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Assessment of genetic diversity in plants intended for enrichment planting compared to natural regeneration: A case study in Fjugstad Nature Reserve

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M.Sc. (Ecology)

Acknowledgement

This project is part of NIBIO's long-term project to characterize and secure ash genetic resources in Norway, in cooperation with Denmark, Sweden, and Lithuania, in an SNS-supported network. The current work is connected to these SNS activities and to a NIBIO research group whose aim is to develop a sustainable forest resource management guide using a multidisciplinary approach through the amalgamation of pathology, physiology, genomics, and biodiversity.

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Abstract:

European ash (*Fraxinus excelsior*) is facing severe population decline due to ash dieback disease, which not only poses a threat to the existence of this keystone species but also to more than 480 other species associated with it directly or indirectly. The disease's spread rate and extent of damage exceed the rate of regeneration, necessitating management actions to conserve the forest. Enrichment planting with relatively resistant plants can boost evolutionary stability and mitigate the dieback crisis. However, it's essential to genetically assess the enrichment planting materials to ensure their representativeness and sufficiency in diversity compared to the degraded forest.

Our study analyzed the genetic diversity of 100 samples from Danish origin grown in nurseries for enrichment plantation using 12 microsatellite loci. We compared results from these samples with 99 samples from Fjugstad Nature Reserve, 50 collected before the heavy dieback infestation in 2012, and 49 from 2022. The Fjugstad population's genetic diversity did not change before and after the heavy infection, but the coefficient of inbreeding was lower after the infestation, possibly due to the clearing of the canopy and middle layer in the forest, facilitating better pollen flow, seed dispersal and hence gene flow. The genetic diversity of the Fjugstad and Danish populations was also similar, except for higher private allelic richness and expected heterozygosity in the Danish population. Moreover, both Fjugstad and Danish population had overlapping allele frequency which complements the genetic diversity of the Fjugstad forest. Additionally, the higher private allelic richness in the Danish population may offer tolerance against dieback, contributing to the forest's genetic tolerance and boosting its population. These findings suggest that Danish reproductive materials are representative and complementary to the genetic diversity of the Fjugstad population, aiding in conservation efforts and evolutionary stability of the declining ash forest in Fjugstad.

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Introduction

Ash dieback – a threat to Norwegian ash populations

Climate change poses a significant threat to global biodiversity, as it triggers alien species invasion, increases pest activities, declines in productivity, and ultimately leads to biodiversity loss. Its impact is predicted to increase in future climate scenarios (Seidl et al., 2018), creating a new environment and, thus, a new relationship among coexisting or newly introduced species, often triggering antagonisms (Grünig et al., 2020). There have been outbreaks of pathogens devastating entire forests and their functional ecosystems. For example, American chestnut (*Cantanea dentata*), one of the major hardwood forests in the south-eastern USA, was eliminated by Asian chestnut blight (*Cryphonectria parasitica*). A similar tragedy was faced by Butternut, whose population was exterminated by butternut canker disease (*Sirococcus clavigigenti-juglandacearum*), and American elm by Dutch elm disease (*Ophiostoma ulmi and O. nova-ulmi*) (Schlarbaum et al., 1998). The ecological significance of the pest becomes highly pronounced when evolutionarily successful pathogens invade keystone species thus creating a long-term momentum of its pathogenicity and therefore cascading the loss in overall forest diversity (Loo, 2009). European ash (*Fraxinus excelsior*), one of the keystone species supporting more than 483 other species directly or indirectly (Hultberg et al., 2020) as reported by a study conducted in Sweden, is threatened by the dieback disease, thus raising concern about not only ash but also the species associated through obligate or facultative relationships.

The devastation of ash forests is caused by a fungus, *Hymenoscyphus fraxineus* which is thought to be introduced in Europe from East Asia (Zhao et al., 2013). The disease this fungus causes is referred to as ash dieback, and the pathogenicity and spread are of major concern due to its speed of invasion and mortality of the infected trees. The disease was first detected in Poland in 1990s (Przybył, 2002) and has invaded the entire distribution range of ash in Europe. It was first reported in Norway in 2008 (Solheim & Hietala, 2017). Since then, it progressed so rapidly that 89.2% of the trees were severely damaged between 2009 and 2016 (Timmermann et al., 2017), resulting in a change in the conservation status of ash from "near threatened" in 2010 to "vulnerable" in 2015, and finally to "endangered" in 2021 (Solstad et al., 2021). Mortality of the forest is higher than the rate of regeneration (Díaz-Yáñez et al., 2020), thus compromising the future of the forest and the genetic resources. Since ash is a keystone species the rapid decline of the forest will have an insurmountable and often irreversible impact on biodiversity, creating an imbalance in the ecosystem. Therefore, controlling the damage caused by the dieback pathogen is needed to protect the ash forest ecosystem.

Forest management and ash genetics

Despite the devastation, forest conservation seemed to be possible due to tolerance against the dieback disease observed in ash (McKinney et al., 2014). A low proportion of the plants ranging from 1-5% of the total population are genetically tolerant to the disease (Kjær et al., 2012). Further

studies showed heritability of 0.4-0.5 of this resistance (Lobo et al., 2014; McKinney et al., 2011; Munoz et al., 2016), which seemed to be polygenetic (Stocks et al., 2019). The level of heritability present in the tolerant ash indicated a possibility of a good response to selection based on individual phenotypes. Since young plants respond to dieback quicker under high infection pressure (Timmermann et al., 2017), the identification of resistant plants is eased and selection time can be significantly reduced for the development of resistant reproduction materials, for the management of the degrading forest. However, the propagation of forests through naturally or artificially selected healthy trees may lead to a decrease in genetic diversity, as trees with lower susceptibility to disease are favored. Due to natural selection in the forest, trees that exhibit greater resistance have a higher chance of survival, which can lead to a reduction in genetic diversity as fewer trees without resistance are eliminated. Regardless of the nature of selection, the random distribution of a small percentage of resistant ash trees across geography may lead to spatial variation in the genetic structure, which can impede the forest's reproducibility, particularly due to limitations in seed and pollen dispersal. This can reduce the effective population size and consequently lead to inbreeding depression (Lienert, 2004). Therefore, careful consideration must be given to the selection of tolerant propagation materials and methods of introducing them into the forests.

Enrichment plantation through tolerant plant materials representative of forest genetic diversity is an ecologically and financially viable option for creating a diverse forest where the propagules are introduced in the canopy gap of a secondary forest that is otherwise unable to grow or is ecologically vulnerable (Lamb et al., 2005; Thomas et al., 2014). It has been successfully tested for restoring degraded forests in the tropics, e.g., (Bertacchi et al., 2016; Montagnini et al., 1997). Trials are underway in Europe as well for the restoration of degraded ash forests which have shown initial effectiveness in promoting late and mid-successional species (Haupt et al., 2022). Enrichment plantation is effective especially when the source of seeds declines, like the current situation ash forest is facing with the rapid decline in the mother trees supplying seed for forest regeneration. Moreover, selecting seeds from provenances expected to be adapted to future climate has the potential of mitigating the impact of climate change on forest biodiversity (Aitken et al., 2008; Finkeldey, 2010). Therefore, enrichment plantation offers a good and sustainable forest management option against the complete turnover of species composition and is appropriate for tackling global change and associated ecological threats, including pathogens, herbivory, and climate-induced species loss, (Haupt et al., 2022). Despite having good prospects for restoring degraded forests, enrichment plantations demand some caution, especially in selecting propagules, to avoid the potential risk of genetic diversity loss and hence the fitness of the forest against environmental and other anthropogenic odds.

The recolonization history of a population plays a crucial role in shaping the genetic structure, leading to variations in gene flow and unique patterns of genetic diversity, structure, and its evolutionary stability. Recolonization of ash from glacial refugia in Iberia, Italy, the eastern Alps, and the Balkan peninsula defines the population structure as revealed by analysis of cpDNA (Heuertz et al., 2004). Northward expansion following different routes to meet in central Europe

makes the population of this region genetically more diverse. In this regard, the Danish reproduction materials can be expected to contain high genetic diversity than that of Norwegian population. In addition, the seeds collected from the Danish mother trees in the seed orchard were more tolerant to ash dieback. The plants adapted to more southern climates will help to better adapt to the changed climatic situation as they move northward to Norway. In this regard, the use of seeds and plants from dieback tolerant Danish trees can potentially be an effective forest management option in Fjugstad Nature Reserve.

Aims and objectives

To ensure healthy and stable ash populations in Fjugstad Nature Reserve, it is crucial to confirm that the plantation materials are diverse enough to supplement the genetic diversity of ash in the Reserve. Evaluating the genetic diversity of both the plantation materials and the degraded forest can determine their representativeness. Dieback infection in the forest may result in compromised genetic diversity through natural selection, with resistant plants more likely to survive while susceptible ones likely to die off. Assessing the genetic diversity before and after the heavy infection of the forest can provide information on how diversity has been affected due to dieback. In this regard, the thesis aims to explore the impact of dieback on the genetic diversity of the ash forest in Fjugstad and assess the representativeness of Danish reproductive materials intended for enrichment planting to the diversity of the ash forest in Fjugstad. Specifically, the thesis aims to:

1. assess the change in the genetic diversity of the ash forest in Fjugstad Nature Reserve between 2012 and 2022, before and after the heavy infection of the forest due to dieback.

2. compare the genetic diversity of the Fjugstad ash population with the Danish reproductive materials to evaluate their appropriateness for use in enrichment plantation in Fjugstad Nature Reserve.

Materials and Methods

Study area

The study was conducted in Fjugstad Nature Reserve, located close to Horten in Vestfold Telemark County, southeastern Norway. The reserve extends to an area of 267 acres and an altitude of 0-50 meters above mean sea level. It is designated as a conservation unit for ash genetic resources and is probably the largest continuous natural ash forest in Scandinavia (Miljødirektoratet, 2022). However, the forest is severely affected by the ash dieback, posing a serious concern for the future of the forest. NIBIO is conducting trials in Fjugstad to assess the forest ecosystem which includes study on forest diversity, environmental and climatic variables, forest pathogens including *Hymenoscyphus fraxineus*, and enrichment plantation of resistant ash reproduction materials in a

canopy-cleared and fenced trial plot to explore the effective options for forest conservation. This thesis forms a part of the big and long-term effort of conserving the forest in Fjugstad.

Table 1. Number of samples collected from different populations and crown damage score for mother/ putative mother trees

Population	Crown damage in mother tree (%)	Number of samples
Dan		
Dan 5	10-25%	11
Dan 19	10-25%	8
Dan 30	<10%	12
Dan 42	<10%	12
Dan 50	<10%	12
Dan 53	10-25%	11
Dan 60	<10%	11
Dan 64	No damage	11
Dan 71	<10%	12
Fjug12*	Putative mother trees with potential little infection	50
Fjug22*	Putative mother trees with potential heavy infection	49
	Total	*199*

Texts in bold indicate population, indented light texts indicate families within the population, populations marked with * were not scored for crown damage and the level of infection was assumed in relation to infection pressure in Fjustad in 2012 and 2022 respectively.

Materials

The study materials consist of three different sets of samples which have been treated as three populations during the data analysis.

1. **Fjugstad12:** These samples were collected before the heavy infection of dieback disease in the forest in 2012, which included leaves from seedlings, saplings, and the trees. covering the whole forest (Fig 1). The already collected data was provided by NIBIO for comparative analysis.
2. **Fjugstad22:** The samples were collected in the summer of 2022, after 14 years of heavy infection of the forest by ash dieback. There were hardly any ash juveniles established to seedling or adults in the sampling area. Therefore, the samples consisted only of leaves

from juveniles collected in a small area designated for the enrichment plantation of Danish reproductive materials.

3. **Dan**: The samples were leaves from plants belonging to tolerant Danish mother trees originating from seed orchards and grown in nurseries intended for enrichment plantation in Fjugstad. The samples were collected in the summer of 2022.

