Gene flow from planted common ash (*Fraxinus excelsior* L.) stands to old-growth forests – implications for conservation and use of genetic resources affected by ash dieback

Dissertation

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"Life is either a daring adventure or nothing at all."

— *Helen Keller, The Open Door*

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PREFACE

This PhD thesis studies gene flow and mating patterns in common ash (*Fraxinus excelsior*) in order to understand the effects of habitat fragmentation, plantations, and ash dieback. It aims also to provide guidance for the use and conservation of ash genetic resources confronted with ash dieback.

The fieldwork was conducted in three sites in two countries: 'Rösenbeck' in Germany, and 'Valby Hegn' and 'Tuse Næs' in Denmark. The research conducted in these study sites founded the basis for three scientific papers. Understanding the influences of fragmentation and urban plantations on native old-growth ash was the main focus of the study conducted in Rösenbeck. The ash trees in the study sites in Denmark were severely affected by ash dieback, which gave the opportunity to investigate the effect of the disease on individual reproductive success. The Valby Hegn study benefited from the presence of a plantation from a known origin. This allowed the studying of gene flow in the surrounding forest and to consider enrichment plantings as an option for improving the fitness of future ash forests. As a clonal seed orchard, Tuse Næs was a suitable site to determine the influence of ash dieback susceptibility on male and female reproductive success.

The thesis starts with the background and objectives, followed by the general methods used in the study, summary of the results in a paper based format, conclusions, and future perspectives. The following chapters are the scientific papers produced during the study period. The thesis ends with a synopsis written in three languages, English, German and Danish.

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1   Background and objectives

1.1   Common ash (*Fraxinus excelsior* L.)

Natural distribution and ecology

A deciduous tree species, common ash (*Fraxinus excelsior*, hereinafter ash), belongs to genus *Fraxinus*, which is one of the 24 extant genera of Oleaceae and comprises 43 species occurring in temperate and subtropical regions of the northern hemisphere (Wallander 2008). It is one of the most widely distributed ash species in Europe, extending from the Atlantic coast to the Volga River and from Norway to Northern Spain, Italy, Greece and Iran (FRAXIGEN 2005; Figure 1).
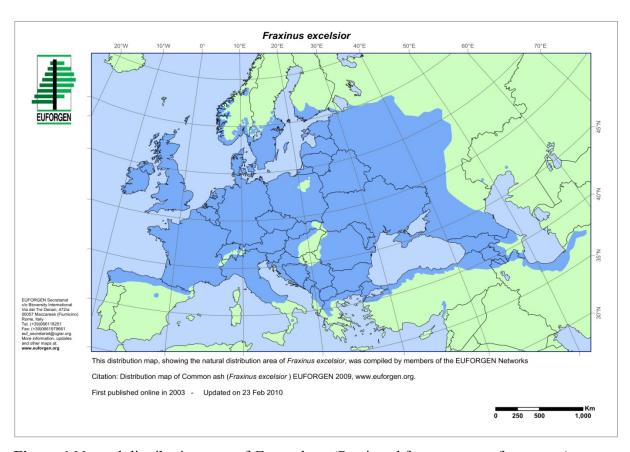
Figure 1 Natural distribution map of *F. excelsior* (Retrieved from: www.euforgen.org)

Ash is intermediate between a pioneer species and a permanent forest component, and its competition ability is strong when the ecological requirements are met (Pliūra & Heuertz 2010). The species often occurs in mixed broadleaved forests, mostly in a group admixture with oak, beech and alder (Dobrowolska et al. 2008). Although ash trees can thrive in various site types, it prefers dry, shallow calcareous, and moist, fertile, non-acidic alluvial soil types (Skovsgaard et al. 2017). It can tolerate soils with a pH as low as 4.5 and seasonal

waterlogging, and is generally cold hardy when dormant (Pliūra & Heuertz 2010) but sensitive to drought (Dobrowolska et al. 2008). Climate has less influence on the growth of ash compared to soil; therefore, it can grow well under a wide range of climatic conditions as long as the soil is suitable (FRAXIGEN 2005).

