørring:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}_a\mathbf{a} + \mathbf{Z}_b\mathbf{b} + \mathbf{e}$$

where $\mathbf{y}$ is the combined vectors of CSNN data for two sites; $\boldsymbol{\beta}$ is the combined vector of fixed effects, including the grand mean and site, with its design matrix $\mathbf{X}$; $\mathbf{a}$ is the combined vector of random genetic effect and genotype-by-environment ($G \times E$) interaction effect with its design matrix $\mathbf{Z}_a$, following a normal distribution with zero mean and covariance matrix $\mathbf{A}\sigma_a^2$, where $\mathbf{A}$ is the average numerator relationship matrix derived from the pedigree information (Henderson 1984) and σ_a^2 is the additive genetic variance; $\mathbf{b}$ is a vector of random block effect with its design matrix $\mathbf{Z}_b$ and $\mathbf{e}$ is the combined vector of the random residuals from each site following a normal distribution with zero mean and (co)variance matrix $\mathbf{I}\sigma_e^2$, where $\mathbf{I}$ is the identity matrix and σ_e^2 is the residual variance. The average-numerator relationship A-matrix was computed using pedigree data derived from parentage analysis. The matrix was created in ASReml-R version 4.0 (Butler et al. 2017).

The narrow-sense individual heritability (h^2) was estimated as:

$$h^2 = \frac{\sigma_a^2}{\sigma_g^2 + \sigma_e^2}$$

where σ_a^2 is the additive genetic variance, σ_g^2 is the total genetic variance and σ_e^2 is the residual variance.

The type B genetic correlation r_B was estimated to assess genotype-by-environment interaction. The estimation was based on a multi-environment mixed model fitted in ASReml, where the same trait measured at different sites was treated as correlated but distinct traits.

The correlation was calculated as:

$$r_B = \frac{\sigma_{a12}}{\sqrt{\sigma_{a1}^2 \times \sigma_{a2}^2}}$$

where σ_{a12} is the additive genetic covariance between sites 1 and 2; and σ_{a1}^2 and σ_{a2}^2 are additive genetic variances for sites 1 and 2, respectively.

The theoretical accuracy (Acc) of the predicted breeding values was estimated using the following expression:

$$Acc = \sqrt{1 - \frac{PEV}{\sigma_a^2}}$$

where PEV is the prediction error variance (Mrode 2014) and is calculated as the square of the standard error; and σ_a^2 is the additive genetic variance.

Screening for and selection against CSNN

To screen the parents for their contribution to the affected offspring and select against CSNN, we compared three approaches: 1) ad hoc breeding, where the reference trees were used to estimate breeding values following parentage analysis, 2) assessing parents for their contribution to the affected offspring in the Sel-495 trees, and 3) comparing the distribution of parentage of the Sel-495 trees with the parentage of a random sample of 1545 reference trees from both sites to identify CSO parents with an over-frequency of offspring in the Sel-495 trees. To evaluate and compare these approaches, we defined two selection scenarios based on parent rankings: identifying the top 10% and 20% of parents most strongly associated with CSNN susceptibility. In each case, this was based on either low breeding values or high offspring representation in the affected population, depending on the ranking method. Lastly, we examined whether the two latter frequency-based approaches resulted in thinning candidates similar to those in the ad hoc approach.

Results

Parentage

The number of SNP loci successfully genotyped meeting the imposed ANGSD criteria was 338 in the CSO parents, 394 in the Mariager reference samples, 401 in the Tørring reference samples, and 359 in the Sel-495 samples from the Mariager site. After combining the clonal parents' genotype dataset with each of the three offspring genotype datasets and filtering out loci with >0.2 missing data, the number of SNP loci used for parentage analysis was 271, 242, and 207 in the Mariager reference, Tørring reference, and Sel-495 trees, respectively.

The assignment rates for the reference trees at the Tørring and Mariager sites were 97 and 83%, respectively, while it was almost complete for the Sel-495 trees (99.8%) (Table 1).