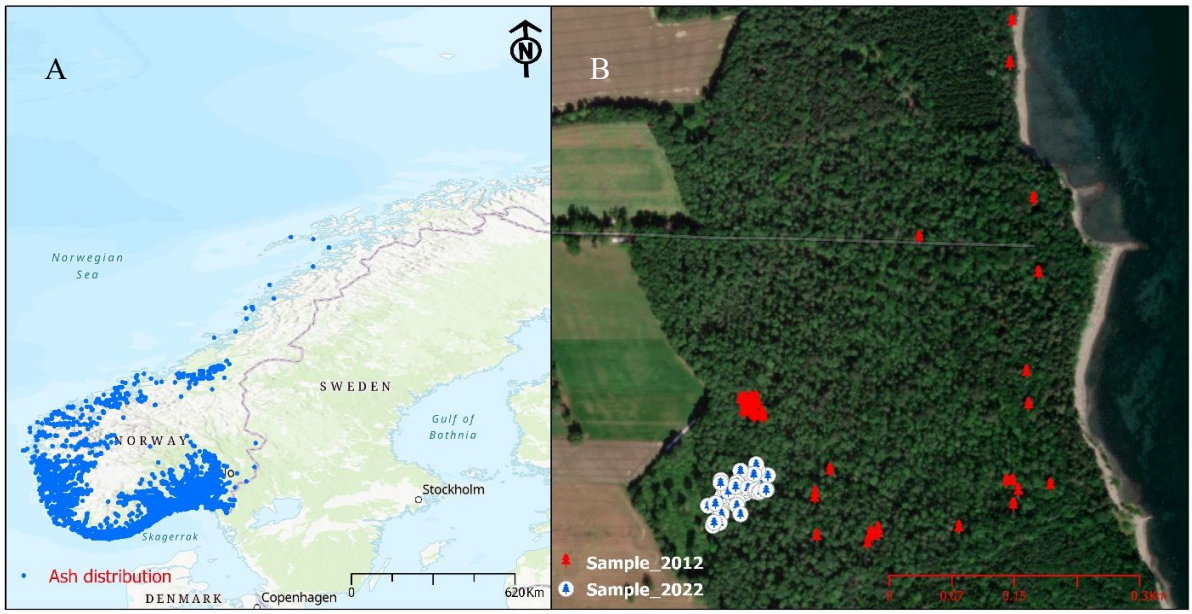

Figure 1. Distribution map of ash and the sample points. *(A) Distribution range of ash in Norway represented by blue dots; GPS points for the distribution of ash were obtained from Global Biodiversity Information Facility (GBIF.Org, accessed on 29 November 2022) and (B) Sampling points in Fjugstad Nature Reserve; samples taken in 2022 represented by white dots and those taken in 2012 by red.*

Sampling and phenotyping

To obtain a representative sample of the Danish material, 100 leaf samples were collected evenly from each of the nine families (Table 1) in August 2022. No signs of ash dieback symptoms were observed during the sample collection period. In Fjugstad, leaf samples were collected from randomly selected plants in the summer of 2022. The GPS location of the sampled plants was recorded to facilitate future follow-up. The plants were scored for the presence of disease, with a score of 0 for the absence of disease and 1 for the presence of disease. There were signs of browsing in the forest, making it difficult to distinguish whether the damage was due to browsing or dieback. The height of the plants was also recorded and assigned to a height class from 1, 2, and 3, with a value of 1 representing seedlings, 2 representing juveniles, and 3 representing trees. Seedlings and trees were absent in 2022, so only juveniles were represented in

the sampling plot. Details on the disease scored samples are presented in Appendix 1. The sampled leaves were dried and preserved in silica gel before DNA extraction.

Methods

DNA Extraction

We used 20 milligrams of dried leaf material for DNA extraction. The material was transferred to a 2-milliliter tube containing a tungsten carbide bead, dipped into liquid nitrogen, and finely crushed using Qiagen MM300 Retsch Tissuelyser at a frequency of 20 Hz for 1-2 minutes. Total DNA was extracted using QIAGEN's DNeasy Plant Mini Kit following the manufacturer's guidelines, with some modifications to enhance the purity of the eluate and concentration of the DNA. The modifications to the protocol included increasing the centrifugation time to 3 minutes and washing and eluting only once with 60 µL of the AE elution buffer. The concentration of extracted DNA was measured using a Nanodrop spectrophotometer. For samples with a DNA concentration of less than 10 ng/µL, DNA was re-extracted. The extracted DNA was stored at 4 °C before conducting PCR.

DNA amplification

A combination of different primers previously tested and used by various researchers were used to target the SSR loci (see details in Table 2). Before amplification, isolated DNA was diluted to 10 ng/µL using nuclease-free water. Fluorescently labeled forward and reverse primers were used to target 13 microsatellite loci and the corresponding DNA segments to be amplified. Different primers with similar annealing temperatures were multiplexed, following Sutherland et al. (2010) and Tollefsrud et al. (2016), as well as earlier work at NIBIO. The microsatellite loci were amplified in a 12.5 µL volume containing 6.25 µL of Type-it multiplex Microsatellite kit by QIAGEN, 1.25 µL of the multiplex primer mixture (final concentration presented in Table 2), and 10 ng of DNA (i.e., 1 µL). PCR was conducted on an Applied Biosystems thermal cycler (GeneAmp PCR System model no. 9700). Details of the labeled primers, multiplexes, and reaction concentrations are presented in Table 2.

For all PCR reactions, the initial denaturation cycle was 95 °C for five minutes. Specific PCR reaction conditions are provided in Table 3. The presence of PCR products was checked using gel electrophoresis with a 2% agarose gel.

Table 2. Primer sequence and details on PCR multiplex mixes for 13 microsatellite loci used

Sn	Primer	Size	Primer sequence	Dye	Multiplex	Concentration
1	M2-30	198-238	(Brachet et al., 1999)	pet	Mix 1	0.45
2	Femsatl 4	166-2280	Developed by Lefort et al. (1999) and modified by Sutherland et al. (2010)	ned	Mix 1	0.3
3	Femsatl 19	169-210	Developed by Lefort et al. (1999) and modified by Sutherland et al. (2010)	hex	Mix 1	0.3
4	Femsatl 8	150-192	Developed by Lefort et al. (1999) and modified by Sutherland et al. (2010)	fam	Mix 1	0.3
5	Femsatl 11	180-222	Developed by Lefort et al. (1999) and modified by Sutherland et al. (2010)	fam	Mix 2	0.2
6	Femsatl 16	182-200	Developed by Lefort et al. (1999) and modified by Sutherland et al. (2010)	hex	Mix 2	0.23
7	EST-SSR 380	198-204	(Aggarwal et al., 2010)	pet	Mix 3	0.2
8	EST-SSR 520	325-331	(Aggarwal et al., 2010)	pet	Mix 3	0.2
9	EST-SSR 528	275-299	(Aggarwal et al., 2010)	ned	Mix 3	0.2
10	Femsatl 12	184-214	Developed by Lefort et al. (1999) and modified by Sutherland et al. (2010)	fam	Amplified alone	0.4
11	fm06- for	288-295	ACGCTAAAGTCTGGTGTCTCT*	pet	Mix 3	0.2
12	fm06- rev		GAAGCCACTCCCTCCATCTT*			
13	fm08- for	253-294	TGGTTGCTTCACGTTCTTCC*	ATTO 550	Mix 3	0.2
14	fm08- rev		ATTTTCATCACCTCGCTCGC*			
15	fm13- for	182-207	ACATCCCTGGTCATGGTGTG*	hex	Mix 3	0.2
16	fm13- rev		CATTCCTGCAAACCAAGCCT*			

Concentration of the primer is given in µM * markers originally developed by Goto and Lian unpublished, (Hu et al., 2010), kindly provided to NIBIO by Susumo Goto and modified by researchers at NIBIO.

Table 3. Conditions for different multiplexes and singleplex PCR

	Temperature and timing of different PCR stages			No of cycles	Final elongation
	Denaturation	Annealing	Extension		
Mix 1	95 °C for 30 s	60°C for 90 s	72 °C for 30 s	28	60 °C for 40 min
Mix 2	95 °C for 30 s	57 °C for 90 s	72 °C for 30 s	30	60 °C for 30 min
Mix 3	95 °C for 30 s	58°C for 90 s	72 °C for 30 s	28	60 °C for 30 min
Femsatl 12	95 °C for 30 s	56 °C for 90 s	72 °C for 30 s	30	60 °C for 30 min

s= seconds, min= minutes

Sizing

One µL of PCR product was mixed with 0.24 µL of GeneScan™ 500 LIZ™ (Applied Biosystems) size standard and 10.00 µL of Hi-Di formamide. The mixture was subjected to denaturation for 2 minutes using the SimpliAmp Thermal cycler (Applied Biosystems). The fragments were then separated in the form of electropherograms using the 3730xl DNA Analyzer (Applied Biosystems) with the POP-7™ Polymer used as a separation matrix. Plates were analyzed using different injection times of 2 or 4 s/2 kv to optimize the peak quality. An injection time of 2 sec for mix 1 and mix 2 and 4 seconds each for mix 4 and the multiplex of mix 3 and Femsatl 12 was chosen as it produced well-defined peaks with less background. Peaks were analyzed and fragment lengths were determined using Gene Mapper6, Applied Biosystem. Mix1 showed inconsistent sizing peaks, therefore PCR and sizing were repeated. Electropherograms generated with the Femsatl8 marker had background noise creating difficulties in scoring and were omitted from further analysis.

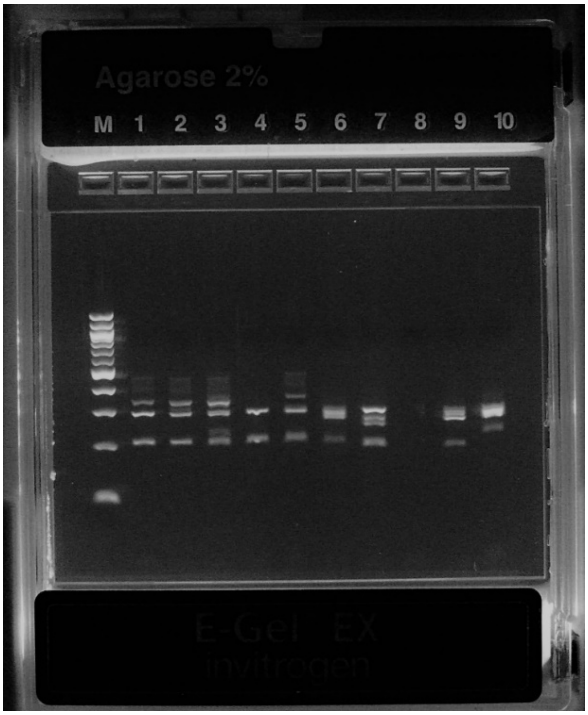

Figure 2. Gel-electrophoresis of PCR products. *Bands in the extreme left was from the ladder and each of the other bands were from PCR products of different samples.*

To ensure consistency in binning, the raw data obtained from the samples collected in 2012 were analyzed together with the data obtained in 2022. Size standards for all the individual samples were manually checked, and corrections were made when necessary. The resulting scores were compared with the previously scored data to check for the consistency of the binning. All peaks and bins were manually checked for accurate scoring by manual calling.

Statistical analysis

Genetic diversity and differentiation

To analyze genetic diversity, we calculated allelic richness and the average number of alleles per locus using FSTAT (Goudet, 2003), and observed and expected heterozygosity using Arlequin v 3.5.2.2 (Excoffier et al., 2005). The estimation of allelic richness was based on the lowest sample size of 38 individuals. We used the Brookfield (1996) method and the web version of Genepop 4.7.5 (Raymond & Rousset, 1995; Rousset, 2008) to calculate null allele frequency. Private allelic richness was computed using the hierarchical rarefaction method with HP-Rare (Kalinowski, 2005). Locus-wise allelic richness, private allelic richness, and observed and expected heterozygosity were averaged to the population level. Confidence intervals of the means were calculated using the percentile method with 10,000 bootstraps, utilizing the "boot" package in R. The comparative results of the diversity measures were presented in a box plot using the "ggplot" package in R (Team, 2021).