Ecological and economical significance

Ash stands out as a keystone species in European forest ecosystems (European Commission 2007). A total of 953 species, including 44 obligate and 62 highly associated, are associated with ash (Mitchell et al. 2014). The species is important in both primary and secondary succession (Dobrowolska et al. 2011). As a hardwood species in European forest ecosystems and landscapes, ash provides timber and supports landscape aesthetics, ecosystem services, and biodiversity (FRAXIGEN 2005, Dobrowolska et al. 2011; Skovsgaard et al. 2017). It is one of the most economically important tree species in Europe (Dobrowolska et al. 2008) due to its wood, which is strong, durable, resilient and easily bent; making it particularly suitable for furniture, house interiors and sports equipment (Pliūra & Heuertz 2003). Since large radial increments do not compromise good mechanical properties, it is possible to grow valuable timber in short production time with large tree rings (Dobrowolska et al. 2008). Chemical compounds in ash bark and leaf have medicinal benefits (Pliūra & Heuertz 2003), and leaves are nutritious as animal feed (FRAXIGEN 2005).

Reproductive biology

Ash is a wind-pollinated tree with small, simple flowers lacking nectar, scent and petals (FRAXIGEN 2005). Leaf flush occurs after flower formation in spring. Fruits, distally winged samaras, are wind dispersed (Tapper 1996). For northern and central parts of Europe, the pollination period is within April–May (d'Amato et al. 1988). Following a maturation period between October and February, most of the seeds disperse (Schütt et al. 1995), but some may stay on the trees until the following summer (Tapper 1992). Seeds experience dormancy and therefore do not germinate until the second year after maturation (Binggeli & Power 1991). Trees start fruiting regularly at about 20 years of age, but masting shows irregularity among years (Bacles et al. 2005). Fruiting phenology varies depending on latitude, altitude and temperature (FRAXIGEN 2005).

Ash has a polygamous reproductive system and consists of phenotypes including purely male, purely female and a range of intermediate hermaphrodites (Binggeli & Power 1999;

Wallander 2001), although it functions as dioecious (Wallander 2008). Hermaphrodites are self-fertile but less successful as fathers compared to males (Bochenek & Eriksen 2011).

1.2 Ash dieback: an emerging fungal disease

Emerging infectious diseases (EIDs) are infectious diseases caused by pathogens, which have been newly evolved or recognized, or have increased in occurrence, range, and severity (Anderson et al. 2004). EIDs are a serious threat to biodiversity and a major challenge for species conservation. They can cause high mortality in their host species and even drive them to extinction (Anderson et al. 2004), because there exists no host-pathogen co-evolution. International trade and transport are responsible for globalization of pathogens and thus for an increasing number of EIDs year by year (Fisher et al. 2012). Among them, fungal and fungal-like pathogens are known to be the main threats to forest trees (Santini et al. 2013). Ash dieback is an emerging fungal disease, severely threatening not only the species itself but also biodiversity in continental Europe. Marçais et al. (2017) stated that ash dieback is one of the most serious health issues that European forests have faced in the last decade.

Aetiology, symptoms and spread

The ascomycete *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz and Hosoya – previously known as *H. pseudoalbidus* and *Chalara fraxinea*– is the primary causal agent of ash dieback (Baral et al. 2014; Gross et al. 2014). There is genetic support that this pathogen originates from East Asia (Zhao et al. 2013; Zheng & Zhuang 2014). Cleary et al. (2016) also reported the presence of *H. fraxineus* on healthy, asymptomatic Asian *Fraxinus* species, Manchurian ash (*F. mandshurica*). Infected trees show a range of symptoms, including dieback of woody tissues and premature leaf shedding in the crown, necrosis and discoloration on leaves and bark, and epicormic shooting (McKinney et al. 2014). The disease was first noticed in North-western Poland in 1992 (Kowalski 2006) and spread all over Europe within a decade (McKinney et al. 2014; Pautasso et al. 2013). Ash dieback symptoms were first noted both in Germany and Denmark in 2002 and soon after became widespread (Schumacher 2011; Skovsgaard et al. 2010; Thomsen & Skovsgaard 2012).

