



# Gene flow and reproductive success in ash (*Fraxinus excelsior* L.) in the face of ash dieback: restoration and conservation

Devrim Semizer-Cuming<sup>1,2,4</sup> · Igor Jerzy Chybicki<sup>3</sup> · Reiner Finkeldey<sup>1,5</sup> · Erik Dahl Kjær<sup>2</sup>

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## Abstract

• **Key message** Ash dieback decreases individual reproductive success of ash trees leaving healthy ash overrepresented as seed and pollen parents for next-generation seedlings. Substantial gene flow over hundreds of meters combined with superior fertility of healthy trees creates optimism for the species' future in European forests.

• **Context** *Hymenoscyphus fraxineus* is causing high mortality in European ash (*Fraxinus excelsior* L.). Due to inheritable resistance to the pathogen, natural selection is likely to act in favour of improved resistance in ash forests following natural regeneration. Still, the frequency of healthy trees is low, and the effect of natural selection will depend on survival, reproductive success and the dispersal capacity of healthy trees under natural conditions.

• **Aims** We aim to test whether healthy trees contribute more to the next generation and to infer their potential for dispersing progenies across the forested landscape.

• **Methods** Using parentage modelling, we estimate mating parameters and dispersal distances of seeds and pollen and compare realised reproductive success of healthy trees to that of unhealthy ones.

• **Results** Healthy trees are overrepresented as the parents of randomly sampled seeds and seedlings in the forest, although that is more pronounced on the female side. We observe long dispersal events and estimate the mean seed and pollen dispersal distances as 67 m and 347 m, respectively.

• **Conclusion** Variation in reproductive success results in selection in favour of lowered susceptibility to ash dieback. The large dispersal capacity decreases the risk of genetic bottlenecks and inbreeding and allows resistant trees to disperse their genes into the neighbourhoods of substantial sizes.

**Keywords** Ash dieback · Enrichment planting · *Fraxinus excelsior* · Gene flow · Pollen and seed dispersal · Reproductive success

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✉ Devrim Semizer-Cuming  
devrim.semizer-cuming@forst.bwl.de

Igor Jerzy Chybicki  
igorchy@ukw.edu.pl

Reiner Finkeldey  
praesident@uni-kassel.de

Erik Dahl Kjær  
edk@ign.ku.dk

<sup>1</sup> Department of Forest Genetics and Forest Tree Breeding, Georg-August University of Göttingen, Büsgenweg 2, 37077 Göttingen, Germany

<sup>2</sup> Department of Geosciences and Natural Resource Management, University of Copenhagen, Rolighedsvej 23, 1958 Frederiksberg C, Denmark

<sup>3</sup> Department of Genetics, Institute of Experimental Biology, Kazimierz Wielki University, Chodkiewicza 30, 85-064 Bydgoszcz, Poland

<sup>4</sup> Forest Research Institute of Baden-Wuerttemberg, Wonnhaldestraße 4, 79100 Freiburg im Breisgau, Germany

<sup>5</sup> University of Kassel, Mönchebergstrasse 19, 34109 Kassel, Germany

## 1 Introduction

Ash (*Fraxinus excelsior* L.) in Europe is severely affected by ash dieback, an emerging fungal disease, caused by the ascomycete *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz and Hosoya (Baral et al. 2014; Gross et al. 2014). Dieback of shoots, twigs and branches in the crown, wilting and premature shedding of leaves, necrotic lesions on leaves and in cambium, discoloration in bark and epicormic shooting are typical symptoms of the disease (McKinney et al. 2014; Skovsgaard et al. 2017). Dieback symptoms were observed in the northwest of Poland in 1992 (Kowalski 2006), but the disease has since spread over most of the native range of the species (McKinney et al. 2014; Pautasso et al. 2013). In Denmark, symptoms were first noticed in 2002 (Thomsen and Skovsgaard 2012), but the disease was already widespread by 2005, particularly in young stands (Skovsgaard et al. 2010).

Ash dieback is currently regarded as a serious threat to European biodiversity (Pautasso et al. 2013; Marçais et al. 2017; Enderle et al. 2019). There is overwhelming evidence of the severity of the problem across Europe (reviewed in Coker et al. 2019). Based on the surveys conducted in France and Belgium in 2010, Marçais et al. (2017) reported up to 35% annual mortality in young ash stands (diameter at breast height (DBH) < 5 cm) 5–6 years after the pathogen arrival. In Lithuania, mortality rate in three different 8-year-old progeny trials reached 90% in 2010, only 5 years after their establishment in 2005 (Pliūra et al. 2011). A dramatic decline from 2009 to 2012 was reported in ash tree populations in an 8-ha-old forest patch in Estonia, where 48% of overstorey and 39% of subcanopy trees died (Lõhmus and Runnel 2014). High mortality rates were also reported in the stands in north-western (21–39%) and south-eastern (30–95%) Germany (Enderle et al. 2017; Lenz et al. 2016). In 2015, the fungus affected 50% of the ash trees (DBH ≥ 10 cm) in 22 forest observation plots in Switzerland (Queloz et al. 2017). Likewise, tree mortality rates in the Scandinavian monitoring sites have also been escalating (see Børja et al. 2017 for Norway; Bengtsson and Stenström 2017 for Sweden and McKinney et al. 2014 for Denmark).

Clonal and progeny trials of ash in Europe have revealed genetic variation in susceptibility to ash dieback with both narrow- and broad-sense heritability often reported above 0.4 (Enderle et al. 2015; Kjær et al. 2012; Lobo et al. 2014; 2015; McKinney et al. 2011; Muñoz et al. 2016; Pliūra et al. 2011; Stener 2013). This verifies that disease resistance is partially under genetic control. Assuming that the heritability of resistance is 0.5 and the frequency of resistant trees in pre-diseased ash forests is about 10%, model simulations estimated that ash populations in the