Inbreeding coefficients (Fis) were calculated following the Weir & Cockerham (1984) method in FSTAT to determine the extent of inbreeding within populations. As the null alleles were detected, Fis was calculated and adjusted to the presence of null alleles using INEST 2.0 (Chybicki & Burczyk, 2009). Full model was used to construct a null model that accounted for null alleles, inbreeding coefficients, and genotyping failures. INEST provides an unbiased estimation of Fis values that are robust to the presence of null alleles. The Exact test (Guo & Thompson, 1992) was used to analyze deviations from Hardy-Weinberg equilibrium for each locus in the web version of Genepop 4.7.5 (Raymond and Rousset, 1995; Rousset, 2008). The Markov chain parameters were set to 1000 dememorizations with 100 batches and 1000 iterations per batch. A sequential Bonferroni correction was applied to test the significance of the Fis value.

To analyze the genetic differentiation among Dan, Fjug 12, and Fjug 22, pairwise Fst values were calculated. Similarly, pairwise Fst values were computed for all nine Dan families to study extent of differentiation among the families. Arlequin v 3.5.2.2 (Excoffier et al., 2005) was used to calculate Fst. A permutation test with 1023 iterations was used to assess the significance of the Fst values at 0.05 level of significance. The comparison of Fst among the different Danish populations was visualized in a Corrplot using R 4.2.2 (Team, 2021).

Population genetic Structure

To infer population structure, we used the STRUCTURE software (Pritchard et al., 2000), which employs a Bayesian clustering approach to identify genetically distinct subpopulations based on multilocus genotype data. We ran STRUCTURE with the following parameters: a burn-in of 100,000, a run length of 100,000 MCMC iterations, a single genetic cluster (K = 1), a prior for the degree of clustering set to 10, and a total of 10 iterations following admixture model. Structure harvester (Earl & VonHoldt, 2012), a web implementation of the Evanno-method (Evanno et al., 2005), was used to find the most likely number of K in the dataset. Clumpak (Kopelman et al.,

2015) was used to visualize the population clustering in the form of a barplot. DAPC, a multivariate, dimensionality reduction approach of population clustering was used to further analyze the overlap among different clusters of populations using the adgent package in R (Jombart, 2008). The data was first transformed into principal components and DAPC was run into the first 10 retained PCs and the first 3 discriminant axes to find the most informative axis.

To analyze the pattern among disease symptoms, developmental stage of ash, and the population structure, principal component analysis (PCA) was conducted using the adegenet package in R (Jombart, 2008). First, we constructed a genotype matrix by converting the raw genotypic data to binary allelic states. We then conducted PCA on this matrix using the "makepca" function with the developmental stage of ash, symptoms of the disease, and the population separately. All analyses were conducted in R version 4.0.3 (Team, 2021)

Detection of Bottleneck

To check the evidence of past bottleneck events, populations with the reduction in heterozygosity excess were tested with sign test, under the assumption of three different mutation models viz. infinite alleles model (IAM), stepwise mutation model (SMM), and two-phase model (TPM) using BOTTLENECK (Piry et al., 1999). The variance of TPM was defined as 30, allowing 70% of SSM while calculating TPM with 1000 iterations. A qualitative test of the model shift was done visualizing the allele frequency distribution along different allele classes specifically to check if there has been any loss of low-frequency alleles.

Results

Locus-wise genetic diversity and differentiation

Twelve microsatellite loci from 199 individuals gave a total of 225 alleles with 18.75 alleles per locus. The number of alleles per locus ranged from 6 for EST-SSR 380 to 37 for Femsatl-4 (Table 4). The size of the PCR products corresponding to the alleles ranged from 162 to 343 nucleotide base pairs. The average allelic richness (AR) was 13.72, with the highest allelic richness recorded for M2-30 and the lowest for EST-SSR 380. Similarly average private allelic richness was 1.87 with the highest value of 5.0336 for Femsatl 4 and the lowest value of 0.44193 for EST-SSR 380 calculated based upon 76 genes. The highest number of null alleles were present in Femsatl 12 with a frequency of 0.305, whereas no null alleles were detected for Femsatl 11, fm 08. The average value of observed heterozygosity was 0.6, ranging from 0.92 for M2 30 to 0.12 for EST SSR 520. Similarly, average values of expected heterozygosity (He) were 0.778, with the highest value of 0.947 for M2 30 and the lowest, 0.468 for EST SSR 520. The mean inbreeding coefficient (Fis) was 0.25, ranging from 0.65 for Femsatl12 to -0.035 for fm 08, six loci showed a sign of inbreeding and deviated significantly from Hardy Weinberg equilibrium (Table 4). The average Fst value was 0.019 ranging from 0.03 for Femsatl 11 to 0.012 for M2 30 and Femsatl19.

Table 4. An overview of the allelic characteristics and diversity across different loci

Locus	A	Allelic size	AR			Ho	He	Fis	*P* value	Fst	PAR	NAF
			Dan	Fjug22	Fjug12							
Femsatl 4	37	162-264	19.878	17.154	20.226	0.77	0.817	0.04	0.459	0.022	5.0336	0.0143
Femsatl 19	27	168-224	17.43	16.648	17.091	0.83	0.922	0.091	0	0.012	2.39663	0.0187
M2 30	36	198-276	22.47	20.723	22.955	0.92	0.941	0.005	0.026	0.012	3.31563	0.0039
Femsatl 11	19	182-238	13.226	12.18	10.142	0.83	0.82	-0.038	0.75	0.03	2.19267	0
Femsatl 16	8	175-198	6.424	3.99	6.222	0.27	0.626	**0.558**	0	0.014	0.8175	0.2425
EST-SSR 380	6	188-206	5.313	3.99	4	0.45	0.526	**0.13**	0	0.019	0.44193	0.0423
EST-SSR 520	8	303-343	5.022	5.905	4.704	0.12	0.462	**0.582**	0	0.028	0.77597	0.1829
EST-SSR 528	13	264-303	9.362	7.258	8.458	0.54	0.644	**0.144**	0	0.016	0.99303	0.0701
Femsatl 12	15	182-214	10.487	13	11.63	0.29	0.868	**0.648**	0	0.035	1.07293	0.3049
fm 13	19	177-221	14.383	11.752	13.902	0.48	0.884	**0.448**	0	0.017	2.0631	0.2311
fm 06	17	276-298	12.736	10.7	15.675	0.83	0.887	0.052	0.01	0.013	1.39733	0.0218
fm 08	20	243-299	13.762	12.074	13.958	0.86	0.852	-0.035	0.726	0.02	2.02207	0
Mean	*18.75*		*12.54*	*11.28*	*12.41*	*0.6*	*0.771*	*0.199*		*0.019*	*1.88*	

A- average number of alleles per locus; AR- Allelic richness, measured based on 38 diploid individuals; Ho - average proportion of observed heterozygosity; He- Expected heterozygosity, average inbreeding coefficient, the bold value indicates significant deviation from hardy Weinberg equation; Fst- coefficient of differentiation, PAR- Private allelic richness NAF- Null allele frequency

Comparison of genetic diversity in Fjugstad before and after heavy dieback infection

The findings suggest that there was similar genetic diversity in Fjug12 and Fjug22, indicating no significant change in the genetic composition of the forest during the dieback crisis (Fig 3). The average number of alleles was slightly higher in Fjug12 than in Fjug22 (Table 5). In case of allelic richness, the confidence intervals Fjug12 (mean = 12.413, CI = 9.22-15.75) and Fjug22 (mean = 11.281, CI = 8.46-14.18) overlapped, so the difference was not significant. Private allelic richness was, however, higher in Fjug12 (mean = 1.71, CI = 0.99-2.57) compared to Fjug22 (mean = 1.04, CI = 0.54-1.65). The expected heterozygosity for Fjug12 (mean = 0.726, CI = 0.68-0.86) and Fjug22 (mean = 0.756, CI = 0.63-0.81), as well as the observed heterozygosity for Fjug12 (mean = 0.59, CI = 0.44-0.76) and Fjug22 (mean = 0.61, CI = 0.45-0.74), were similar since the confidence intervals overlapped and the differences were not significant.

Comparison of genetic diversity between Danish and Fjugstad populations

The Danish population displayed higher genetic diversity measures for some parameters and was similar to Fjugstad for others (Fig 3). For instance, the average number of alleles was highest in the Danish population (184) compared to Fjug12 and Fjug22 (Table 5). Even though mean allelic richness for the Danish population (mean = 12.54, CI = 9.52-15.59) was not significantly different from that of Fjug12, as the confidence intervals overlapped, the private allelic richness was higher (mean = 2.88, CI = 1.78-4.11) compared to both Fjug12 and Fjug22. Moreover, the Danish population had a higher expected heterozygosity (mean = 0.782, CI = 0.68-0.86) but similar observed heterozygosity (mean = 0.609, CI = 0.44-0.76) compared to Fjugstad.

The presence of null alleles appeared to inflate Fis (coefficient of inbreeding) with a significant value for all three populations (Table 5). However, when a correction was applied for the presence of null alleles using INEST it led to a different result. In this case, absence of inbreeding was indicated for Dan, as the Deviance Information Criterion (DIC) value was higher than the random model. However, significant inbreeding was observed for Fjug12 and Fjug22. Additionally, the FiNEST value for Fjug12 was higher than that of Fjug22, indicating a higher degree of inbreeding in Fjug12 (Table 5).

Table 5. Comparison of the number of alleles and inbreeding coefficient among three populations

Population	A	Fis	FisINEST
Dan	184	**0.222**	0.0067
Fjug22	142	**0.178**	**0.0167**
Fjug12	159	**0.194**	**0.0226**

A- Number of alleles detected; **Fis**- average inbreeding coefficient, before accounting for null alleles, bold indicates significant deviation from Hardy Weinberg equation after sequential Bonferroni correction; FisINEST- Fis corrected for null allele, bold numbers signify the values are statistically significant.

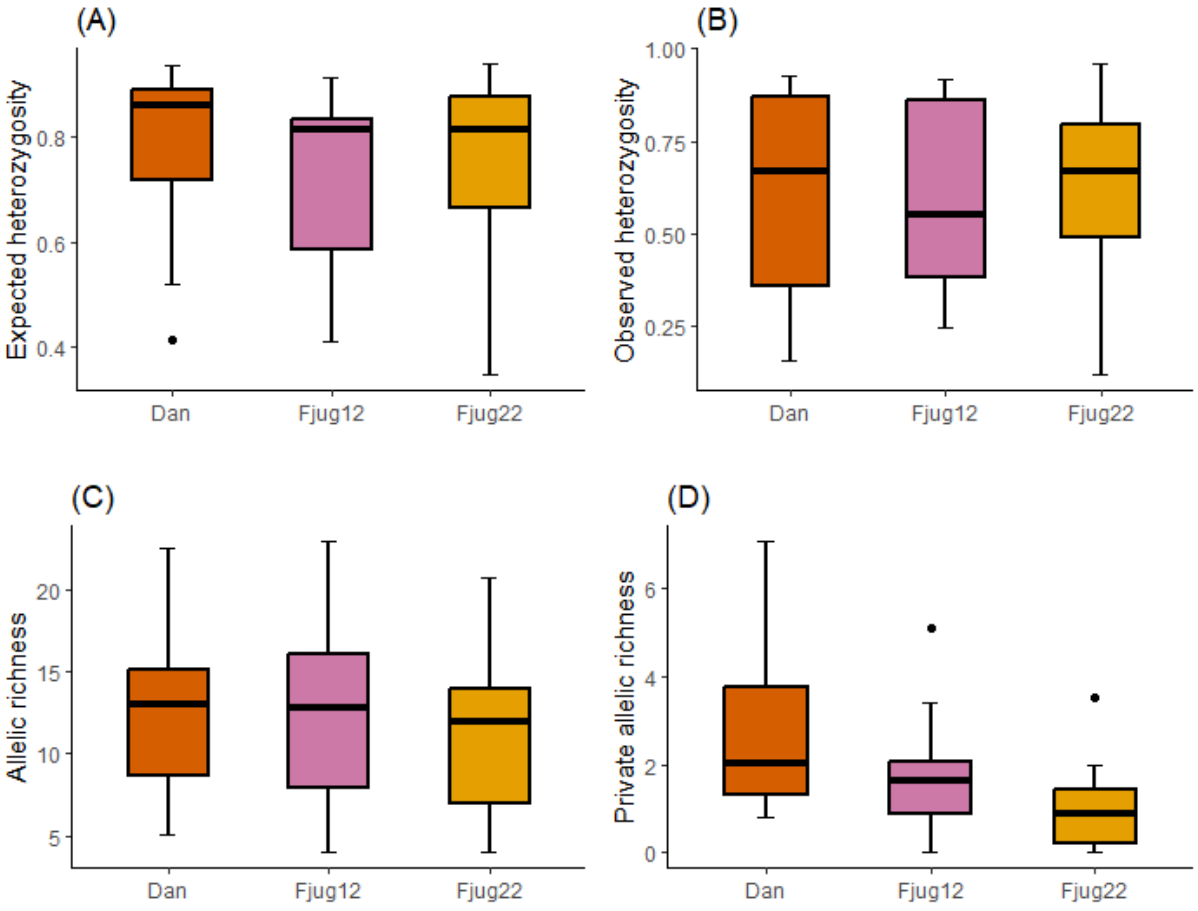

Figure 3. Box plot comparing different population parameters *A. Expected heterozygosity, B Observed heterozygosity, C. Allelic richness, and D. Private allelic richness for Dan, Fjug12, and Fjug22 populations. The horizontal line within each box indicates the median, while the whiskers extend to the minimum and maximum values.*