Parentage analysis revealed that the parentage distribution at the two sites showed the same pattern ($r=0.77$, $p<0.001$) (Fig. 3). As a result, we combined the parentage data of the reference trees at the two sites for further analysis, including an assessment of the relative contribution of the clonal parents to the affected offspring and quantitative genetic parameter estimation.

The total number of offspring with significant assignments in the combined reference data was 1371, with a highly positively skewed parentage distribution (skewness=2.7). The average number of offspring per parent was 19.6. The clone with the maximum number of offspring was C148, which had 125 offspring (4.6% of all parentages),

Table 1 Results from parentage analysis: assignment rates and number of CSO parents assigned in the three datasets

Site	No. of trees analyzed	Assignment rate at 95% CL (%)		Number of trees assigned	Number of clones assigned as parents
		Observed	Expected		
Tørring	689	97	100	665	139
Mariager	856	83	100	706	139
Sel-495	495	99.8	100	494	132

CL confidence level, *Mariager* reference trees from Mariager, *Tørring* reference trees from Tørring

followed by C58 and C120, which had 80 (2.9%) and 76 (2.8%) offspring, respectively (Fig. 4). Only one clone (Clone C155) did not have any offspring, while the remaining 139 were assigned parentage.

Even though the proportion of affected offspring per parent was highly variable, 36% of the parental clones had moderately to severely affected offspring in the reference dataset (CSNN score ≥ 4). The remaining 64% had only offspring with minor or no damage (CSNN score ≤ 3).

Parentage distribution in CSNN-selected (Sel-495) data

The parentage in the Sel-495 trees from Mariager was assessed to identify the relative contribution of the clonal parents to these moderately to severely affected and selected individuals. In the Sel-495 trees, 132 parents contributed to parentage. However, their contributions were highly variable, with 36.6 and 55.6% of affected individuals coming

Fig. 3 The relative parentage contribution of the CSO parents in FP.266 (in percentage) to the Tørring reference trees (y-axis) plotted against the relative parentage contribution to the Mariager reference trees (x-axis). Blue dots represent clonal parents. The dotted line marks $x=y$, where parentage would be exactly the same in the two stands

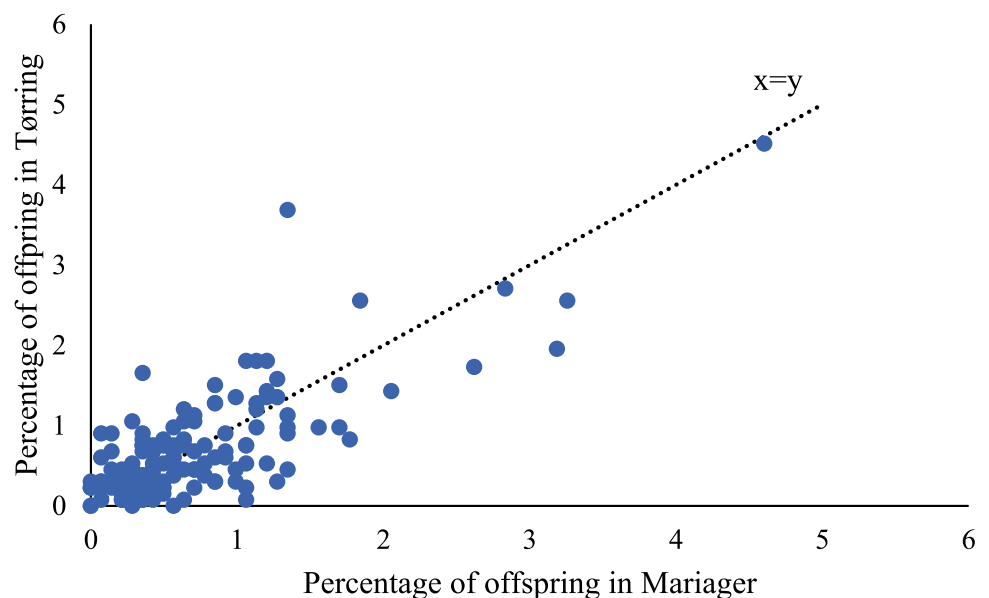
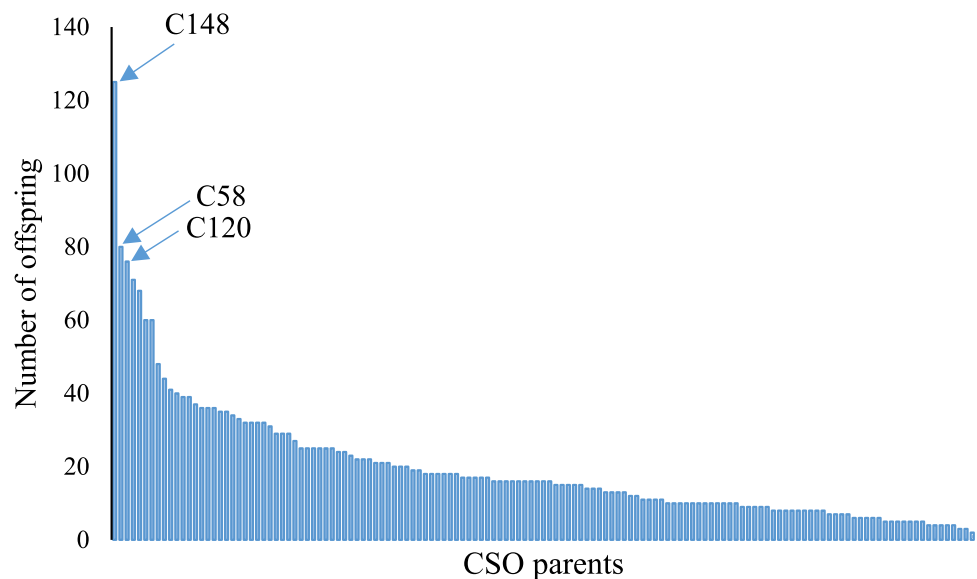