UK woodlands would still exist in 100 years but decrease to about one-third of current levels (Evans 2019). It has thus been suggested that genetic variation in resistance may prove to be a long-term solution to ash dieback if natural selection is coupled with artificial selection (McKinney et al. 2014; Evans 2019). However, the frequency of ash trees with reasonable to high levels of genetic resistance (defined as crown damage below 10%) within natural populations is low, 1–5% (Kjær et al. 2012; McKinney et al. 2014), and due to their long lifetime, no studies have yet measured the realised lifetime fitness of healthy trees with respect to their more susceptible neighbours in the surroundings (Kjær et al. 2012). The relative fitness of healthy trees can be negatively influenced if ash dieback damage triggers strong flowering among weakened trees. Our previous findings in a Danish clonal seed orchard of ash fortunately indicated a positive correlation between resistance to ash dieback and reproductive success (Semizer-Cuming et al. 2019). However, studies conducted in natural or semi-natural forests with mature trees of different size and age, spread over a heterogeneous area, have been lacking so far. The issue of fitness in natural ash populations is further complicated if the offspring of resistant trees are affected by increased inbreeding. This could be the case in the future if the majority of surviving trees in a genetically isolated area were the offspring of only a few old, resistant trees. Therefore, the recovery of ash forests will also rely on effective gene flow including seed dispersal at the forest and landscape scale (Semizer-Cuming et al. 2017). Gene flow is a complex process that incorporates biotic and abiotic interactions that affect mating, propagule production, dispersal, establishment, survival and maturation (Burczyk et al. 2004). Phenological synchrony and relative fecundity of individuals (Adams and Birkes 1991; Bochenek and Eriksen 2011; Chybicki and Burczyk 2013; Gleiser et al. 2018), propagule size and shape (Hintze et al. 2013), spatial distribution of individuals and populations (Klein et al. 2006; Robledo-Arnuncio et al. 2007), population size (Ellstrand and Elam 1993), topography of sites (Trakhenbrot et al. 2014) and atmospheric conditions (Jackson and Lyford 1999; Heydel et al. 2015) are among the factors influencing dispersal patterns of pollen and seeds. All these factors will shape the spatial genetic structure of the next generation of ash trees and thereby influence the long-term outcome of the ongoing natural selection in European ash forests.

Given the above background, the objectives of the present study are to estimate how ash dieback symptoms influence individual reproductive success and to assess seed and pollen dispersal in an ash forest suffering from ash dieback. Our hypothesis is that tolerant trees have higher reproductive success compared with damaged trees and therefore contribute

disproportionally more to the next-generation recruitments. We applied a spatially explicit mating model to determine mating parameters and dispersal kernels of pollen and seeds. Sex expressions of the adult trees were modelled explicitly in order to reflect differences in a priori contribution of trees to seed and pollen pool. Flower intensity, fruit set, crown dieback intensity, height and basal area of the adult trees were incorporated into the model estimations to examine their effects on individual reproductive success, as well as to properly account for any differences in fecundity between trees when modelling the impact of crown damage on reproductive success.

## 2 Materials and methods

### 2.1 Study site and sampling design

The study site is located in Valby Hegn, Helsingør, Denmark (56°03'17" N 12°13'52" E) (Fig. 1). The sampling was conducted in the northern part of a 362.4 ha mixed continuous forest. Ash is an important species in the area, mainly as a mosaic of several smaller stands but also as single trees scattered within dominating beech and oak stands (Fig. 1). The ash stands in the forest are of different origin and age (Semizer-Cuming et al. 2020). The two stands in the west (1.0 ha) and in the north east (0.4 ha) include ash trees planted in 1958 based on seeds from an old-Danish seed orchard ('FP202' consisting of 8 clones) (Nielsen et al. 2009). Most other mature ash trees in the surveyed area are from 1934 to 1956 and of putative Danish origin, either from natural seeding or plantings with seeds procured from other Danish ash forests. There is a single record of a very small area (<0.1 ha) planted with ash seedlings originated from Wiesbaden, Germany (unpublished records, The Nature Agency, Ostrupgaard, DK-3230 Græsted). The classical silvicultural practice for ash in the area has been planted with 2–4-year-old seedlings, but natural regeneration has also most likely been important and abundant natural regeneration is widely observed.

Bud and wood tissues were sampled from 469 reproductively mature ash trees in the stands (approximately 80% of the total number of mature ash trees in the study area). The ash trees are mainly located in the wetter areas and therefore not evenly distributed across the study area. The average density was calculated to about 20 mature ash trees per ha. The sampling of the mature trees in the 1.0-ha seed orchard offspring in the west (hereinafter W (FP202); Fig. 1) was exhaustive. Leaves from 399 naturally regenerated seedlings were sampled along two main transects stretching from the W (FP202) stand towards older

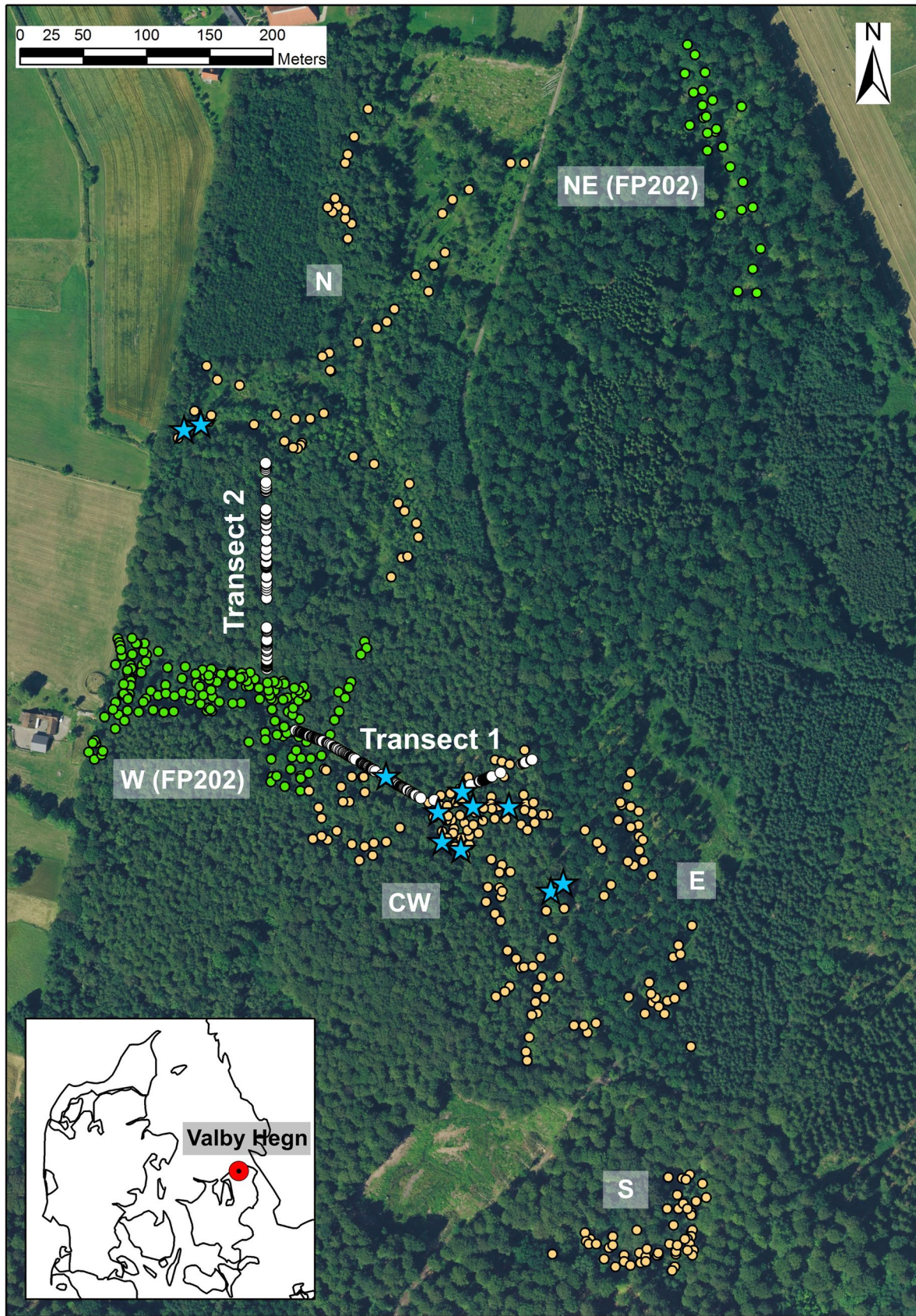
trees in the forest (Fig. 1). Transect 1 (~210 m) was placed in east–west direction whereas transect 2 (~180 m) was placed in north–south direction (Fig. 1) as they represented the recruitment. The position of the seedlings along the two transects and their heights was recorded. Their age was estimated as 1–2 years but some could be older because ash seedlings can be repeatedly browsed by wildlife. Due to the small size of the seedlings (average height = ~12 cm) and the absence of observable symptoms, we assumed that ash dieback has not caused significant mortality among the sampled plants after they germinated. Finally, seeds were collected from 11 mother trees in order to obtain precise estimates of realised pollen dispersal using seeds from known mothers (Fig. 1).