Disease development and tree mortality

Even though surveys on disease development and mortality in sites are still in progress, previous studies have already documented the varying intensity of ash dieback across Europe. For example, according to a survey conducted in 8 monitoring plots in Norway, 51.7 % of the

ash trees were dead in 2015 compared to 2009 (Børja et al. 2017). Also, the rate of tree mortality increased from 5 % to 70 % between 2006 and 2013 in a Danish field trial (McKinney et al. 2014). In Sweden, the mortality rate of veteran trees in a monitoring site was 11 % in 2015 (Bengtsson & Stenström 2017). In France and Belgium, annual mortality was higher (35 %) in young ash stands compared to old ones (3.2–11 %), reported in 2010 by Marçais et al. (2017). Similarly, Lenz et al. (2016) reported a higher frequency of tree mortality in young stands (95 %) and in pole stands (78 %) relative to matured forests (30 %) in southeastern Germany between 2010 and 2014. Enderle et al. (2017) found the mortality rates in the German ash stands of various ages to be between 1.5 % and 39 % in 2015. The frequency of symptomatic ash escalated from 13 % to 88 % in 2007–2011 in a provenance trial in southwestern Germany (cited in McKinney et al. 2014). Based on the surveys performed between 2008 and 2010 in Austria, the average intensity of ash dieback in mature ash trees was 5 % (Kessler et al. 2012, cited in Kirisits & Freinschlag 2012). The fungus had already infected 50 % of the trees in 22 forest monitoring plots in Switzerland in 2015 (Queloz et al. 2017). Pliūra et al. (2011) reported a 90 % mortality rate in 2010 in three 8-year old progeny trials in Lithuania, whereas Pliūra et al. (2017) reported lower disease intensity (56.8 %) in 2008 in 25 monitoring plots in Lithuania with ash trees between 40 and 90 years of age. From 2011 to 2015, a 69 % rise was observed in disease intensity in 6 different regions in Ukraine (Davydenko & Meshkova 2017). In Estonia, 48 % of the overstory and 39 % of the subcanopy trees died in an 8 ha forest fragment between 2009 and 2012 (Lõhmus & Runnel 2014).

Artificial selection for disease tolerance

Previous studies conducted in clonal ash stands have reported that variation in dieback susceptibility among ash trees is at high levels (Cleary et al. 2014; McKinney et al. 2010; Stener 2013). Quantitative genetic studies, performed in progeny trials, estimated high levels of narrow sense heritability in dieback tolerance (Kjaer et al. 2012; Lobo et al. 2014; Muñoz et al. 2016; Pliūra et al. 2011). This means that disease resistance is genetically controlled and therefore can be inherited from parents to offspring. McKinney et al. (2014) suggested that this might provide a long-term solution to ash dieback based on natural selection assisted by artificial selection. However, in order to estimate the likelihood of the recovery of ash in European forests, the relationship between ash dieback and reproductive fitness should be identified by studying gender, health, parentages and dispersal capacities of seed and pollen.