Fig. 4 Number of offspring per CSO parent in the combined reference tree dataset



from only 10% and 20% of these parents, respectively. Overall, the parentage contribution in Sel-495 (similar to the contribution in the reference trees) was highly positively skewed (skewness = 1.9). Figure 5 displays the relative contribution of the CSO parents to the Sel-495 trees.

To identify CSO parents with offspring overrepresented in the Sel-495 trees, we compared the parentage distribution in the reference trees with the parentage distribution in the Sel-495 trees. Based on the difference between the parentage distributions (ratios between parentage fractions) in the two datasets, CSO parents with offspring over-frequency in the Sel-495 trees were identified. Twenty-two CSO parents showed more than a two-fold increase in parentage fraction in the Sel-495 trees compared to the reference trees with three CSO parents (C181, C143, and C70) showing more than a five-fold increase. Figure 6 shows the differences in parentage contribution per CSO parent between the Sel-495 trees and the reference trees.

Genetic parameter estimates

Linear mixed model analysis revealed that the type B correlation between Mariager and Tørring was 1 and did not indicate a genotype-by-environment ($G \times E$) interaction for CSNN. For the multi-site analysis of CSNN data, the site effect was significant (p -value < 0.001). Mariager had the highest average CSNN damage, with a least square mean CSNN score of 0.57 (0.09), compared to 0.23 (0.04) at Tørring (see Supplementary Table 1 for CSNN damage score

data at the two sites). This was mirrored by additive variance estimates, where the estimate for the Mariager site was 0.16 (0.10), compared to 0.05 (0.03) for trees from the Tørring site (Table 2). The across-site narrow-sense heritability (h^2) was calculated as 0.08 (0.04).

The prediction accuracy (theoretical) of the breeding values was moderate, with average values of 0.32 ± 0.12 in Mariager and 0.35 ± 0.12 in Tørring.