Absence of population bottleneck in all three populations

Sign test of significance for the reduction in heterozygosity excess based on IMM (infinite mutation model), TPM (two-phase model), and SMM (stepwise mutation model) models showed the absence of recent bottleneck events in all three populations tested, i.e., Fjug12, Fjug22, and Dan (Table 6). The SMM model best describes the data (Table 6). The mode-shifted curve for all three populations was normal L-shaped (Fig 4), with low-frequency classes more abundant, further suggesting no recent loss of low-frequency alleles, supporting the absence of bottleneck.

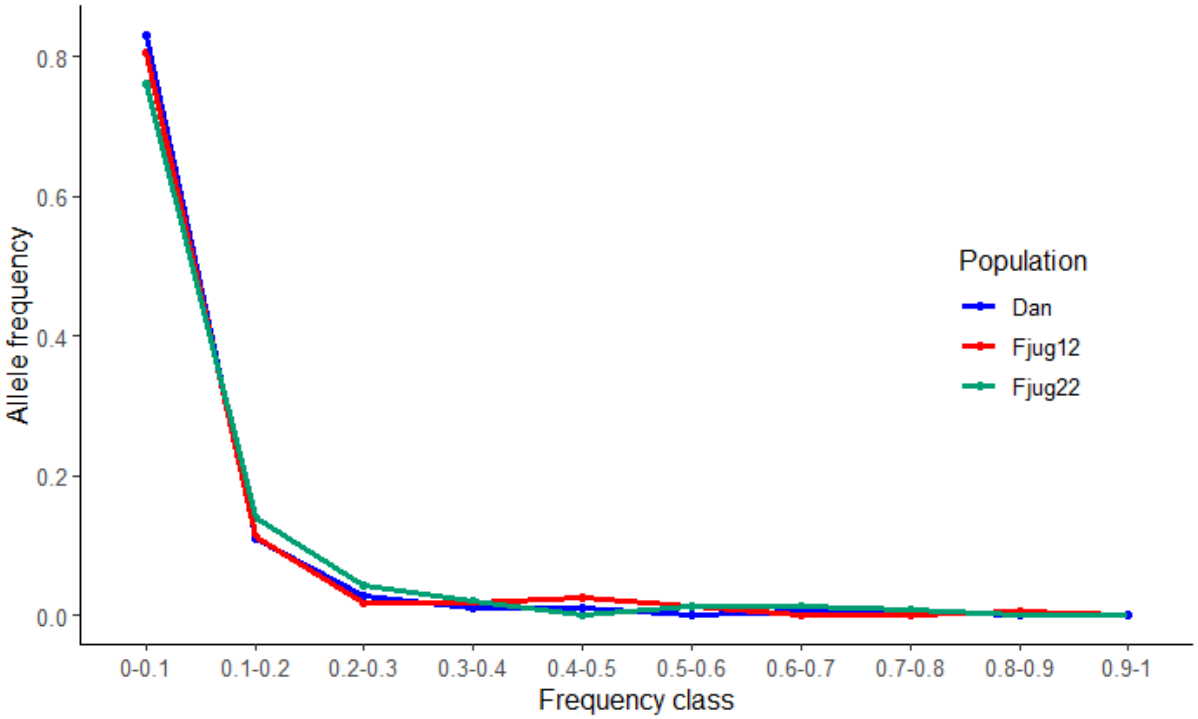

Figure 4. A line graph showing Mode-shift curve. *L shaped curve of Dan, Fjug12, and Fjug22 showed prevalence of low frequency allele.*

Table 6. *P* values for sign test of significance for reduction in heterozygosity excess for testing bottleneck under the infinite allele model (IAM), two phase model (TPM) and the stepwise mutation model (SMM), assuming mutation-drift equilibrium at the null hypothesis using Bottleneck.

	IAM	SMM	TPM
Fjug 22	0.21206	0.00038	0.01763
Fjug 12	0.340	0.003	0.318
Dan	0.44903	0.00002	0.16769

Overlapping allele frequency between Danish and Fjugstad populations

The results showed genetic differentiation among all three populations viz. Dan, Fjug12 and Fjug22 ($P < 0.001$), with Fst values ranging from 0.022 to 0.037 (Table 7). The highest Fst value was observed between populations Dan and Fjug22 (Fst = 0.037), indicating the highest genetic differentiation between these two populations among the three pairwise comparisions. The Fst value between populations Dan and Fjug12 was intermediate (Fst = 0.025), while the lowest Fst value was observed between populations Fjug12 and Fjug22 (Fst = 0.022). Significant Fst value

between Fjug12 and Fjug22 indicated differentiation in population since the infection of the forest by dieback.

Table 7. Pairwise comparisons of Fst values between Danish and Fjugstad populations

	Dan	Fjug12	Fjug22
Dan	0		
Fjug12	0.025	0	
Fjug22	0.037	0.022	0

Based upon 1023 iterations of permutations tests, all the Fts values differed significantly from zero.

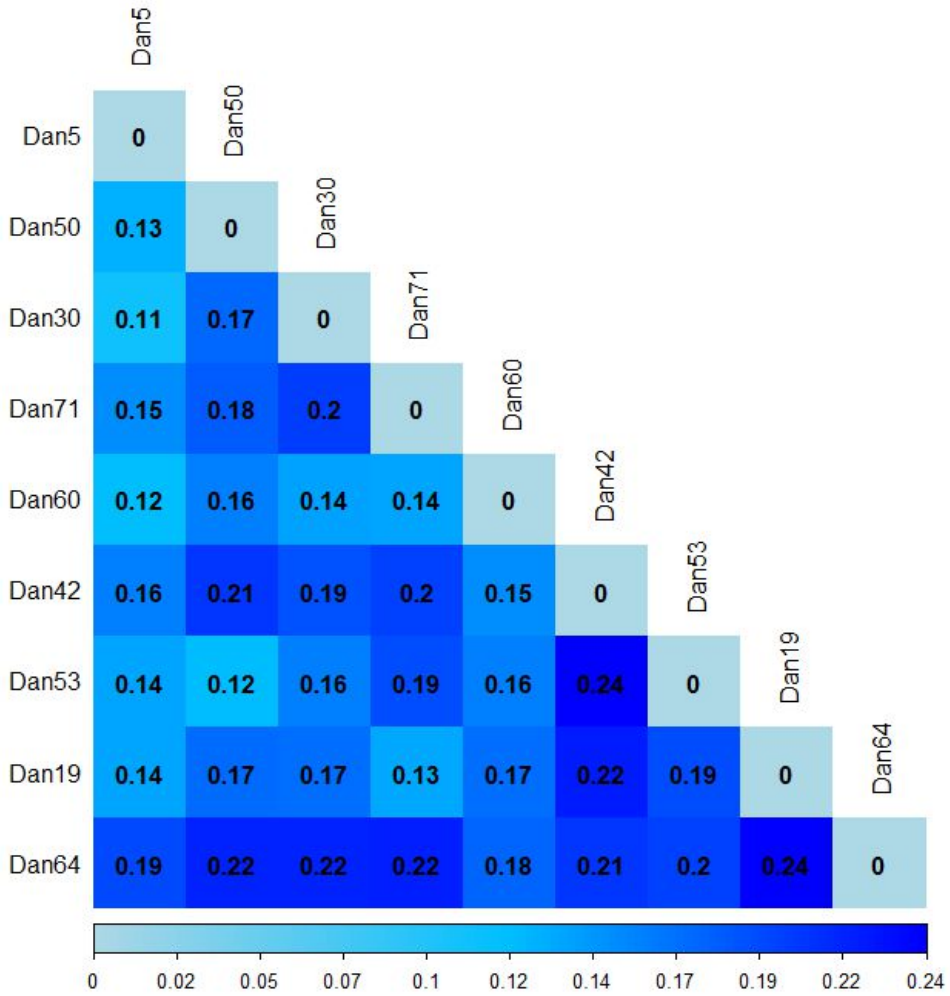

Figure 5. Corrplot with pairwise comparison of Fst values among 9 Danish families. *The numbers in the boxes represent pairwise Fst values and the color intensity correlates with the Fst value. All the Fst values were significantly different from 0 based upon 1023 iterations of permutation test.*

Pairwise comparisons of Fst values among nine different Danish families suggested higher differentiation within themselves (Fig 5). The lowest Fst value among Danish families was between Dan 5 and Dan 30 (Fst=0.1), which was higher than the Fst values between the overall Danish population and Fjug12 (Fst=0.022) or Fjugstad22 (Fst=0.037), indicating an asymmetric distribution of allele frequency in the population and higher differentiation within the Danish families.

The DAPC analysis of Fjug12, Fjug22, and nine families from the Danish population treated separately showed that Fjug12 and Fjug22 clustered together with significant overlap and hence can be assigned the same grouping. Similarly, some of Danish families, Dan30, Dan5, Dan19 and Dan 53 which had closer Fst values to Fjugstad (Fig 5), clustered together indicating their affinity to this population (Fig 6). However, majority of the Danish families (Dan60, Dan64, Dan42, Dan 71, and Dan50) clustered farther apart, indicating a high level of differentiation (Fig 6).