### 2.2 Characterisation of mature trees

Geographical coordinates, heights and DBH of the mature trees were recorded, and basal areas were calculated based on DBH assuming circular stems (Semizer-Cuming et al. 2020). Flowering was observed in May 2014: flower types were categorised as male, female or hermaphrodite, and flowering intensities were estimated based on a logarithmic scale, from 1 to 8, according to Kjær and Wellendorf (1997). Crown damage (in all cases identified as ash dieback symptoms) was assessed individually for each tree in the summer 2014 as percent damage score (PDS) in seven classes: class 0 = no damage, while classes from 1 to 5 represent increasing levels of damage (<10%, 10–25%, 25–50%, 50–75% and 75–100%), and class 6 represents dead trees (100% crown damage). Fruit set was also scored in the same summer as relative abundance, from 0 to 9, where 0 means absence of fruits, while 1–3 indicates low, 4–6 is medium and 7–9 represents high abundance of fruits in crowns.

### 2.3 DNA extraction and microsatellite analysis

Total DNA was extracted from leaf, bud and embryo tissues using the DNeasy® 96 Plant Kit (Cat. no. 69181) and from wood tissues using the DNeasy® Plant Mini Kit (Cat. no. 69104 and 69106) according to the manufacturer's protocol (Qiagen, Hilden, Germany). The extraction protocol for the cambium included slight modifications: (1) AP1 + RNase + polyvinylpyrrolidone (up to 10% end concentration) and (2) overnight incubation at 65 °C. Seeds (50 per mother tree) were embedded in water for 1–2 days for rehydration after their pericarp tissues were removed. Embryos were then carefully extracted to avoid endosperm or seed coat. Leaf, bud and embryo samples were ground



**Fig. 1** Map of the study site and sampling design. Adult trees (green circles, FP202 seed orchard offspring planted in 1958; orange circles, ash trees established before 1958); progenies (blue stars, mother or seed trees; white circles, seedlings collected along two main transects); bottom left, location of the study site in Denmark. The maps were created using ArcMap™ 10.4.1, Copyright © ESRI

in 96-well plates with liquid nitrogen and 3 mm stainless steel beads in TissueLyser (Qiagen), whereas wood samples were prepared separately in Eppendorf tubes. Microsatellite analyses were carried out with 13 primers including FEMSATL8, FEMSATL11 and FEMSATL19 (Lefort et al. 1999), ASH2429 (Bai et al. 2011), FRESTSSR279, FRESTSSR308, FRESTSSR427 and FRESTSSR528 (Molecular Ecology Resources Primer Development Consortium et al. 2011) and Fp12378, Fp18437, Fp20456, Fp21064 and Fp104136 (Noakes et al. 2014) in four multiplexes. The reaction mixtures were subjected to PCR conditions combining a hot start with a touchdown procedure. The details of the microsatellite analysis concerning multiplexing, PCR conditions, allele size calling and binning can be found in Semizer-Cuming et al. (2017).

## 2.4 Data analysis

Number of alleles ( $A$ ), effective number of alleles ( $A_e$ ), observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) were calculated using GenAEx 6.502 (Peakall and Smouse 2006). Allelic richness ( $A_R$ ) and private allelic richness ( $PA_R$ ) were computed using rarefaction method in HP-Rare (Kalinowski 2005), considering sampling size differences. INEST 2.2 (Chybicki and Burczyk 2009) was used for unbiased multilocus estimates of inbreeding coefficients within population ( $F_{IS}$ ). The software jointly estimated inbreeding coefficients, null allele rates and genotyping failures based on Bayesian approach at the parameter settings of 200,000 number of cycles, 2000 thinning and 20,000 burn-in.

We aimed to characterise the dispersal process as well as to test if there is any relationship between the crown damage due to ash dieback (scored as PDS; see the previous section) and the reproductive success of trees, treating female and male reproductive functions separately. For this purpose, we used the neighbourhood model (Adams and Birkes 1991; Burczyk et al. 2006), implemented in the  $NM\pi$  software version 1.2 (Chybicki 2018). The model has the advantage of taking simultaneously into account the effect of dispersal process (*dispersal kernel*) as well as the other confounding factors of reproductive success related to individual fecundity. Using a specifically designed regression analysis (Adams and Birkes 1991; Chybicki 2018), the method provides estimates of effect slopes

(known as selection gradients; Morgan and Conner 2001) for putative covariates of reproductive success. In addition, the model treats unsampled parents (mostly due to either pollen or seed immigration) as well as self-fertilisation explicitly, so that effect slope estimates are also robust to the origin of progeny, which is treated as an unknown state (except for the cases when a parent is known explicitly, as in the case of seeds collected from mother trees).