Selection by different approaches

To compare different approaches and make selections, parents were ranked based on three approaches: breeding values from an ad hoc approach, the proportion of offspring per parent in the Sel-495 trees, and the over-frequency of offspring per parent in the Sel-495 trees compared to their parentage contribution in reference trees. Here, the 'top (worst)' 10% and 20% refer to parents most associated with CSNN susceptibility, depending on the ranking method. We then compared the breeding value-based rankings with the two frequency-based rankings to determine overlap in identifying the top (worst) candidates. The breeding values and Sel-495 proportion approaches shared 38 and 42% of their thinning candidates in the top (worst) 10% and top (worst) 20% selection scenarios, respectively (Table 3). The ranking based on the over-frequency of offspring in Sel-495 shared slightly fewer (negative) selection candidates with the breeding value ranking (Table 3). Figure 7 illustrates the changes in rankings between the compared approaches.

Fig. 5 Percentage of offspring per CSO parent in the Sel-495 trees, where all individuals had a CSNN score of ≥ 4 . The first broken line (left to right) marks the percentage contributed by the top 10% of contributors out of the 132 assigned CSO parents, while the second one marks the top 20% of contributors

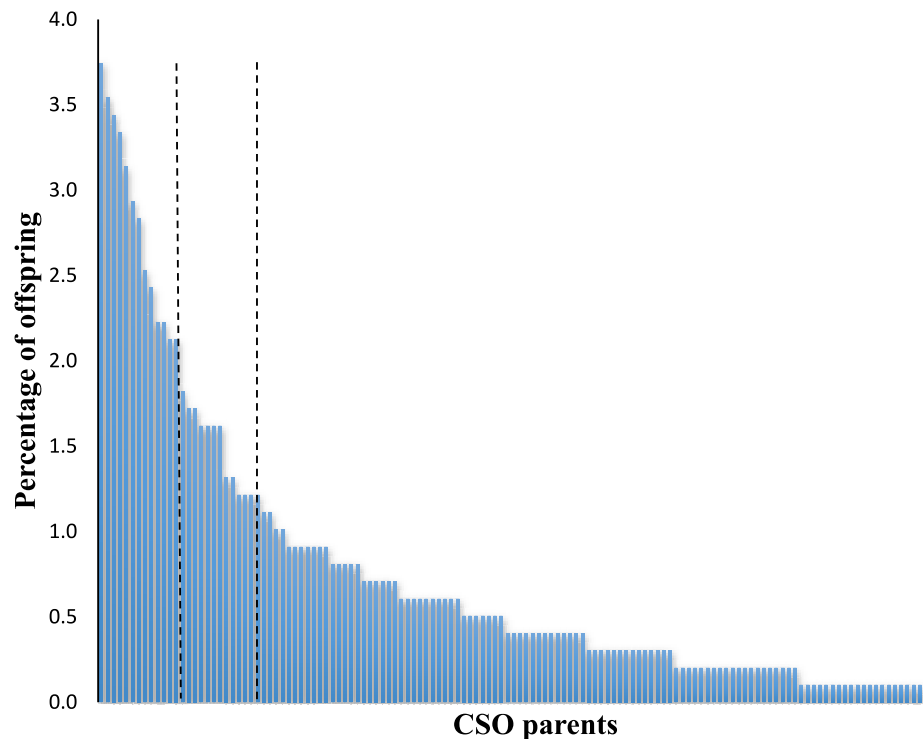


Fig. 6 Comparison of parentage distributions in the reference trees and the Sel-495 trees. **(a)** shows the percentage of offspring per parent in the Sel-495 trees plotted against the percentage of offspring per parent in the reference trees. **(b)** shows the ratio of parentage fractions in Sel-495 and reference trees, showing the increase or decrease in parentage contribution in Sel-495 relative to the contribution in the reference trees. The blue dots represent the parent clones. The broken line in **(b)** marks a two-fold increase in the parentage fraction in Sel-495