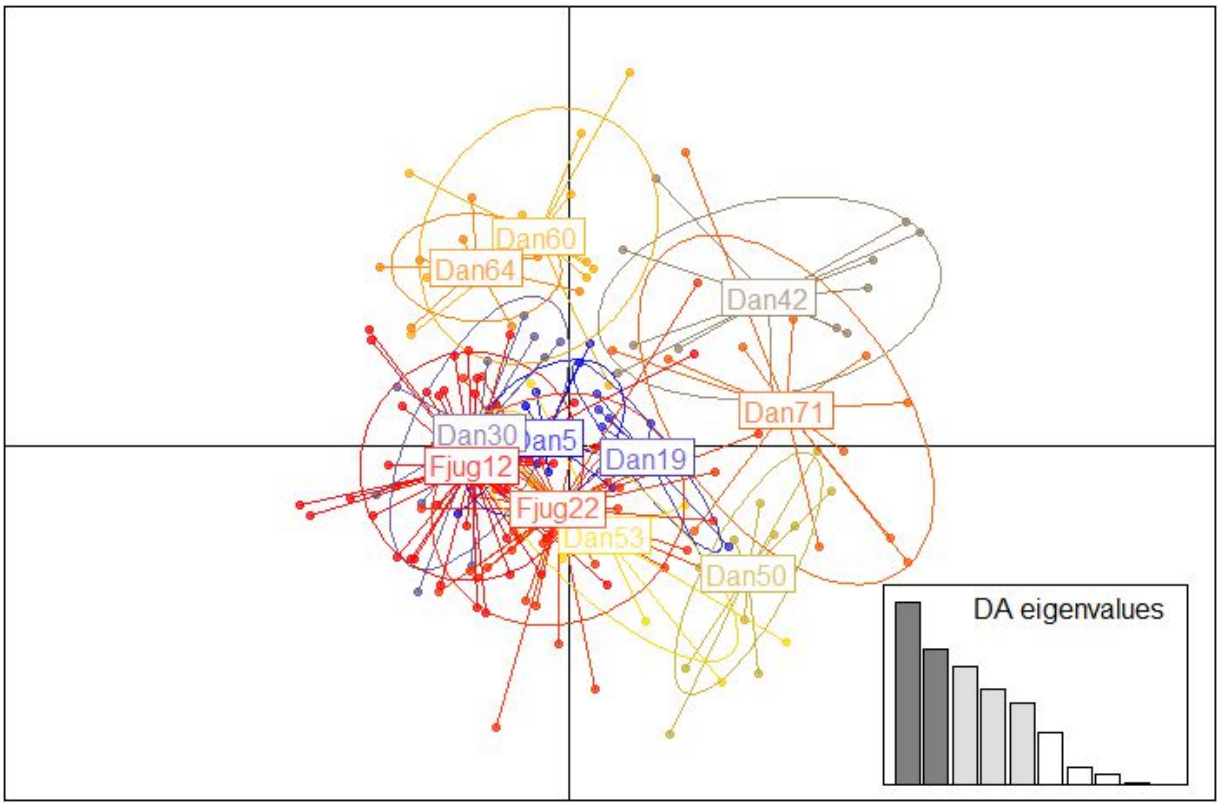

Figure 6. DAPC scatter plot showing the clustering among Fjug12, Fjug22 and the nine Danish families. *Samples belonging to different Danish families were also treated separately to analyze their clustering. The overlapping ellipses showed shared allele frequency among different populations and the distance between the two ellipse depicts the differentiation between the two.*

Bayesian clustering using STRUCTURE supported population differentiation, with a peak in the graph between the rate of change of log probability (ΔK) and the number of clusters (K) at K=2 and K=4 (Fig 7). At K=2, Fjug12 and Fjug22 formed a well-clustered group, while the Danish

samples had a mixture of the two different ancestral components (Fig 8). This pattern persisted until K=5, beyond which the clustering became unstable. At K=5, the Danish population was divided into four distinct clusters sharing three different ancestral genetic components (Fig 8). Similar clustering was obtained with DAPC clustering (Fig 6) thus supporting the number of clusters.

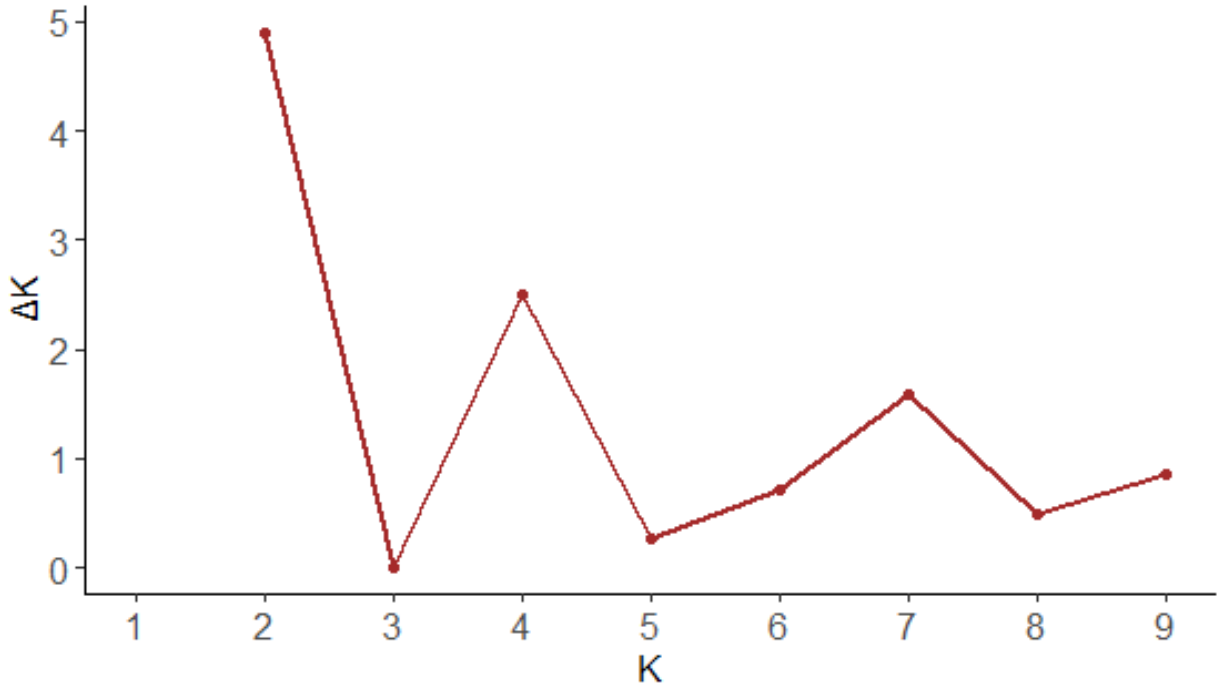

Figure 7. STRUCTURE line graph, showing the rate of change of likelihood of clusters (K) at different values of Ks. *A peak was formed at ΔK=2 and the next highest peak at ΔK=4, indicating the number of possible clusters in the population.*

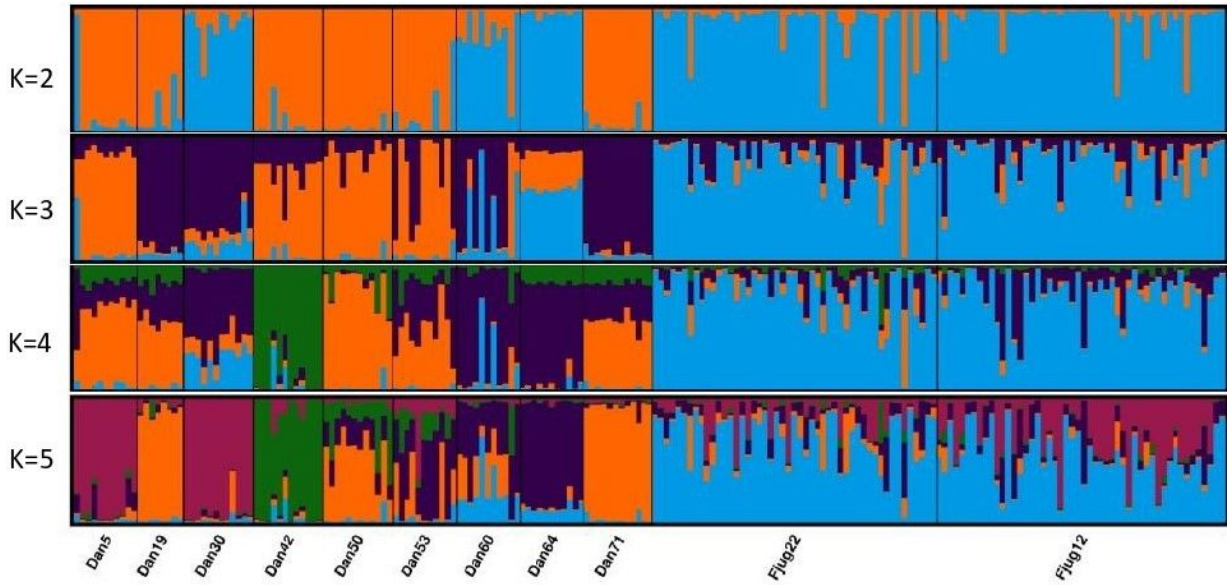

Figure 8. STRUCTURE bar plot for different values of Ks. *Each bar represents a different individual, each of the populations and families of the Dan populations were separated by a black line. The colors represent the probability of membership of different individuals in each genetic cluster.*

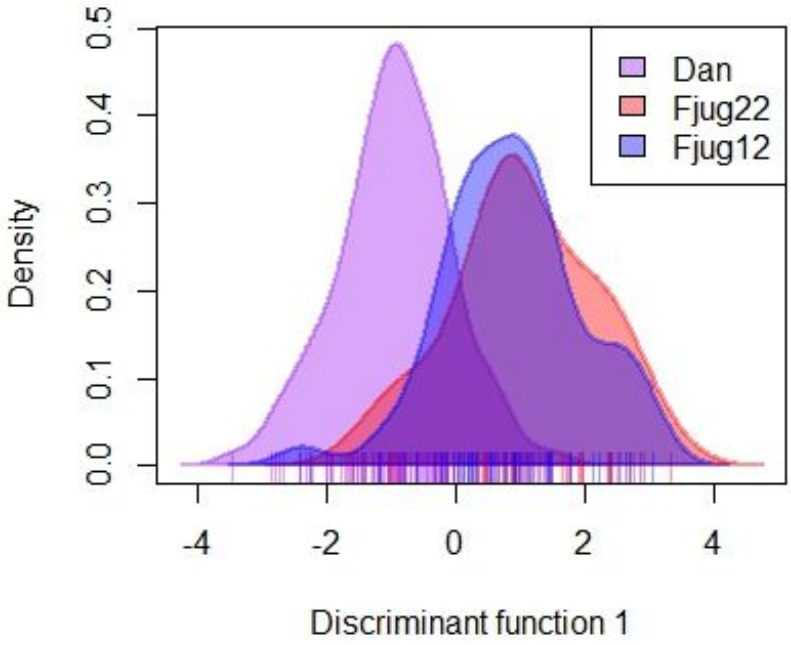

Figure 9. DAPC density plot, highlighting the clustering of samples belonging to 3 populations, Fjug12, Fjug22 and Dan.

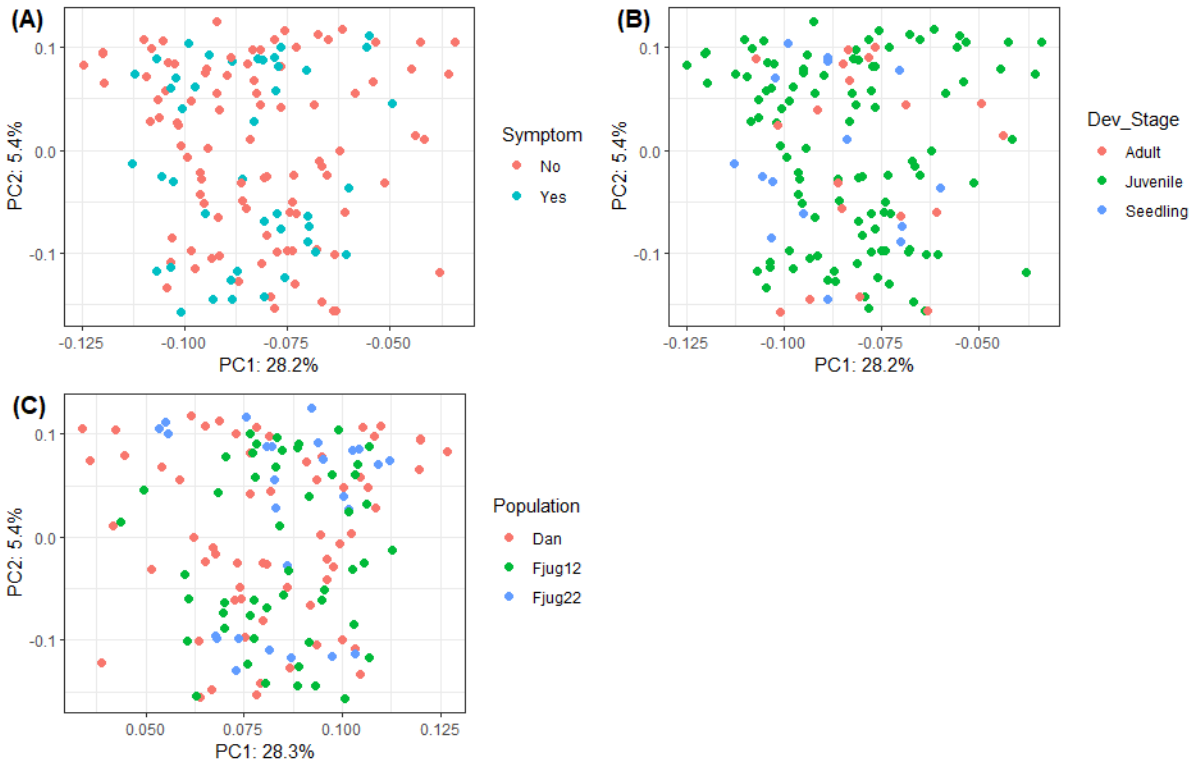

Figure 10 PCA of microsatellite allele counts. *Scatterplots of PC1 and PC2 from 225 microsatellite allele counts are shown and colored according to (A) presence and absence of disease (B) – Developmental stage of the plants and (C)- different populations. Dan represents Danish population, Fjugstad 12 and Fjugstad22 respectively.*

The PCA analysis of the genotypes, based on the presence or absence of the dieback symptom, did not reveal any discernible pattern. PC1 accounted for 28.2% of the variation, while PC2 explained 5.4% of the variation (Fig 9A). We also performed PCA based on the developmental stage of the plants, in which PC1 explained 28.1% of the variation, and PC2 explained 5.4% of the variation (Fig 9B). Similarly, we ran PCA based on the population type, in which PC1 explained 28.3% of the variation, and PC2 explained 5.4% of the variation. However, none of the grouping variables described showed any identifiable pattern of genotypic distribution.