The estimation of effect slopes for female and male reproductive success requires a sample of so-called “dispersed” progeny, i.e. the progeny with unknown both parents, such as naturally regenerated seedlings (Chybicki 2018). However, due to seed and pollen dispersal, seedlings typically provide much more information about female reproductive success than male reproductive success. Therefore, in order to balance the information content for each sex, to enable robust conclusions, we took the advantage of  $NM\pi$  to treat “dispersed” and “non-dispersed” (seeds assigned to mother trees) progeny jointly in a single model. It should be stressed that seedlings represented mostly up to 2-year-old individuals, so that their survival was believed to be not affected by the pathogen, and that the estimated effect slopes reflected only differences in female and male reproductive success at the adult stage.

The following characters were included into the model as potential covariates of reproductive success: (i) flowering intensity (for male reproductive success only), (ii) fruit set (for female reproductive success only), (iii) height (both female and male function), (iv) basal area (both female and male function) and (v) PDS (both female and male function). All phenotypic characters were treated as quantitative variables and standardised prior to the estimation. Including flowering intensity, fruit set, height and basal area in addition to the main covariate of interest (PDS) was motivated by our a priori expectation that these characters are directly linked to individual fecundity and can exhibit individual variation due to factors not related to PDS. Such variation can reflect intrinsic (genetic) differences between individuals as well as differences in micro-site quality (e.g. nutrients, light, exposition to wind, etc.). Consequently, obtained effect slopes for PDS should be treated as rather conservative estimates because a fraction of the total PDS effect is likely captured by the effects of the fecundity-related covariates, especially the effect of flowering intensity and fruit set.

In the model, the gender expressions of the putative parents was incorporated as suggested in the program manual (1 denoted female, 0 described male and 0.5 indicated hermaphrodite or unknown gender, according to field observations) (see the Eq. 2a and 2b in Chybicki 2018 for more details). Incorporation of sex data allowed us to avoid potential bias in dispersal kernels and effect slopes that could result from uniform expectations of the female

and male reproductive output for true hermaphrodites and male- or female-skewed individuals. To model the seed and pollen dispersal process, we adopted the exponential-power kernel, offering enough flexibility to fit different shapes and scales of the actual probability distribution of dispersal distances (Nathan et al. 2012; Bullock et al. 2017). It should be stressed that including the distance effect into the model was necessary and also a means to account for the distance effect, which otherwise could affect the estimates of effect slopes for the covariates of reproductive success. In this way, we were able to disentangle the effect of spatial proximity of a parent and the effect of phenotypic characters. It is worth noting that the estimated dispersal kernels remain independent of the size and shape of the sample plot, providing a robust description of the dispersal process, especially when compared with empirical distributions of dispersal distances obtained through parentage reconstruction (which are strongly plot-dependent) (Nathan et al. 2012). Also, unlike empirical distribution, estimated dispersal kernels provide estimates of the true (i.e. not plot-constrained) mean dispersal distance as well as the other quantiles of dispersal kernel. Because some candidate parents in the study plot were not included in the sample, the so-called seed and pollen immigration rates should be considered as a sum of the true immigration rate and the frequency of maternity or paternity of unsampled candidates. Therefore, we focused on dispersal kernels as the main source of information about gene flow.

A backward elimination approach (Arnold 2010) was applied to select the best-fitting model, with statistically significant effect slopes for phenotypic variables (Table 7 in Appendix). The backward approach started with the full model including all phenotypic variables and successively refitted the reduced models after excluding the least significant variable, i.e. the variable for which the selection gradient was characterised by the highest  $p$  value using the likelihood ratio test (Chybicki 2018). The procedure ended when any further elimination produced a significantly worse model ( $\alpha=0.05$ ). In addition, the Akaike information criterion (AIC; Burnham and Anderson 2002) was used to compare model fit after penalizing model dimensionality.

In addition to the modelling approach, we also performed some direct comparisons as follows. Based on the best fitting model, for each progeny, the two most likely genealogies were reconstructed, and their posterior probabilities were estimated (Chybicki 2018). The inferred genealogy was considered as true when the probability was  $\geq 0.8$ . Seedling genealogies were used to determine the realised contribution of the parents to the gamete pool, specifically pollen and seed dispersal from the W (FP202) stand to the seedlings distributed along the transect lines in the neighbouring forest (cf. Fig. 1).

**Table 1** Adult population gender demographics and mean crown damage levels

Stand ID	<i>N</i>	Gender (m/h/f)	Mean PDS
NE (FP202)	11	m	30.0
	7	h	33.6
	8	f	36.2
	26		33.3
W (FP202)	55	m	33.7
	66	h	65.6
	52	f	35.9
	173		45.1
N	24	m	33.1
	26	h	63.6
	9	f	42.2
	59		46.3
CW	47	m	30.9
	55	h	54.0
	18	f	41.2
	120		42.0
E	11	m	38.6
	16	h	93.0
	10	f	54.0
	37		61.9
S	15	m	52.5
	33	h	73.8
	6	f	58.3
	54		61.7

*NE* north east, *W* west, *N* north, *CW* central west, *E* east, *S* south, *N* number of individuals, *Gender (m/h/f)* male, hermaphrodite and female genders, *Mean PDS* mean percent damage score

The adult trees in the stands ‘NE and W’ are originated from the FP202 seed orchard

The average health (PDS) of the parents of the progenies (seeds and seedlings) was calculated and compared with that of all mature trees in order to determine if the identified parent trees represent the healthier group within the mature trees. We applied a randomisation approach (999 permutations) to test whether it is likely that the observed parents (either mothers or fathers) were simply drawn from the population of all candidate parents of the given gender, regardless of their PDS values.