Many countries in Europe have already started their artificial selection programmes to obtain tolerant ash genetic material: e.g. Austria (Heinze et al. 2017), Belgium (Sioen et al. 2017), Czech Republic (Rozsypálek et al. 2017), Denmark (Kjær et al. 2017), Germany (Enderle et al. 2017), Great Britain (Clark & Webber 2017), Ireland (McCracken et al. 2017), Lithuania (Pliūra et al. 2017), Poland (Gil et al. 2017), Slovakia (Longauerová et al. 2017), Sweden (Cleary et al. 2017), Switzerland (Queloz et al. 2017) and the Netherlands (De Vries & Kopinga 2017). These programmes aim to replace unhealthy ash forests by establishing new plantings with selected healthy material. However, as an alternative, existing unhealthy ash forests may be enriched with healthy material, if they are intermixed with each other. This may facilitate gene exchange and allow the spread of resistance genes against the disease. In order to evaluate enrichment plantings as an option, the extent of gene flow from plantations to surrounding forests should be studied.

1.3   Gene flow

Importance, risks and benefits

Gene flow is one of the main factors determining the genetic architecture of populations (Burczyk et al. 2004). It is a critical factor for the spatial distribution of genetic variation, since high gene flow homogenizes genetic structures, whereas low gene flow allows non-random distribution of alleles and genotypes (Stefenon et al. 2008). Long distance dispersal (LDD) is critical to many aspects of plant biology, including population dynamics, diversity, evolution and biological invasions (Cain et al. 2000). Understanding the extent and patterns of gene flow is fundamental to predicting the responses of plant populations to ecosystem disturbances, such as landscape change, climate change and EIDs.

Habitat fragmentation may result in small population sizes over generations, leading to a decrease in genetic variation within populations and an increase in genetic differentiation among populations, because of increased genetic drift, inbreeding, and limited gene flow (Young et al. 1996). On the other hand, in some species, it can also aid gene flow among fragments (e.g. Bacles et al. 2006; White et al. 2002). Keeping fragments connected while avoiding unwanted gene flow is important for gene conservation (Adams & Burczyk 2000; DiFazio et al. 2012; Sork & Smouse 2006).

Local adaptation results from the balance between selection and gene flow, when populations exchange genes (Savolainen et al. 2013). In case of cultivation of large-scale plantations close to interfertile native trees, gene flow into natural stands may have negative impacts; i.e. it may potentially reduce diversity and adaptability of future generations (Adams

& Burczyk 2000). On the contrary, gene flow from plantations may also impede genetic erosion of small native populations by recovering genetic variation and lowering inbreeding depression (Ingvarsson 2001; Richards 2000). Nonetheless, gene flow from plantations to native forests is not well studied in many tree species.

EIDs may also cause loss of genetic diversity and local adaptive patterns in their host species through high mortality (Fisher et al. 2012). Gene flow among survivors may facilitate the establishment of healthier future populations and therefore the recovery of the species.

Factors affecting gene flow

In plants, the process of gene flow is perplexing, because it incorporates biotic and abiotic interactions affecting mating, propagule production, dispersal, establishment, survival and maturation (Burczyk et al. 2004). There are many factors influencing dispersal patterns of seeds and pollen, such as phenological synchrony and relative fecundity of individuals (Adams & Burczyk 2000; Bochenek & Eriksen 2011), propagule size and shape (Hintze et al. 2013), spatial distribution of individuals and populations (Klein et al., 2006; Robledo-Arnuncio & Gil 2005; Robledo-Arnuncio & García 2007), population size (Ellstrand & Elam 1993), topography of sites (Trakhtenbrot et al. 2014) and atmospheric conditions (Heydel et al. 2015; Jackson & Lyford 1999).

Particularly, wind conditions are expected to play an important role in shaping seed and pollen dispersals in wind dispersed species. For example, dry and windy weather has been repeatedly reported as a facilitator of LDD of seeds (Greene 2005; Heydel et al. 2015; Howe & Smallwood 1982; Schippers & Jongejans 2005), while clear and warm weather with low relative humidity has been stated to aid pollen release (Whitehead 1983; Curtis & Lersten 1995; Jackson & Lyford 1999). Weather data can therefore be useful to understand dispersal patterns in a particular landscape (Kremer et al. 2012).