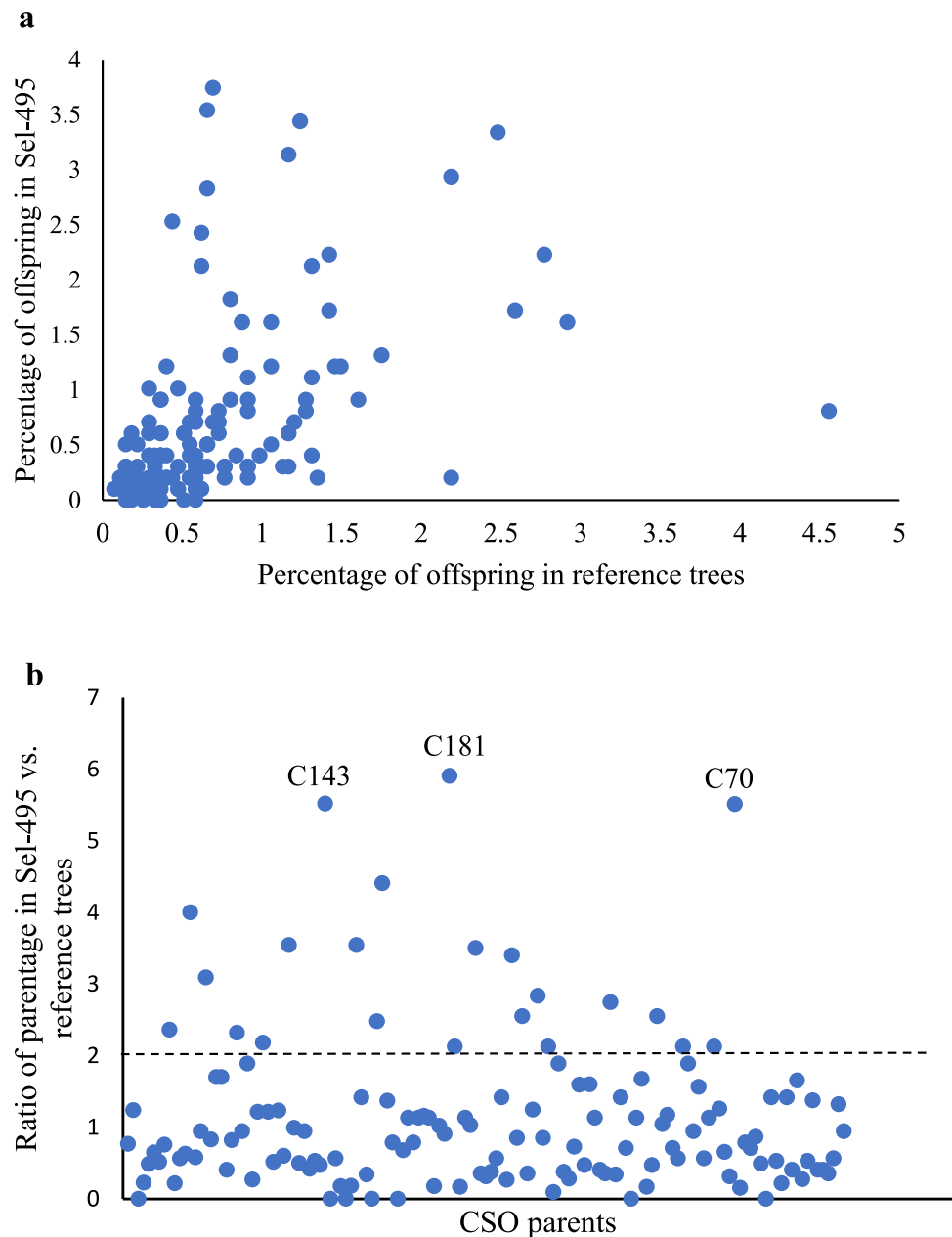


Table 2 Genetic parameter estimates for CSNN in the reference trees

Additive variance ($\pm$ SE)		Residual variance ($\pm$ SE)		Narrow-sense heritability ($\pm$ SE)
Mariager	Tørring	Mariager	Tørring	
0.16 (0.10)	0.05 (0.03)	1.83 (0.13)	0.46 (0.03)	0.08 (0.04)

Table 3 Comparison of the different selection approaches. Proportion of shared candidates among the top 10 and 20% negative selection candidates

	Selection scenario	
	10%	20%
BV vs Sel-495	0.38	0.42
BV vs Overfreq. in Sel-495	0.31	0.31