### 3 Results

Adult population gender demographics and mean crown damage levels (PDS) are summarised in Table 1. Except for the stand in the north east, crown damage was higher in hermaphrodites than in females and males in all stands, and

**Table 2** Genetic diversity parameters for the adult trees and progenies

	<i>N</i>	<i>A</i>	<i>A<sub>R</sub></i>	<i>PA<sub>R</sub></i>	<i>A<sub>e</sub></i>	<i>H<sub>o</sub></i>	<i>H<sub>e</sub></i>	<i>F<sub>IS</sub></i>
Adults								
NE (FP202)	26	5.923	5.880	0.210	3.486	0.418	0.515	0.174
W (FP202)	173	8.385	5.690	0.210	3.510	0.473	0.510	0.047
N	59	7.154	5.990	0.330	3.768	0.516	0.530	0.033
CW	120	7.615	5.640	0.170	3.630	0.530	0.541	0.042
E	37	5.846	5.480	0.190	3.479	0.425	0.505	0.136
S	54	6.231	5.290	0.220	3.193	0.449	0.536	0.160
Progenies								
SDL	399	8.538	5.660	0.180	3.616	0.494	0.524	0.017
SD	550	8.538	5.360	0.120	3.802	0.524	0.535	0.002

*NE* north east, *W* west, *N* north, *CW* central west, *E* east, *S* south, *SDL* seedlings, *SD* seeds, *N* number of individuals, *A* mean number of alleles, *A<sub>R</sub>* allelic richness corrected for sample size, *PA<sub>R</sub>* private allelic richness corrected for sample size, *A<sub>e</sub>* effective number of alleles, *H<sub>o</sub>* observed heterozygosity, *H<sub>e</sub>* expected heterozygosity, *F<sub>IS</sub>* mean inbreeding coefficient (Avg Fi)

The adult trees in the stands 'NE and W' are originated from the FP202 seed orchard

overall mean crown damage levels ranged between 33.3 and 61.9% (Table 1).

Genetic diversity parameters for the adult trees and progenies are given in Table 2. Of 469 adult trees, only 10 could not be genotyped at a single locus whereas no genotyping failure existed either of the progeny data set. Population genetic parameters related to allelic richness or heterozygosity showed no signs of reduction in the progenies compared with the parental generation, and the estimated inbreeding coefficients were low in the progenies compared with the parents (Table 2).

The full model incorporating all selection gradients performed better ( $AIC = 37,406.1$ ,  $p < 0.001$ ) when compared with the null model without any selection gradients ( $AIC = 37,798.9$ ) (Table 3). The backward elimination of the selection gradients starting from the full model (Table 7 in Appendix) indicated that the model without tree height and female basal area performed best ( $AIC = 37,402.5$ ; Table 3).

The mating model parameters and the standard errors computed based on the best model are given in Table 4. The estimated dispersal kernel parameters and the approximate

95% confidence interval around them are given in Table 5. According to the model estimations, the frequency of seed and pollen immigration from the surrounding sources were 24.3% and 40.4%, respectively, and there was no self-fertilisation (Table 4). Both seed and pollen dispersal kernels followed a fat-tailed distribution ( $b_s = 0.672$ ,  $b_p = 0.229$ ; Tables 4 and 5). The mean dispersal distances of seed ( $\delta_s$ ) and pollen ( $\delta_p$ ) were estimated at 67.2 m and 346.7 m, respectively (Tables 4 and 5).

The cumulative probability distributions of seed and pollen dispersals based on the best model (Fig. 2) predict that 50% of the seeds disperse within 50 m, whereas less than 5% seeds travel further than 180 m, while the likelihood for dispersal beyond 300 m is less than 1%. For pollen, the model predicts that 50% of the pollen successfully disperses up to 140 m, 5% beyond 1.3 km and 1% even further than 3 km from the source tree.

Based on the best model predicted for the study population, the observed fruit set is positively related to realised female reproductive success (0.482), and similarly, flower intensity is also positively related to realised male

**Table 3** Model comparison parameters for the null, full and the best model

Model	LogL	npar	AIC	<i>p</i> -val (LR)	FS_g	PDS_g	H_g	BA_g	FI_b	PDS_b	H_b	BA_b
Null	- 18,880.5	19	37,798.9	-	0	0	0	0	0	0	0	0
Full	- 18,676.1	27	37,406.1	2.43E-83*	1	1	1	1	1	1	1	1
Best	- 18,677.2	24	37,402.5	0.497**	1	1	0	0	1	1	0	1

*LogL* log-likelihood, *npar* number of parameters, *AIC* Akaike information criterion, *p*-val (LR) *p* value of the likelihood ratio test; selection gradient series: *FI* flower intensity, *FS* fruit set, *PDS* percent damage score, *H* height, *BA* basal area, *g* (*gamma*) female selection gradient, *b* (*beta*) male selection gradient, *I/O* included/excluded selection gradient

\**p* value of the LR test for the difference in fit between the null and full model

\*\**p* value of the LR test for the difference in fit between the full and the best model according to the backward elimination procedure

**Table 4** Mating model parameters based on the best model

Parameter	s	ms	mp	$1/\delta_s$	$1/\delta_p$	$b_s$	$b_p$
Estimate	0	0.243	0.404	0.015	0.003	0.672	0.229
Std. Error	-	0.0259	0.0196	0.0020	0.0016	0.1086	0.0522

$s$  probability of self-fertilisation,  $ms$  probability of seed immigration,  $mp$  probability of pollen immigration,  $\delta_s$  mean distance of seed dispersal (m),  $\delta_p$  mean distance of pollen dispersal (m),  $b_s$  shape of seed dispersal kernel,  $b_p$  shape of pollen dispersal kernel

reproductive success (1.246) (Table 6). The relationship between crown damage (PDS) and reproductive success is negative for both female ( $-0.674$ ) and male ( $-0.238$ ), i.e. the healthiest trees (low PDS) have the highest likelihood of contributing gametes to the next generation. Tree size (BA) is negatively related to male reproductive success ( $-0.361$ ). All of the estimates were statistically significant ( $p < 0.05$ ).

Based on the best-fitting model, 180 (45%) most likely maternal and 285 (52%) most likely paternal genealogies were, respectively, inferred for the seedlings and seeds, which possessed at least the minimum threshold probability of 0.8. The signs of gene flow from the W (FP202) stand could be observed in the surrounding forest. The maternal profile of the seedlings along the two transects (Fig. 1) showed that 39% of the seedlings that were successfully assigned parentage originated from the seeds dispersed from the trees in the W (FP202) stand, while 61% of them came from the other trees in the area. The paternal contribution from the W (FP202) stand to the seedlings with assigned parentage was quite similar (31%), whereas the other trees in the area sired 69% of the seedlings. These are the average figures for the two transects, but there was a clear pattern where the contribution from the trees in the W (FP202) stand decreased rapidly with the distance from the stand, as would be expected (Fig. 3). A total of 7% of the seeds collected from the trees 100–200 m east from the border of the W (FP202) stand (see Fig. 1) were sired by the trees in the W (FP202) stand.