Estimation of gene flow

Estimating seed and pollen dispersal is necessary in order to anticipate the response of species to ecosystem disturbances. Species' dispersal capacities can give an idea about their recovery potentials. Parentage assignment methods allow for tracking of seed and pollen dispersal in terms of distance and directionality (García et al. 2007).

Categorical approaches to parentage analysis (reviewed in Jones et al. 2010) can confidently assign candidate parents to their progenies using genetic information and enable to identify the distances and directions of realized dispersals afterwards. Paternity analysis is

particularly useful to assign candidate fathers to the offspring with known mothers, while parent pair analysis is used to assign both seed and pollen parents to offspring. However, assignment rates are fully dependent on the power of microsatellite markers and sampling scheme in study sites. On the other hand, the neighbourhood model (Adams and Birkes 1991; Burczyk et al. 2002; 2006) is less biased, because it estimates mating model parameters incorporating spatial distributions and phenotypic characters of trees, and infers parentages after parameter fitting. The model allows the assessment of gene flow potential of species and is particularly useful when individual reproductive success is of concern. Combining these two approaches can improve gene flow estimations.

Reliable estimation of dispersal distances and directions depends on the identification of maternal and paternal trees. In the case of seeds, when sampled from known mothers, pollen dispersal distances and directions can easily be determined. In the case of seedlings, however, knowing the genders of both parents is necessary. One approach to this problem is to assume that the nearest parent is the mother, but such a *priori* assumption is potentially misguiding (Ashley 2010). The other solution is to use uniparentally inherited cytoplasmic markers (e.g. Beatty et al. 2015), however, in angiosperms polymorphism levels at regional scales are generally low (Heuertz et al. 2004). Alternatively, repeated observation of flowers can provide reliable information on gender.

Previous studies on gene flow of ash

In general, the patterns of gene flow are poorly understood in both fragmented and non-fragmented forest ecosystems (Kramer et al. 2008). Using different approaches to parentage analysis, only a few gene flow studies have been conducted so far in *F. excelsior*. The neighbourhood model estimated 53 % of pollen immigration from outside of a 3 km radius in remnant ash populations in Scotland with a mean dispersal distance of 328 m (Bacles et al. 2005). In the same study site, the maximum observed seed dispersal distance was 1.4 km and at least 46 % of seed-mediated gene flow was estimated to occur from outside of the sampling area (Bacles et al. 2006). Based on the categorical approach, Thomasset et al. (2014) studied pollen mediated gene flow among native and non-native *F. excelsior*, *F. angustifolia* and hybrid ash (*F. excelsior* x *F. angustifolia*) populations in Ireland and detected more than 30 % of pollen coming beyond 400 m. Beatty et al. (2015) reported a lack of population genetic structure, and frequent seed dispersal over 100 m in natural and semi-natural ash populations in Ireland. However, gene flow patterns are highly affected by intrinsic and extrinsic factors as well as the method of analysis and thus vary among populations. For this reason, it is

important to better understand these patterns in different ash populations, particularly which are highly threatened by habitat fragmentation, plantations and ash dieback.

1.4 Objectives

The overall objective of the study is to understand gene flow and sexual selection in ash in order to guide the conservation and management of ash genetic resources in the face of ash dieback. The specific objectives are to (1) study dispersal distances and directions of ash seeds and pollen in small and large forests, (2) determine the effect of wind on seed and pollen dispersal, (3) investigate gene flow from planted ash trees into surrounding ash forests, and (4) estimate how ash dieback influences the reproductive success of ash trees.

This thesis is based on three case studies designed to address the objectives. The first case study (Paper 1) focuses on dispersal patterns of ash seed and pollen in a fragmented landscape in Rösenbeck, where ash trees occur in small forest patches and as alley trees. The objective was to investigate genetic connectivity among ash trees and the influence of wind on seed and pollen dispersal, assuming that fragmentation, nearby alley trees and local wind conditions influence mating patterns.