Discussion

No change in genetic diversity in Fjugstad during the dieback infection

The genetic composition of the ash forest in Fjugstad Nature Reserve is likely shaped by the continuity of the same mother trees contributing to the seed pool and the generational time of ash. We did not observe any significant change in the genetic diversity of the forest before and after the heavy dieback infection, except that private allele decreased after the dieback crisis (Fig 3, Table 5). DAPC population clustering and STRUCTURE (Fig 8) assigned both Fjug12 and Fjug22

to the same cluster, further corroborating the result. This study is the first of its kind as a comparative study on the genetic diversity of ash before and after heavy dieback infection is lacking. It takes time for a species with a long generational time to show the effect on genetic diversity (Nielsen & Kjær, 2010). A study conducted on elm with herbarium collections spanning over 100 years, before and after infection of Dutch elm disease, showed no significant change in allelic diversity and heterozygosity (Brunet et al., 2016). Similar studies on Dutch elm could not attribute the loss of genetic diversity to Dutch elm disease (Bertolasi et al., 2015; Brunet et al., 2016; Buiteveld et al., 2016; Nielsen & Kjær, 2010). Since both Dutch elm and Fraxinus excelsior have a long generation time and high pollen-mediated gene flow, the results are comparable. Despite the devastation caused by the disease, it may be too early to detect major changes in the genetic diversity of ash.

Systematic differences in tree age, developmental stage, and probable natural selection against dieback could potentially bias population genetics results. In our study, Fjug22 samples were collected only from juveniles in a small plot designated for ash restoration trials, while Fjug12 included older trees, seedlings, and juveniles covering the entire forest (Fig 1). The PCA analysis did not show any patterns of genetic differentiation across the plant developmental phase or with disease symptoms. It is therefore reasonable to assume that the inclusion of adults and the difference in sampling had little effect on the results between 2012 and 2022. Additionally, the field observations in 2022 we observed many seedlings on the forest floor, but no trees as all the juveniles died before reaching tree size. The sampled plants also showed symptoms of damage, although it was not clear whether the damage was due to dieback or browsing. We did not test for the tolerance of sampled plants against dieback; however, previous studies have shown that only a few plant individuals possess tolerance against the disease due to natural selection (McKinney et al., 2014). Therefore, the disease causes selective mortality, with specific genetic traits that confer resistance to the disease are more likely to survive, while others die. This process can lead to a loss of genetic diversity and a reduction in allelic richness. Our observation of low private alleles in Fjug22 (Fig 3) may be due to the selection in favor of the tolerant plants. This is concerning, specifically because private alleles are associated with the adaptive potential of the plant (Vilas et al., 2015), and their loss may compromise the long-term fitness of the population.

The extent of inbreeding within a population can be influenced by environmental factors that facilitate pollen mediated gene flow (Semizer-Cuming et al., 2017). We observed inbreeding in both Fjug12 and Fjug22, with Fjug22 exhibiting lower Fis values than Fjug12 (Table 5). Tollefsrud et al. (2016) reported no inbreeding in Fjugstad in a previous study using six SSR markers, including the one used in current study. Since, most of the additional markers used in our study had a relatively higher percentage of null alleles (Table 4), which might have slightly inflated the Fis value. Despite this, the current measurements were still significant after accounting for the effect of null alleles. Wind speed has a positive correlation with pollen-mediated gene flow, which is enhanced with the clearing of canopy and the intervening vegetation (Semizer-Cuming et al., 2017). In a severely degraded ash forest, the pollen-mediated gene flow was effective up to 1.9 km

(Bacles & Ennos, 2008). With increasing mortality of ash forests (Díaz-Yáñez et al., 2020; Timmermann et al., 2017), it is possible that the opening of the canopy and the intervening vegetation layer facilitated pollen flow and dispersal of winged ash seeds, contributing to the reduction of the coefficient of inbreeding in Fjug22.

Genetic diversity in Danish reproductive materials and their use for enrichment plantation

The genetic diversity of a population is influenced by various factors, such as population history and colonization which deserve consideration while studying the genetic makeup of a population. The Danish population showed similarities to the Fjugstad population in some of the genetic diversity parameters, except for higher private allelic richness and expected heterozygosity (Fig 3). Both the Norwegian and Danish ash share a common ancestry from the glacial refugia in Iberia, Italy, the eastern Alps, and the Balkan Peninsula, colonized following different routes (Heuertz et al., 2004). Populations that are closer to the source population generally have more genetic diversity. The Danish population, being in proximity to the source population, input from the refuge, and effective mixture of ash from different source populations, it is natural to have a higher diversity. Tollefsrud et al. (2016) reported the presence of highest number of cpDNA haplotypes in Danish population among different European populations indicating the range of diversity it harbors. Interestingly, our findings suggested similar genetic diversity in both Norway and Denmark. It is worth noting that Danish samples collected for this work, were collected from plants grown from seeds belonging to mother trees from seed orchard selected for dieback tolerance. Therefore, the plants selected, both naturally and artificially, may contain only a subset of the genetic diversity present in the natural population, which may result in similarity in some of the genetic diversity parameters between Fjugstad and Danish populations.

Private alleles are influenced by adaptive and selection pressures mediated both artificially and naturally in a population. Denmark is implementing an ash breeding program to establish a resilient and resistant forest. It selects resistant trees from various parts of the country and promotes their seeds (McKinney et al., 2014). So far, 214 trees have been selected (Kjær et al., 2017). To maximize diversity and avoid potential inbreeding depression, only one healthy-looking tree per stand is selected for breeding. The program also avoids selecting trees from the old seed orchard to minimize potential inbreeding depression from the selected breeds while maintaining genetic diversity (Kjær et al., 2017). In this context, both natural and artificial selection are active in the Danish population, promoting the plants that potentially carry resistant alleles. This has likely influenced the higher count of private allelic richness.

Despite the similarity in genetic diversity measures, the Danish and Fjugstad populations were differentiated from one another, but with some overlap in allelic frequency. Interestingly, even among the Danish families, there was a higher level of differentiation, as revealed by pairwise Fst comparison (Fig 5) and DAPC clustering (Fig 6). The results of the pairwise comparison between Danish and Fjugstad populations are consistent with those reported by Tollefsrud et al. (2016). However, current finding on the level of differentiation observed among Danish populations was

even higher than those of populations from other European countries compared among themselves. For example, the Bulgarian population measured based on Femsatl19, Femsatl11, Femsatl4, Femsatl16 and M2 30 had a Fst value of 0.087 (Heuertz et al., 2001), the German population measured based on Femsatl14, Femsatl18, Femsatl112, and Femsatl16 had 0.012 (Hebel et al., 2006), and the French population measured based on M2–30, Femsatl4, Femsatl5, Femsatl11, and Femsatl19 had 0.043 (Morand et al., 2002). The differentiation observed in Danish samples may be due to the samples collected from plants belonging to seeds from resistant mother trees grown in a seed orchard for ash breeding program and the tree selection protocol (Kjær et al., 2017). This may have led to an increased number of alleles offering resistance against dieback.

One of the preconditions for using propagules for enrichment planting is that their genetic composition should be similar to that of the target forest. Although there is differentiation between the Fjugstad and Danish populations, both DAPC (Fig 6 and 9) and STRUCTURE analysis (Fig 8) showed an overlapping allelic frequency between them, suggesting that the Fjugstad population contained parts of the genetic composition present in the Danish population, with shared allelic frequency. This may be due to a shared ancestry between these populations. The fact that the Danish materials have this shared allelic frequency makes them suitable for use in the enrichment plantation in Fjugstad forest.

Protocols for the selection of reproductive materials and plantation methods are crucial for successful management of degraded forests. In previous enrichment plantations, decisions on propagule selection were based on macro-scale indicators such as species diversity and composition, plant survival rate, growth, development, canopy size, and gaps, resulting in some positive outcomes (Asanok et al., 2013; Jayawardhane & Gunaratne, 2020; Marshall et al., 2021). Some plantations, where seeds and propagules were selected carefully, seemed to conserve genetic diversity well, despite not screening restoration materials for genetic diversity (Cooper et al., 2023). However, introducing genetic resources without careful assessment risks introducing entirely new genetic materials and consequent outbreeding depression. However, information on the practice of genetic analysis of reproductive materials prior to plantation is lacking, probably due to cost and technical know-how associated with it. Our work is probably the first of its kind and can be established as a basic protocol for enrichment plantation. Plantation with complete turnover of the species creates uniform climatic conditions, however canopy gaps in enrichment plantations creates substantial habitat heterogeneity, providing a favorable microclimate for growth and development of plants, as evident in the successful restoration effort of European ash (Haupt et al., 2022). Enrichment plantation may offer a better method of introducing new ash plants in Fjugstad.

Artificial selection combined with natural selection can accelerate the development of a resistant and robust forest. However, the use of maladapted or non-native genetic materials in enrichment plantations can lead to compromise in local adaptability (Frankham, 2015). Therefore, knowledge on the adaptive variation within a population linked by geneflow is needed to maintain the evolutionary potential of a population (McKay et al., 2005). Moreover, introducing reproductive

materials from sources adapted to different climates is concerning for plants with high gene flow, as it can quickly contaminate the entire population and undermine genetic resilience in stochastic events such as pathogenicity or climate change (Aitken & Whitlock, 2013). Studies from NIBIO (unpublished) suggest similar phenology in materials from southern Norway and Lithuania, indicating that transferring materials from the south of Norway to southern Norway would work well in terms of adaptability to the climate. This suggests that Danish materials may be compatible with Fjugstad in terms of local adaptability. Furthermore, our observation of similar genetic diversity (Fig 3) and overlapping allelic frequencies (Fig. 9) between Danish and Fjugstad populations suggests that adding Danish reproductive materials would complement the diversity of the Fjugstad ash forest and introduce low susceptibility traits present in the Danish samples. As Fjugstad population has low inbreeding it should be capable of purging recessive deleterious genes through selection as indicated by McKay et al. (2005). Moreover, higher rare and private allelic richness (Fig. 3) present in the Danish population, selected for low susceptibility, could offer tolerance to the plants against dieback, which made it a better option for enrichment planting materials in Fjugstad Nature Reserve and for creating a resilient ash forest.