**Table 5** Dispersal kernel parameters estimated at 95% confidence interval ( $Q$ )

Parameter	Estimate	$Q$ (2.5%)	$Q$ (97.5%)
$\delta_s$	66.6	54.1	86.7
$b_s$	0.672	0.407	0.938
$\delta_p$	346.7	154.1	ND
$b_p$	0.229	0.101	0.357

$\delta_s$  mean of the seed dispersal kernel (m),  $b_s$  shape parameter of the seed dispersal kernel,  $\delta_p$  mean of the pollen dispersal kernel (m),  $b_p$  shape parameter of the pollen dispersal kernel, ND not determined

The mean crown damage level (i.e. mean PDS) of the assigned fathers of the seedlings was only 26% compared with a general damage level of 51% among the assessed males in the population (Fig. 4a, b). The difference was similarly large for the female trees, where the assigned mothers of the seedlings showed an average damage level of 25%, again much lower than the general damage level of 57% among females in the population (Fig. 4c, d). Based on the randomisation test, the differences between the groups were highly significant ( $p < 0.001$ ).

### 3.1 Discussion

The most important finding in our study is that crown damage due to ash dieback reduces individual reproductive success. Although this is in line with our previous findings in a Danish seed orchard of ash (Semizer-Cuming et al. 2019), here we showed for the first time that naturally recruited ash seedlings tend to have seed and pollen parents among healthy trees. Additionally, as in the seed orchard study, the intensity of the effect appeared to be stronger in females ( $-0.674$ ) than males ( $-0.238$ ). The replicated observation of difference between sexes strongly suggests that ash dieback influences male and female reproductive functions differently. This may result from intrinsic differences between maternal and paternal investments because the cost of reproduction is usually higher for

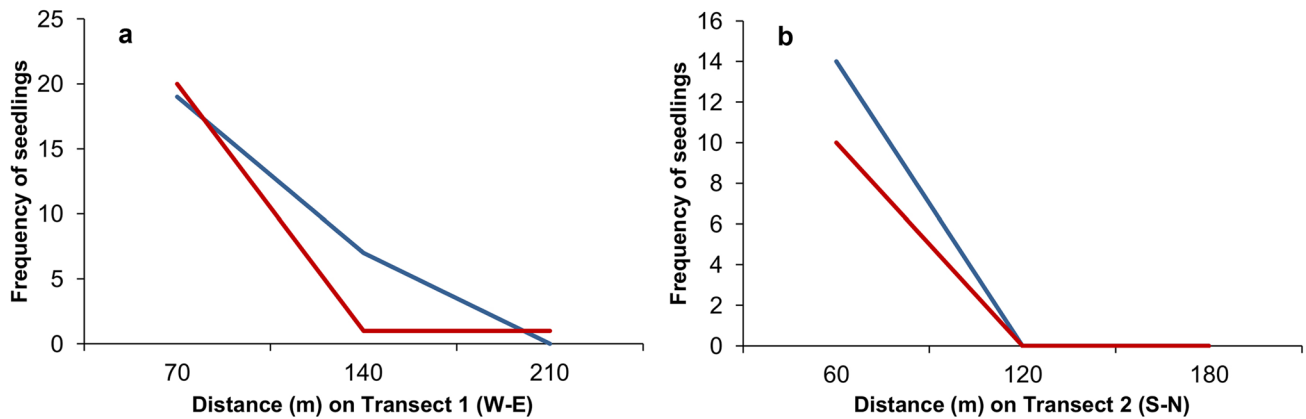
**Table 6** Selection gradients for the phenotypic variables of individual reproductive success within the study population

	FS	FI	PDS		BA
	Female	Male	Female	Male	Male
Estimate	0.482*	1.246*	$-0.674^*$	$-0.238^*$	$-0.361^*$
Std. error	0.064	0.142	0.114	0.088	0.104

FS fruit set, FI flower intensity, PDS percent damage score, BA basal area

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.005$





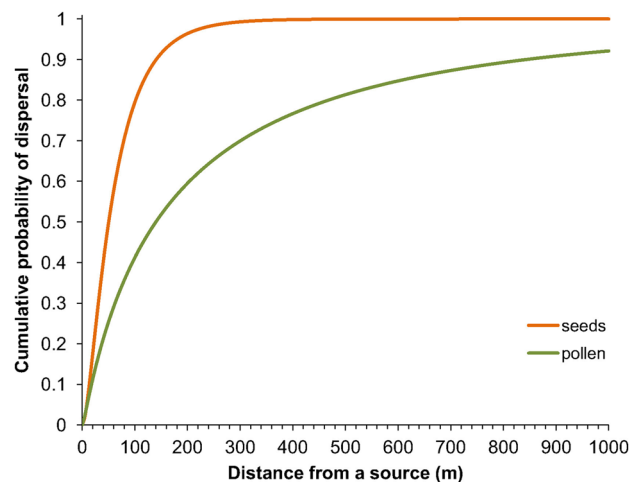
**Fig. 2** Gamete dispersal curves along transects 1 (a) and 2 (b) from the W (FP202) stand into the surrounding ash populations. Blue lines, both parents are from the planted stand; red lines, one parent is from the planted stand

females than for males (Obeso 2002). If resources are limited due to crown damage and reproduction is much costlier for females, unhealthy females cannot produce enough mature seeds while some of their male counterparts can still produce enough viable pollen to reproduce. Stress conditions can occasionally enhance flowering as an emergency response in order to produce the next generation (Wada and Takeno 2010), and biotic stress caused by *H. fraxineus* could lead ash individuals to compromise between reproduction and defence response (Denancé et al. 2013). Since dieback tolerance is partly inherited, increased flowering of unhealthy trees could, in theory, slow down the process of natural selection. However, our study points towards a substantial positive correlation between health and reproductive success. It is worth noting that the regression model allows variation in flowering intensity and fruit set to explain variation in reproductive success. Both variables are likely to capture a fraction of the total effect of pathogen, and the net effect of pathogen on reproductive success can therefore be even stronger. With this kept in mind, our results show that variation in reproductive success can increase the speed of natural selection in favour of tolerant ash trees, because they have not only lower risk of mortality but also higher chance of producing offspring (most pronounced on the female side).

The assessment of selection based on the paternity analysis of seeds only reflects variation in male reproductive fitness, while estimates of variation in reproductive success based on seedlings may include a minor effect of early selection created by mortality due to ash dieback. However, we expect that the parentage

analysis of the seedlings mainly reflects reproductive variation among mature trees because the young seedlings had no observable disease symptoms when they were sampled.