The second case study (Paper 2) focuses on gene flow and reproductive success of ash in a large forest of Valby Hegn, where ash trees are severely affected by ash dieback. One main objective was to estimate dispersal distances of ash seeds and pollen, specifically from planted trees to the surrounding older stands. The other main objective was to estimate the effect of ash dieback on reproductive success, hypothesizing that ash dieback negatively affects the reproductive success of the ash parents.

The third case study (Paper 3) estimates the effect of ash dieback susceptibility on male and female reproductive success in a clonal trial at Tuse Næs, based on the hypothesis that healthy trees are more successful parents in comparison to unhealthy trees.

2 General methods

2.1 Study sites and sampling methods

The fieldwork of the study was conducted in three sites (Figure 2): one site was located in Germany (Rösenbeck, 51˚24'36" N 8˚40'12" E) and two others in Denmark (Valby Hegn, 56˚03'17" N 12˚13'52" E; Tuse Næs, 55° 45' 57.99" N 11° 42' 47.48" E).
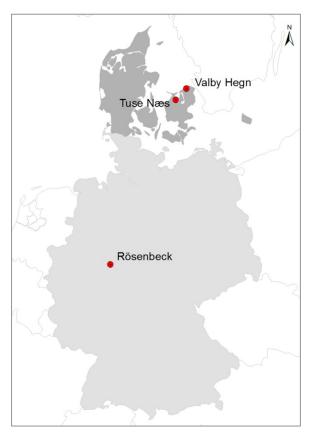
Figure 2 Location of the study sites (ArcMap™ 10.4.1, Copyright © ESRI).

The fragmented landscape of Rösenbeck comprises agricultural and forest patches with a highway stretching the middle. The cultivated alley trees span along both sides of the highway (see Figure 1 in Paper 1). Northern Valby Hegn is a 362.4 ha mixed continuous forest, where ash mainly exists as small stands as well as single trees scattered within dominating beech and oak stands. Two planted ash stands, 1 ha and 0.4 ha in size, are located in the west and northeast from the central part of the forest (see Figure 1 in Paper 2). The planted trees originated from a seed orchard (FP202) in Denmark. Tuse Næs is a clonal seed trial, established in 1998 based on a randomized complete blocks design with 39 individual *F. excelsior* genotypes (clones). Each clone was grafted onto rootstocks and replicated approximately 25 times (ramets).

The sampling of reproductively mature trees in the two study sites (Rösenbeck and Valby Hegn) was carried out in the winter of 2014. Seedlings were sampled along transects in the spring of 2014, and seeds were collected on mother trees in October 2014. In Tuse Næs, the clones were sampled in the spring of 2016. The seeds were harvested in October 2012 from the ramets of the three open pollinated female clones (Clone nos. 30, 33, 35) that are positioned in the middle of the trial area. The seeds were germinated and grown for two years in a greenhouse.

2.2 Phenotypic assessments

Geographical coordinates, heights and diameters at breast heights (DBH) were measured for all mature trees. Flower types were recorded as male, female or hermaphrodite. Flower intensities were scored based on a logarithmic scale from 1 to 8 (Kjær & Wellendorf 1997). Fruit set was scored as relative abundance at 9 scales: 0 means no fruit, while 1–3 indicates low, 4–6 is medium and 7–9 represents high abundance of fruits. Ash dieback symptoms were recorded in terms of crown damage, and assessed as percentage damage score (PDS) in 7 classes: Class 0 indicates no damage, while Classes 1 to 5 represent increasing levels of damage (< 10 %, 10–25 %, 25–50 %, 50–75 %, and 75–100 %), and Class 6 denotes dead trees (100 % crown damage). A reduced 5-class PDS (Class 0: 0 %, Class 1: < 10 %, Class 2: 10–50 %, Class 3: > 50 %, Class 4: 100 %) was applied in Tuse Næs in order to analyse the data together with that collected since 2007.