Limitations

Microsatellite markers are widely used to study genetic diversity, but their limited number of loci may not provide sufficient resolution to detect fine-scale changes in the population. In the case of Fjugstad, although we did not detect any major change in genetic diversity before and after heavy dieback infection, field observations suggested that only old mother trees were contributing to the seed bank in the forest, with no new seedlings being established to contribute to genetic diversity. This may have implications for genetic diversity on a fine scale. Further, microsatellite markers use allelic size rather than base pair sequences, which means that any mutations involving a mutational base, like homoplasy will not be counted. Homoplasious alleles are expected to occur frequently in microsatellite markers due to the allele size constraints (Estoup et al., 2002; Kimura & Crow, 1964). As a result, the actual diversity may be higher than estimated. To capture a more accurate estimation of the diversity and differentiation, it is helpful to have high-resolution genotyping, such as SNP markers.

Future plans and follow up

Monitoring forest management actions has two major goals: measuring the success or failure of the action and restoring the same population (McKay et al., 2005). As the mother trees of the plantation materials showed some disease symptoms (Annex: 1), regular monitoring to assess the health of the plants will provide information on the performance of the enrichment planted materials. Selective pressure from the disease favors plants with high genetic tolerance against dieback disease (Kjær et al., 2012; Lobo et al., 2014; Lobo et al., 2015; McKinney et al., 2014; Munoz et al., 2016). It will be interesting to see how the Danish reproductive materials planted in Fjugstad will enhance the remaining ash forest through long-term genetic monitoring. However, short-term follow-up on how the plants introduced in Fjugstad establish themselves in the

Norwegian climate in the field trial is important. This follow-up will be conducted by NIBIO as part of a larger project on ash trials, which includes clearing of the canopy and fencing, as well as regular follow-up to facilitate the establishment of the planted materials.

Conclusions

This study conducted a comparative genetic analysis of an ash forest in Fjugstad Nature Reserve before and after heavy infection due to dieback disease and assessed the genetic diversity of the plantation materials intended for enrichment plantation in the reserve. The results showed that there was no significant change in genetic diversity before and after the heavy dieback infection. Both Fjugstad and Danish populations exhibited similar levels of genetic diversity, with slightly higher private allelic richness in the Danish populations and overlapping allele frequencies, making Danish reproductive materials suitable for enrichment plantation in Fjugstad. This study likely represents the first documentation of genetic considerations in enrichment planting the reproductive materials from different provenances for the management of degraded forest. The results offered assurance of genetic compatibility of plantation materials with the Fjugstad forest. More importantly, the current work will help to define a protocol for genetic consideration before introducing plantation materials, protecting the forest from potential inbreeding, or outbreeding depression and creating a more resilient and evolutionarily stable forest.

Reference

Aggarwal, R. K., Allainguillaume, J., Bajay, M., Barthwal, S., Bertolino, P., Chauhan, P., Consugra, S., Croxford, A., & Dalton, D. L. (2010). Permanent genetic resources added to molecular ecology resources database 1 August 2010–30 September 2010. *Molecular ecology resources*, *11*(1), 219-222.

Aitken, S. N., & Whitlock, M. C. (2013). Assisted gene flow to facilitate local adaptation to climate change. *Annual review of ecology, evolution, and systematics*, *44*, 367-388.

Aitken, S. N., Yeaman, S., Holliday, J. A., Wang, T., & Curtis-McLane, S. (2008). Adaptation, migration or extirpation: climate change outcomes for tree populations. *Evolutionary applications*, *1*(1), 95-111.

Asanok, L., Marod, D., Duengkae, P., Pranmongkol, U., Kurokawa, H., Aiba, M., Katabuchi, M., & Nakashizuka, T. (2013). Relationships between functional traits and the ability of forest tree species to reestablish in secondary forest and enrichment plantations in the uplands of northern Thailand. *Forest Ecology and Management*, *296*, 9-23.

Bacles, C. F., & Ennos, R. A. (2008). Paternity analysis of pollen-mediated gene flow for Fraxinus excelsior L. in a chronically fragmented landscape. *Heredity*, *101*(4), 368-380.

Bertacchi, M. I. F., Amazonas, N. T., Brancalion, P. H., Brondani, G. E., de Oliveira, A. C., de Pascoa, M. A., & Rodrigues, R. R. (2016). Establishment of tree seedlings in the understory of restoration plantations: natural regeneration and enrichment plantings. *Restoration Ecology*, *24*(1), 100-108.

Bertolasi, B., Leonarduzzi, C., Piotti, A., Leonardi, S., Zago, L., Gui, L., Gorian, F., Vanetti, I., & Binelli, G. (2015). A last stand in the Po valley: genetic structure and gene flow patterns in Ulmus minor and U. pumila. *Annals of Botany*, *115*(4), 683-692.

Brachet, S., Jubier, M.-F., Richard, M., Jung-Muller, B., & Frascaria-Lacoste, N. (1999). Rapid identification of microsatellite loci using 5'anchored PRC in the common ash Fraxinus excelsior. *Molecular ecology*.

Brookfield, J. (1996). A simple new method for estimating null allele frequency from heterozygote deficiency. *Molecular ecology*, *5*(3), 453-455.

Brunet, J., Zalapa, J., & Guries, R. (2016). Conservation of genetic diversity in slippery elm (Ulmus rubra) in Wisconsin despite the devastating impact of Dutch elm disease. *Conservation genetics*, *17*, 1001-1010.

Buiteveld, J., Vanden Broeck, A., Cox, K., & Collin, E. (2016). Human impact on the genetic diversity of Dutch field elm (Ulmus minor) populations in the Netherlands: implications for conservation. *Plant Ecology and Evolution*, *149*(2), 165-176.

Chybicki, I. J., & Burczyk, J. (2009). Simultaneous estimation of null alleles and inbreeding coefficients. *Journal of heredity*, *100*(1), 106-113.

Cooper, S. L., Bundock, P. C., & Nock, C. (2023). Comparative analysis of genetic diversity in wild and restored subtropical rainforest trees: Argyrodendron trifoliolatum, Ficus watkinsiana and Diploglottis australis.

Díaz-Yáñez, O., Mola-Yudego, B., Timmermann, V., Tollefsrud, M. M., Hietala, A. M., & Oliva, J. (2020). The invasive forest pathogen Hymenoscyphus fraxineus boosts mortality and triggers niche replacement of European ash (Fraxinus excelsior). *Scientific reports*, *10*(1), 1-10.

Earl, D. A., & VonHoldt, B. M. (2012). STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation genetics resources*, *4*, 359-361.

Estoup, A., Jarne, P., & Cornuet, J. M. (2002). Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. *Molecular ecology*, *11*(9), 1591-1604.

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular ecology*, *14*(8), 2611-2620.

Excoffier, L., Laval, G., & Schneider, S. (2005). Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary bioinformatics*, *1*, 117693430500100003.

Finkeldey, R. (2010). Genetik, Ökologie, Forstwirtschaft: Zusammenhänge und Perspektiven| Genetics, ecology, forest management: connections and perspectives. *Schweizerische Zeitschrift für Forstwesen*, *161*(6), 198-206.

Frankham, R. (2015). Genetic rescue of small inbred populations: Meta-analysis reveals large and consistent benefits of gene flow. *Molecular ecology*, *24*(11), 2610-2618.

Goudet, J. (2003). FSTAT (version 2.9. 4), a program (for Windows 95 and above) to estimate and test population genetics parameters. *Department of Ecology & Evolution, Lausanne University, Switzerland*, *53*.

Grünig, M., Mazzi, D., Calanca, P., Karger, D. N., & Pellissier, L. (2020). Crop and forest pest metawebs shift towards increased linkage and suitability overlap under climate change. *Communications biology*, *3*(1), 1-10.

Guo, S. W., & Thompson, E. A. (1992). Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics*, 361-372.

Haupt, K. S., Mausolf, K., Richter, J., Schrautzer, J., & Erfmeier, A. (2022). The FraDiv experiment: Biodiversity-ecosystem functioning research meets reforestation practice. *Ecological Indicators*, *144*, 109497.

Heuertz, M., Fineschi, S., Anzidei, M., Pastorelli, R., Salvini, D., Paule, L., Frascaria-Lacoste, N., Hardy, O. J., Vekemans, X., & Vendramin, G. (2004). Chloroplast DNA variation and postglacial recolonization of common ash (Fraxinus excelsior L.) in Europe. *Molecular ecology*, *13*(11), 3437-3452.

Hu, L. J., Uchiyama, K., Saito, Y., & Ide, Y. (2010). Contrasting patterns of nuclear microsatellite genetic structure of Fraxinus mandshurica var. japonica between northern and southern populations in Japan. *Journal of Biogeography*, *37*(6), 1131-1143.

Hultberg, T., Sandström, J., Felton, A., Öhman, K., Rönnberg, J., Witzell, J., & Cleary, M. (2020). Ash dieback risks an extinction cascade. *Biological Conservation*, *244*, 108516.

Jayawardhane, J., & Gunaratne, A. (2020). Restoration success evaluation of a thinned and enriched pine plantation in Sri Lanka. *Journal of Tropical Forest Science*, *32*(4), 402-413.

Jombart, T. (2008). adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics*, *24*(11), 1403-1405.

Kalinowski, S. T. (2005). hp-rare 1.0: a computer program for performing rarefaction on measures of allelic richness. *Molecular ecology notes*, *5*(1), 187-189.

Kimura, M., & Crow, J. F. (1964). The number of alleles that can be maintained in a finite population. *Genetics*, *49*(4), 725.

Kjær, E., McKinney, L., Hansen, L., Olrik, D., Lobo, A., Thomsen, I., Hansen, J., & Nielsen, L. (2017). Genetics of ash dieback resistance in a restoration context–experiences from Denmark. *Dieback of European ash*, 106-114.

Kjær, E. D., McKinney, L. V., Nielsen, L. R., Hansen, L. N., & Hansen, J. K. (2012). Adaptive potential of ash (Fraxinus excelsior) populations against the novel emerging pathogen Hymenoscyphus pseudoalbidus. *Evolutionary applications*, *5*(3), 219-228.

Kopelman, N. M., Mayzel, J., Jakobsson, M., Rosenberg, N. A., & Mayrose, I. (2015). Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular ecology resources*, *15*(5), 1179-1191.

Lamb, D., Erskine, P. D., & Parrotta, J. A. (2005). Restoration of degraded tropical forest landscapes. *Science*, *310*(5754), 1628-1632.

Lefort, F., Brachet, S., Frascaria-Lacoste, N., Edwards, K., & Douglas, G. (1999). Identification and characterization of microsatellite loci in ash (Fraxinus excelsior L.) and their conservation in the olive family (Oleaceae). *Molecular ecology*, *8*(6), 1088-1089.

Lienert, J. (2004). Habitat fragmentation effects on fitness of plant populations–a review. *Journal for nature conservation*, *12*(1), 53-72.

Lobo, A., Hansen, J. K., McKinney, L. V., Nielsen, L. R., & Kjær, E. D. (2014). Genetic variation in dieback resistance: growth and survival of Fraxinus excelsior under the influence of Hymenoscyphus pseudoalbidus. *Scandinavian Journal of Forest Research*, *29*(6), 519-526.

Lobo, A., McKinney, L., Hansen, J., Kjær, E., & Nielsen, L. (2015). Genetic variation in dieback resistance in Fraxinus excelsior confirmed by progeny inoculation assay. *Forest Pathology*, *45*(5), 379-387.

Loo, J. A. (2009). Ecological impacts of non-indigenous invasive fungi as forest pathogens. *Ecological impacts of non-native invertebrates and fungi on terrestrial ecosystems*, 81-96.

Marshall, A., McLaughlin, B. P., Zerr, C., Yanguas-Fernández, E., & Hall, J. S. (2021). Early indications of success rehabilitating an underperforming teak (Tectona grandis) plantation in Panama through enrichment planting. *New Forests*, *52*(3), 377-395.

McKay, J. K., Christian, C. E., Harrison, S., & Rice, K. J. (2005). “How local is local?”—a review of practical and conceptual issues in the genetics of restoration. *Restoration Ecology*, *13*(3), 432-440.