Our study also revealed considerable seed and pollen dispersal in the relatively dense stand. Pure ash forests in Denmark between 50 and 100 years typically contain 100–200 mature trees per ha with an average distance between the nearest trees of approximately 7–10 m. However, the distance between very healthy mates would easily become 50–100 m with only 2% of highly resistant trees. Estimated dispersal kernels, as well as the results of previous studies (Bacles et al. 2005;



**Fig. 3** Cumulative probability distributions of seed and pollen dispersal

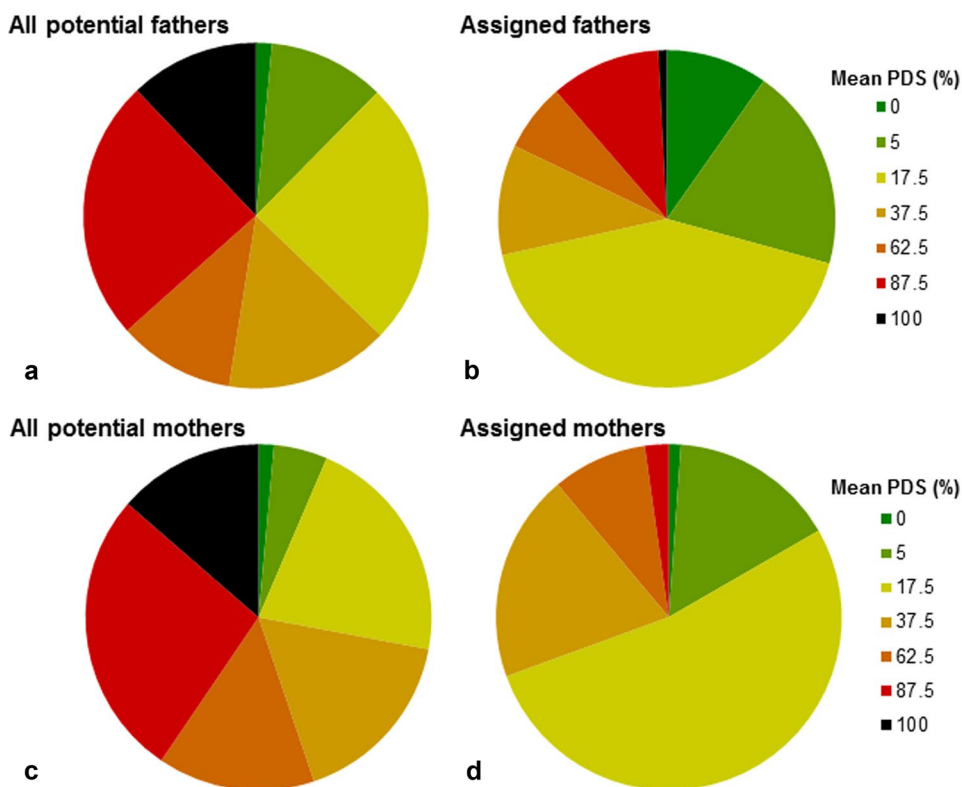
Bacles and Ennos 2008; Semizer-Cuming et al. 2017), suggest that such trees would still be able to mate and be highly successful in transmitting their genes to the next generation compared with unhealthy trees. Our estimated seed dispersal distances (average 67 m) show that 2 to 4 healthy trees per ha will probably be able to disperse seeds onto the forest floor among them. This fits well with our population genetic analyses that revealed no indication of reduced diversity or increased inbreeding in the seedlings compared with the mature trees in the studied forest. However, in mixed stands with a lower frequency of ash trees, distances between putatively resistant trees can be larger than the estimated pollen dispersal distances, and especially, dispersal into suitable microhabitats via seeds may become a limiting factor, at least within a single generation. More studies are required to analyse these dispersal dynamics in such forests.

On a larger spatial scale, pollen and seed dispersal distances are important to prevent the risk of population fragmentation in future landscapes in the absence of long-distance dispersal (LDD). Long-distance seed dispersal is vital for species' colonisation at suitable sites in managed and unmanaged forests and open landscapes (Cain et al. 2000; Kramer et al. 2008). We did not specifically examine LDD in the present study,

but the cumulative probability distributions of dispersal distances suggest that such events are likely in the species (Fig. 3). A study of a severely fragmented *F. excelsior* population in Scotland estimated the mean pollen dispersal distance of 328 m (Bacles et al. 2005) and up to 1.9 km (Bacles and Ennos 2008) with 8% of the effective seed dispersal originating from dispersals between 100 m and 3 km (Bacles et al. 2006). In the fragmented landscape of Rösenbeck in Germany, we have previously estimated that the mean dispersal distance of *F. excelsior* pollen was even greater (571 m), with a proportion of pollen most likely representing LDD events (Semizer-Cuming et al. 2017). A shorter average dispersal distance of pollen (197 m) was estimated in Japanese *F. mandshurica* located in a forest reserve in Hokkaido (Goto et al. 2006). Altogether, the results show that though the majority of the dispersal events in ash occurs in short distances, rare LDD over hundreds and thousands of meters, in particular of pollen, may still keep fragmented ash populations connected with each other in future landscapes.

We observed that healthy trees were overrepresented as parents for the next generation. The assigned parents on average showed half the level of crown damage compared with that of the general damage level in the study population. The implication is that the next

**Fig. 4** Mean crown damage levels (PDS) of the male and female trees in the study population: PDS of **a** all potential fathers *versus* **b** assigned fathers of the seedlings and **c** all potential mothers *versus* **d** assigned mothers of the seedlings



generation will have reduced susceptibility to ash dieback because the trait is known to be partly heritable, as discussed in the introduction. However, estimates of narrow sense heritability have so far been based on the studies of half-sib families grown in plantations. Lobo et al. (2015) applied parent-offspring regression to verify that resistance to ash dieback is inherited but still based on planted offspring grown under field trial conditions. Wohlmuth et al. (2018) studied parent-offspring correlation in damage level under in situ conditions in Austria but did not find significant narrow-sense heritability. However, data from Danish breeding activities revealed that crown damage levels of old mature trees were significantly correlated with their susceptibility levels to ash dieback when tested in a clonal trial (Kjær, personal communication). This supports that healthy mature trees in our study site most likely represent the trees with improved level of genetic resistance. The fact that offspring tend to have parents among healthy trees is very positive because the current frequency of trees with very high level of resistance is generally low in native ash forests. And we do expect that selection in favour of offspring from healthy parents will continue as the seedlings grow older (zygotic selection) because several trials have revealed higher mortality in susceptible families (Kjær et al. 2012; Lobo et al. 2014; Muñoz et al. 2016).