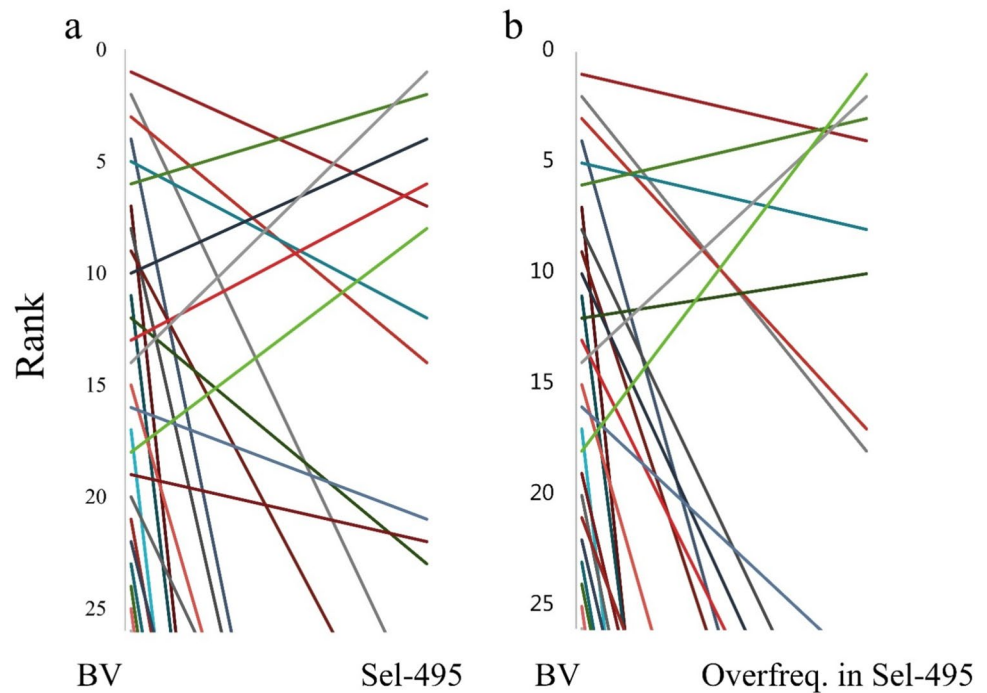
BV ranking based on breeding value, Sel-495 ranking based on the number of offspring in Sel-495, Overfreq. in Sel-495 ranking based on the over-frequency of offspring in the Sel-495 compared to the reference trees

Discussion

Breeding against occasional biotic and abiotic stressors in trees calls for a fast and efficient selection tool to rapidly enhance the frequency of desirable alleles. In this study, we

used CSNN in Nordmann fir as a case study to demonstrate such a selection via parentage analysis and ad hoc breeding in production stands with a known parental population.

Fig. 7 Ranking plots for the top 20% of negative selection candidates comparing different frequency-based selections to the ad hoc approach based on breeding value. The approaches compared to the ad hoc approach are ranking solely based on proportion of offspring in the Sel-495 trees (Fig. 6a) and ranking based on parentage over-frequency in the Sel-495 trees compared to the parentage in reference trees (Fig. 6b). Note: BV=Ranking based on breeding value; Sel-495=Ranking based on number of offspring in Sel-495; Overfreq. in Sel-495=Ranking based on the over-frequency of offspring in Sel-495 compared to the reference trees



Parentage analysis carried out to reconstruct the pedigree showed different assignment rates for the different datasets analyzed. The highest assignment rate was observed for the Sel-495 trees (99.8%), followed by the reference trees from Tørring (97%). In a corresponding study on noble fir, a comparable assignment rate of $\geq 94\%$ was achieved across trials (Hansen et al. 2020). The reference trees from Mariager showed the lowest assignment rate (83%) in this study. Different factors, including genotyping error (Wang 2010), pollen contamination (Jones et al. 2008), and marker density (Strucken et al. 2016), are known to impact parentage results. The almost complete assignment rate of the Sel-495 trees, which originate from the same plantation as the Mariager reference trees, rules out pollen and/or seed contamination as the cause of the lower assignment rate observed in the Mariager reference trees. However, the workflow—from DNA extraction to genotyping—was performed in sequential order where most individuals in the same category were processed together. This suggests that an error occurring at any stage of the workflow, from contamination in the lab to genotyping inaccuracies, could be batch specific. Consequently, such batch-specific issues might have contributed to the lower parentage assignment rate in the Mariager reference trees.