McKinney, L., Nielsen, L., Collinge, D., Thomsen, I., Hansen, J., & Kjær, E. (2014). The ash dieback crisis: genetic variation in resistance can prove a long-term solution. *Plant Pathology*, *63*(3), 485-499.

McKinney, L., Nielsen, L., Hansen, J., & Kjær, E. (2011). Presence of natural genetic resistance in Fraxinus excelsior (Oleraceae) to Chalara fraxinea (Ascomycota): an emerging infectious disease. *Heredity*, *106*(5), 788-797.

Miljødirektoratet. (2022). *Naturbase fact sheet*. Retrieved 11/6/2022 from

Montagnini, F., Eibl, B., Grance, L., Maiocco, D., & Nozzi, D. (1997). Enrichment planting in overexploited subtropical forests of the Paranaense region of Misiones, Argentina. *Forest Ecology and Management*, *99*(1-2), 237-246.

Munoz, F., Marçais, B., Dufour, J., & Dowkiw, A. (2016). Rising out of the ashes: additive genetic variation for crown and collar resistance to Hymenoscyphus fraxineus in Fraxinus excelsior. *Phytopathology*, *106*(12), 1535-1543.

Nielsen, L. R., & Kjær, E. D. (2010). Gene flow and mating patterns in individuals of wych elm (Ulmus glabra) in forest and open land after the influence of Dutch elm disease. *Conservation genetics*, *11*, 257-268.

Piry, S., Luikart, G., & Cornuet, J.-M. (1999). BOTTLENECK: a program for detecting recent effective population size reductions from allele data frequencies. *Journal of heredity*, *90*(4), 502-503.

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, *155*(2), 945-959.

Przybył, K. (2002). Fungi associated with necrotic apical parts of Fraxinus excelsior shoots. *Forest Pathology*, *32*(6), 387-394.

Raymond, M., & Rousset, F. (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of heredity*, *86*(3), 248-249.

Rousset, F. (2008). genepop’007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular ecology resources*, *8*(1), 103-106.

Schlarbaum, S. E., Hebard, F., Spaine, P. C., & Kamalay, J. C. (1998). Three American tragedies: chestnut blight, butternut canker, and Dutch elm disease. In: Britton, Kerry O., ed. Exotic pests of eastern forests conference proceedings; 1997 April 8-10; Nashville, TN. US Forest Service and Tennessee Exotic Pest Plant Council: 45-54.,

Seidl, R., Klonner, G., Rammer, W., Essl, F., Moreno, A., Neumann, M., & Dullinger, S. (2018). Invasive alien pests threaten the carbon stored in Europe’s forests. *Nature Communications*, *9*(1), 1-10.

Semizer-Cuming, D., Kjær, E. D., & Finkeldey, R. (2017). Gene flow of common ash (Fraxinus excelsior L.) in a fragmented landscape. *PLoS One*, *12*(10), e0186757.

Solheim, H., & Hietala, A. M. (2017). Spread of ash dieback in Norway. *Baltic Forestry 23*(1), 144-149.

Solstad, H., Elven, R., Arnesen, G., Eidesen, P., Gaarder, G., Hegre, H., Høitomt, T., Mjelde, M., & Pedersen, O. (2021). *Karplanter: Vurdering av ask Fraxinus excelsior for Norge*. Retrieved 29/04/2022 from

Stocks, J. J., Metheringham, C. L., Plumb, W. J., Lee, S. J., Kelly, L. J., Nichols, R. A., & Buggs, R. J. (2019). Genomic basis of European ash tree resistance to ash dieback fungus. *Nature ecology & evolution*, *3*(12), 1686-1696.

Sutherland, B., Belaj, A., Nier, S., Cottrell, J., P VAUGHAN, S., Hubert, J., & Russell, K. (2010). Molecular biodiversity and population structure in common ash (Fraxinus excelsior L.) in Britain: implications for conservation. *Molecular ecology*, *19*(11), 2196-2211.

Team, R. C. (2021). R: A language and environment for statistical computing. Published online 2020. In.

Thomas, E., Jalonen, R., Loo, J., Boshier, D., Gallo, L., Cavers, S., Bordács, S., Smith, P., & Bozzano, M. (2014). Genetic considerations in ecosystem restoration using native tree species. *Forest Ecology and Management*, *333*, 66-75.

Timmermann, V., Nagy, N. E., Hietala, A. M., Børja, I., & Solheim, H. (2017). Progression of ash dieback in Norway related to tree age, disease history and regional aspects. *Baltic Forestry.* , *23*(1), 150-158.

Vilas, A., Pérez-Figueroa, A., Quesada, H., & Caballero, A. (2015). Allelic diversity for neutral markers retains a higher adaptive potential for quantitative traits than expected heterozygosity. *Molecular ecology*, *24*(17), 4419-4432.

Weir, B. S., & Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. *evolution*, 1358-1370.

Zhao, Y.-J., Hosoya, T., Baral, H.-O., Hosaka, K., & Kakishima, M. (2013). Hymenoscyphus pseudoalbidus, the correct name for Lambertella albida reported from Japan. *Mycotaxon*, *122*(1), 25-41.

Appendix 1

Phenotypic score and geographical location of the Fjugstad and Danish samples taken in 2012 and 2022.

Fjugstad 2012					**Fjugstad 2022**					**Danish mother trees**		
Sample	PH	HC	Lat	Long	Sample	PH	Height (cm)	Lat	Long	Sample	Sample no	CD
Fj1	0	3	59.36197	10.45756	Fjug1	0	30	10.45696412	59.36105	Dan5	12	b
Fj10	0	3	59.36201	10.45751	Fjug2	0	30	10.45704874	59.36101	Dan19	8	b
Fj10C	1	2	59.36200	10.45755	Fjug3	0	28	10.45697214	59.36103	Dan30	12	a
Fj1A	1	2	59.36200	10.45761	Fjug4	0	24	10.45701682	59.36113	Dan42	12	a
Fj1B	0	1	59.36200	10.45760	Fjug5	1	53	10.45701682	59.36113	Dan50	12	a
Fj2A	1	1	59.36201	10.45765	Fjug6	0	48	10.456985	59.36108	Dan53	12	b
Fj2B	1	1	59.36201	10.45759	Fjug7	0	34	10.45684636	59.36097	Dan60	12	a
Fj2C	1	2	59.36205	10.45763	Fjug8	0	20	10.45696324	59.3609	Dan64	12	0
Fj2D	1	1	59.36202	10.45750	Fjug9	0	55	10.45695895	59.36088	Dan71	12	a
Fj3	1	3	59.36197	10.45777	Fjug10	0	25	10.4572058	59.36106			
Fj3A	1	3	59.36199	10.45772	Fjug11		44	10.45703732	59.36115			
Fj3B	1	1	59.36197	10.45769	Fjug12	0	32	10.45730147	59.36116			
Fj3C	1	1	59.36200	10.45769	Fjug13	0	37	10.45734994	59.36114			
Fj3D	1	2	59.36204	10.45774	Fjug14	1	47	10.45737983	59.36114			
Fj4A	1	1	59.36184	10.45789	Fjug16		38	10.45740902	59.36133			
Fj4B	1	1	59.36188	10.45786	Fjug17	0	28	10.45761558	59.36126			

Fj4C	1	1	59.36186	10.45785	Fjug18	0	28	10.45904875	59.36088
Fj4D	1	2	59.36185	10.45784	Fjug19	1	25	10.4590587	59.36091
Fj5	0	3	59.36187	10.45767	Fjug20	1	34	10.45909464	59.36092
Fj58	1	3	59.36061	10.46025	Fjug151	0	29	10.45783033	59.36123
Fj59	0	1	59.36056	10.46026	Fjug152	1	49	10.45783874	59.36122
Fj5B	1	1	59.36185	10.45771	Fjug153	0	13	10.4579526	59.36129
Fj5C	0	2	59.36189	10.45763	Fjug154	0	17	10.45774252	59.36135
Fj5D	1	2	59.36190	10.45762	Fjug155	0	13	10.45770966	59.36141
Fj6	1	3	59.36191	10.45750	Fjug156	1	30	10.45759602	59.3613
Fj60	0	3	59.36054	10.46013	Fjug157	1	29	10.45765619	59.36131
Fj61	0	2	59.36056	10.46010	Fjug158	0	100	10.45745352	59.36109
Fj62	1	2	59.36057	10.46009	Fjug159	0	33	10.45746385	59.36113
Fj63	0	3	59.36046	10.46002	Fjug160	0	19	10.45730724	59.36101
Fj65	1	3	59.36055	10.45895	Fjug161	1	23	10.4572846	59.36097
Fj66	0	1	59.36096	10.45893	Fjug162	1	53	10.45691466	59.3608
Fj67	1	1	59.36101	10.45894	Fjug163	1	109	10.45677069	59.36076
Fj68	1	3	59.36125	10.45926	Fjug164	0	65	10.4567986	59.3608
Fj69	0	2	59.36061	10.46195	Fjug165		173	10.45667793	59.36098
Fj70	0	3	59.36084	10.46311	Fjug166	1	32	10.45683207	59.361
Fj74	0	3	59.36109	10.46298	Fjug167		18	10.4573546	59.36088
Fj75	0	1	59.36109	10.46311	Fjug168	1	38	10.45711002	59.36113

Fj76	0	3	59.36099	10.46322	Fjug169	1	120	10.45696066	59.36125
Fj78	0	3	59.36104	10.46390	Fjug170	1	57	10.45695209	59.36122
Fj7A	1	2	59.36190	10.45746	Fjug171	1	24	10.45737462	59.36135
Fj8	0	3	59.36204	10.45749	Fjug172	1	86		
Fj82	1	1	59.36191	10.46348	Fjug173	0	29		
Fj83	1	3	59.36226	10.46345	Fjug174	0	40		
Fj84	0	3	59.36333	10.46375	Fjug175	1	73		
Fj85	1	2	59.36413	10.46368	Fjug176	0	81		
Fj86	1	2	59.36559	10.46324	Fjug177		26		
Fj87	1	2	59.36604	10.46331	Fjug178	0	32		
Fj88	1	3	59.36374	10.46125	Fjug179	0	52		
Fj8D	1	2	59.36203	10.45741	Fjug180		53		
Fj9	0	3	59.36202	10.45743					

PH- plant health, 0 indicated absence of dieback symptom and 1 indicate presence of symptoms; HC- height class; Lat= latitude, Long = longitude, CD- crown damage class b=10-25%, a= <10% and 0= absence of damage

Appendix 2

Comparative Phenotypic score and geographical location of the Fjugstad and Danish samples taken in 2012 and 2022.

Pop	A	AR	Private AR	Fis	Ho	He	FisINEST
Fjug22	142	11.281, CI= 8.46-14.18	1.04, CI=0.54-1.65	**0.222**	0.61, CI=0.46-0.74	0.756, CI=0.65-0.84	0.0067
Fjug12	159	12.413, CI= 9.22-15.75	1.71, CI=0.99-2.57	**0.178**	0.59, CI=0.45-0.74	0.726, CI=0.63-0.81	**0.0167**
Dan	184	12.541, CI=9.52-15.59	2.88, CI= 1.78-4.11	**0.194**	0.609, CI=0.44-0.76	0.782, CI=0.68-0.86	**0.0226**

Pop- Population, **A**- average number of alleles, **AR**- Allelic richness; Fis- coefficient of inbreeding, **Ho** - average proportion of heterozygotes; **He**- expected heterozygosity, the data is presented as values averaged across loci and the confidence interval at 95% calculated with 10000 bootstraps using percentile method.

Appendix 3

Mean log probability and mean similarity score for different value of clusters (K) obtained from STRUCTURE

K	Mean (LnProb)	Mean (similarity score)
2	-9459.543	0.905
3	-9233.614	0.746
4	-9020.812	0.707
5	-8885.46	0.61
6	-8713.843	0.775
7	-8602.422	0.787
8	-8493.75	0.845
9	-8411.138	0.749
10	-8321.85	0.858

The higher value of similarity score corresponds to the number of clusters that best describes the samples and higher mean log probability values descries better fit of the data to the clustering model.

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