Finally, our findings suggest that plantations with resistant ash trees can modify the gene pool of existing forests not only by replacing them but also by exchanging genes. Gene flow from commercial ash plantings into old-growth forests may have negative implications for gene conservation, including the risk of lost genetic diversity and patterns of local adaptation (Semizer-Cuming et al. 2017). However, in the face of ash dieback, gene flow from new plantations into the existing native forests can become an important process, based on the idea of enrichment planting. Genetic enrichment with improved resistance can be a way to enrich the otherwise rapidly decreasing fitness of native ash populations and thereby restore their ability to compete with other species, while partly preserving existing genetic patterns of local adaptation. Our Fig. 4 shows that the trees with crown damage levels between 10 and 25% (i.e. mean PDS of 17.5%) still maintain a relatively high reproductive success. From an evolutionary perspective, this suggests that the reproductive success of a tree (in its overall life-span) is kept even at 25% of crown damage. Contribution from such trees to next generation will probably reduce the risks of genetic bottlenecks and loss of gene diversity that might be crucial for future adaptive processes, because completely healthy trees are often rare in damaged ash forests. However,

their contribution can also reduce the efficiency of selection in favour of completely healthy ash trees and thereby slow down the build-up of resistance to ash dieback.

We expect that an enrichment plantation, once mature, is likely to create a zone of few hundred meters next to its borders, where a reasonable frequency of next-generation seedlings with one or two parents from the enrichment trees, while a larger zone will contain seedlings with one of the parents originating from the enrichment trees. Thomasset et al. (2014) raised the conservation concern that the dispersal of genes from planted into native populations may decrease local adaptation. It is important that future planted European ash forests remain adapted to local growth conditions. This speaks in favour of developing ash enrichment seed sources based on the multiple population concept (cf. Eriksson et al. 1993) in order to encompass large variation in ecological conditions within the natural distribution of common ash in Europe. This fits well with tree improvement and selection programs initiated simultaneously in many European countries, thereby covering many different eco-regions.

## 4 Conclusion

Our results predict that the next generation of ash trees from natural seed fall will have improved levels of tolerance to ash dieback compared with their parental generation due to the interplay between sexual and zygotic selection. This is promising for the future health of the species in European forests, although we still cannot judge if gained natural resistance will be sufficient to allow the ash trees to maintain their ecological niche in the long run. Our results further suggest that natural recovery can be enriched by plantings with seeds from ash genotypes selected and tested in seed orchards for high level of disease tolerance, because healthy trees in such plantings are expected to spread their seeds and pollen several hundred meters into the surrounding ash stands in managed forests. Human intervention may thus prove a valuable tool to ensure the future existence of ash in European forests and ecosystems when based on sound genetic principles, but our results also provide hope for the natural recovery of protected forests without intervention. It is important to follow the dynamics in the present and other survey areas in the years to come to reveal if and how zygotic selection until maturity can continuously increase the representation of offspring from healthy parents and to provide in situ heritability estimates relevant to natural selection under forest conditions.

## Appendix

**Table 7** Backward elimination of the selection gradients

Step	Model	LogL	df	AIC	FS_g	PDS_g	H_g	BA_g	FI_b	PDS_b	H_b	BA_b	<i>p</i> value
0	M0	-18,676.1	27	37,406.10	1	1	1	1	1	1	1	1	NA
1	M1	-18,704.0	26	37,460.04	0	1	1	1	1	1	1	1	7.47E-14
1	M2	-18,694.6	26	37,441.14	1	0	1	1	1	1	1	1	1.16E-09
1	M3	-18,676.1	26	37,404.16	1	1	0	1	1	1	1	1	0.806496
1	M4	-18,676.1	26	37,404.12	1	1	1	0	1	1	1	1	0.887537
1	M5	-18,727.4	26	37,506.84	1	1	1	1	0	1	1	1	3.82E-24
1	M6	-18,678.9	26	37,409.70	1	1	1	1	1	0	1	1	0.017960
1	M7	-18,677.1	26	37,406.20	1	1	1	1	1	1	0	1	0.147299
1	M8	-18,683.6	26	37,419.10	1	1	1	1	1	1	1	0	0.000108
2	M9	-18,705.5	25	37,461.08	0	1	1	0	1	1	1	1	1.61E-14
2	M10	-18,694.8	25	37,439.58	1	0	1	0	1	1	1	1	9.33E-10
2	M11	-18,676.2	25	37,402.34	1	1	0	0	1	1	1	1	0.639040
2	M12	-18,727.5	25	37,504.90	1	1	1	0	0	1	1	1	3.75E-24
2	M13	-18,678.9	25	37,407.70	1	1	1	0	1	0	1	1	0.018167
2	M14	-18,677.1	25	37,404.22	1	1	1	0	1	1	0	1	0.147299
2	M15	-18,683.6	25	37,417.10	1	1	1	0	1	1	1	0	0.000109
3	M16	-18,707.9	24	37,463.88	0	1	0	0	1	1	1	1	1.57E-15
3	M17	-18,697.1	24	37,442.12	1	0	0	0	1	1	1	1	1.02E-10
3	M18	-18,727.6	24	37,503.18	1	1	0	0	0	1	1	1	3.63E-24
3	M19	-18,679.0	24	37,405.94	1	1	0	0	1	0	1	1	0.017960
3	M20	-18,677.2	24	37,402.48	1	1	0	0	1	1	0	1	0.143502
3	M21	-18,683.7	24	37,415.36	1	1	0	0	1	1	1	0	0.000106
4	M22	-18,709.0	23	37,464.04	0	1	0	0	1	1	0	1	1.56E-15
4	M23	-18,698.4	23	37,442.72	1	0	0	0	1	1	0	1	8.07E-11
4	M24	-18,731.5	23	37,508.96	1	1	0	0	0	1	0	1	2.11E-25
4	M25	-18,681.1	23	37,408.16	1	1	0	0	1	0	0	1	0.005584
4	M26	-18,684.2	23	37,414.48	1	1	0	0	1	1	0	0	0.000183

*LogL* log-likelihood, *df* degrees of freedom, *AIC* Akaike information criterion, *FS* fruit set, *FI* flower intensity, *PDS* percent damage score, *H* height, *BA* basal area, *g* (*gamma*) female selection gradient, *b* (*beta*) male selection gradient, 1/0, included/excluded, *NA* not applicable

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**Data Availability** The dataset generated and analysed during the current study is available in the University of Copenhagen Electronic Research Data Archive (ERDA). <https://doi.org/10.17894/ucph.c39ad0ed-5ef2-43bf-8d06-f94870962f1c>

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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