Among the reference trees, 139 of 140 potential CSO parents were assigned parentage. This could be because the analyzed plantation was established using seeds from a good seed year with large seed production. Similarly, in the Sel-495 trees, 132 CSO parents were assigned parentage. However, the relative contribution of the CSO parents to

these trees (CSNN score ≥ 4) was highly variable. More than half of the offspring (55.6%) came from only 20% of the 132 parents. Some parents showed up to a five-fold increase in their parentage contributions relative to the contributions in the random reference trees. This suggests that substantial improvement can be achieved in the next generation by simply thinning out these parents from the parent pool.

There was higher CSNN damage in Mariager compared to Tørring, with a least square mean of the CSNN scores of 0.57 (0.09) and 0.23 (0.04), respectively. Despite this difference, no $G \times E$ interactions were revealed by the linear mixed model analysis of CSNN in the reference trees from Mariager and Tørring. The discrepancy in CSNN damage between Mariager and Tørring, despite the absence of a $G \times E$ interaction, may suggest variations in causal agents between the sites, necessitating analysis of biotic and abiotic factors suspected to contribute to CSNN for a comprehensive understanding and targeted management strategy.

The occurrence of CSNN at both sites indicates that the causal agent is present at both locations. Thus, the difference in the level of damage could be attributed to varying conditions that favor the development and intensity of the disorder. Talgø et al. (2010) reported the fungus *Sydowia polyspora* as the cause of CSNN but also claimed that unknown abiotic and biotic factors contributed to the severity of CSNN symptoms. Examining prevalent pathogens, pests, environmental conditions, and site-specific management practices at the two sites may reveal the potential factors contributing to CSNN severity, aiding in the development of mitigation strategies to enhance tree health.

The narrow-sense heritability estimated for CSNN using the reference trees was 0.08 ± 0.04 . In 2003, CSNN was assessed in 10 trials established in 1997 with 128 single-tree, open-pollinated offspring from 5 Danish seed sources (Nielsen et al. 2020). Of these 10 trials, only 3 showed genetic variation regarding CSNN, with narrow-sense heritability estimates ranging from 0.31 to 0.34 (unpublished results), which is higher than in the present study. However, in a new assessment the year after (in 2004) the additive genetic variance at these three sites decreased to 0, while a new site showed variation, resulting in a narrow-sense heritability of 0.34. These findings suggest that the genetic parameter estimate for this occasional disorder is highly dependent on the level of trait expression, which varies from year to year and site to site. The additive variance estimates from the present study support this conclusion: the site with more CSNN damage (Mariager) had an estimate of 0.16 (0.10), while the site with less damage (Tørring) had an estimate of 0.05 (0.03). This indicates that more accurate estimates are easier to obtain when the damage is more pronounced or, in other words, when the trait is more expressed.

While heritability provides important insight into the potential for genetic improvement, it is only part of breeder's equation (Lush 1937) used for estimating gain by multiplying selection intensity, heritability and genetic variation. Using backwards selection of parent trees for seed production (White et al. 2007) based on field trials comprising numerous offspring per parent (using family heritability) overcomes very efficiently the issues of low heritability - when large genetic variation is present as exemplified in Nielsen et al. (2020) for improving Christmas tree quality, where heritability was estimated to 0.13 across sites.

The comparison between the rankings of parents based on their BV estimated using the reference trees and the ranking based on the over-frequency of their offspring in the affected individuals showed a moderate overlap. Because of the random selection of the reference trees, the number of offspring per parent was variable, with some parents having few offspring, potentially leading to lower prediction accuracy. Thus, the idea of negative selection of the worst 495 individuals was to complement the standard genetic analysis, which utilized reference trees. Selecting parents based on the prevalence of CSNN in their offspring offers a quick and simple solution. However, the proportion of offspring per parent in Sel-495 was influenced by the parent's overall parentage contribution to an extent (both highly positively skewed and correlated with $r=0.45$, $p<0.001$). This indicates that parents with more offspring are more likely to have offspring in the Sel-495 trees compared to parents with fewer offspring. More offspring also means more chances of exposure to the biotic or abiotic factor causing or favoring the damage. Thus, simplistic ranking based solely on

the Sel-495 trees can lead to misinterpretation and failure to address the underlying issue, especially given the low observed heritability. This is because the correlation between performance and breeding value is determined by heritability (Cassell 2001). To minimize the effect of overall parentage contribution on the ranking based on the Sel-495 trees, relative parentage contribution in the reference trees and Sel-495 trees was compared to identify parents with offspring over-frequency in the Sel-495 trees. Despite the fact that this comparison was performed to mitigate the potential bias due to a skewed parentage contribution, the overlap in identifying the most CSNN prone clonal parents was higher between the BV from the ad hoc approach and the proportion of offspring per parent in the Sel-495 trees than between the BV from the ad hoc approach and the over-frequency of offspring per parent in the Sel-495 trees.

Breeding values offer statistical rigor because statistical models are employed to account for randomness (environmental factors). However, the imperfect prediction accuracy (average in Mariager = 0.32 ± 0.12 ; average in Tørring = 0.35 ± 0.12) indicates that the ranking based on the breeding value is not 100% reliable. Thus, the combination of breeding value estimates and negative selection based on their contribution to the Sel-495 trees (affected trees) seems to be a better solution for selection against CSNN, which showed low genetic control in the present study.

Using realized genomic relatedness instead of the average expected additive relationship could improve the accuracy of genetic parameter estimates (Beaulieu et al. 2022). However, more SNPs might be required to obtain accurate pairwise genomic relatedness values. The use of genomic relatedness analysis can also enable parameter estimation in plantations with unknown parental populations (e.g., El-Kassaby et al. 2012). Increasing the number of reference trees will increase the number of offspring per parent, which can, in turn, lead to better predictive accuracy.

Conclusions

This study demonstrates the efficacy and suitability of ad hoc breeding and direct utilization of parentage results in breeding against occasional biotic and abiotic stressors in trees, exemplified through the case of CSNN in Nordmann fir. By leveraging pedigree reconstruction and production stands to estimate breeding values, genetic improvement can be achieved in a fast and efficient manner. The observed differences in CSNN damage between the Mariager and Tørring sites emphasize the necessity of analyzing biotic, abiotic, and management factors to enhance understanding and management practices. The combination of ad hoc breeding and the examination of parents for their contribution to the Sel-495 trees facilitated

the backward selection of parents based on offspring performance in production stands, indicating its potential to improve tree resilience against specific disorders under changing climate conditions. The use of genetic markers to reconstruct pedigrees is invaluable in assessing the diversity of offspring and revealing potential bias in the expected genetic gain due to uneven flowering and seed setting. Overall, these findings underscore the practical utility of combining genetic tools with field observations to guide selection for traits with unpredictable expression, such as CSNN, where obtaining reliable genetic parameter estimates is challenging. This approach not only supports immediate improvement but also provides a framework for future studies targeting other occasional and unpredictable disorders. As climate change intensifies the unpredictability of stress-related conditions, such integrative, rapid, and flexible breeding methods will become increasingly important for sustainable forest management.

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Author contribution UBN, OKH and JX conceptualized the study. JX and UBN collected samples. UBN, OKH, JX and KO laid down the analytical approach. KO and JX analyzed the data with the help of OKH. KO wrote the first draft, and all authors contributed to development of the paper through methodological advice, comments and edits of the text.

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Data availability The damage score data are provided as supplementary material. Phenotype and pedigree data analyzed in this study are available upon request.

Declarations

Competing interests The authors declare no competing interests.

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