2.3 DNA extraction and microsatellite analysis
DNA extraction

According to the manufacturer's protocol (Qiagen, Hilden, Germany), total DNA was extracted from leaf, bud and embryo tissues using DNeasy® 96 Plant Kit (Cat. no. 69181) and from wood tissues (cambium) using the DNeasy® Plant Mini Kit (Cat. no. 69104 and 69106). The extraction protocol for wood samples was slightly modified: (1) AP1 + RNAse + PVP (up to 10 % end concentration), and (2) incubation at 65°C for overnight. Prior to embryo extraction, randomly picked seeds were embedded in water for 1–2 days after pericarp tissues were removed. After rehydration, seeds were sliced into two halves and embryos were extracted. Before DNA extraction, the surface tissues of the wood samples were removed and the remaining cambium were cut into small pieces.

Microsatellite Analysis

Rösenbeck and Valby Hegn samples were analysed at the Department of Forest Genetics and Forest Tree Breeding in Göttingen using 13 microsatellite primers, previously published for *Fraxinus* species: FEMSATL8, FEMSATL11 and FEMSATL19 (Lefort et al. 1999), ASH2429 (Bai et al. 2011), FRESTSSR279, FRESTSSR308, FRESTSSR427, FRESTSSR528 (Aggarwal et al. 2010) and Fp12378, Fp18437, Fp20456, Fp21064, Fp104136 (Noakes et al. 2014). The primers were labelled with two fluorescent dyes (6-FAM: FEMSATL8, FEMSATL19, ASH2429, FRESTSSR308, and Fp18437, Fp21064, Fp104136; 6-HEX: FEMSATL11, FRESTSSR279, FRESTSSR427, FRESTSSR528, and Fp12378, Fp20456) and mixed in 4 multiplexes (Multiplex-1: FEMSATL11, Fp18437, and Fp21064; Multiplex-2: FEMSATL19, FRESTSSR427, and Fp12378; Multiplex-3: FEMSATL8 and ASH2429, and Multiplex-4: FRESTSSR279, FRESTSSR308, FRESTSSR528, Fp20456, and Fp104136). Each PCR reaction (14 µl) contained 1.5 µl of 1× Reaction Buffer: 0.8 M Tris-HCl (pH 9.0), 0.2 M $(NH_4)_2SO_4$, 0.2 % w/v Tween-20 (Solis BioDyne, Tartu, Estonia), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 µl 1 U of Taq DNA polymerase (HOT FIREPol® DNA Polymerase, Solis BioDyne, Tartu, Estonia), 0.3 µM forward and reverse primer, and ~ 10 ng genomic DNA. PCR amplifications were performed under the following conditions: 95°C for 15 min, 10 cycles at 94°C for 1 min, from 60°C to 50°C (1°C step down for each cycle) for 1 min, 72°C for 1 min. The final annealing step included 25 cycles at 50°C for 1 min, and the final extension step was performed at 72°C for 20 min (see Paper 1 for further details).

Tuse Næs samples were analysed at the Department of Geosciences and Natural Resource Management in Copenhagen with 9 selected primers: FEMSATL11, FEMSATL12 (Gerard et al. 2006), FEMSATL19, ASH2429, FRESTSSR308, FRESTSSR427, FRESTSSR528, Fp18437, and Fp21064. The primers were labelled with four fluorescent dyes (6-FAM: FEMSATL12, Fp18437, and Fp21064, VIC: FEMSATL11, FRESTSSR308, and FRESTSSR528, NED: FEMSATL19 and ASH2429, PET: FRESTSSR427) and mixed in 3 multiplexes (Multiplex-1: FEMSATL11, FEMSATL12, and FEMSATL19; Multiplex-2: Fp18437, Fp21064, and FRESTSSR528; Multiplex-3: FRESTSSR308, FRESTSSR427, and ASH2429). Qiagen Multiplex PCR Kit (Cat. no. 206143) was used for PCR amplifications. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 15 min, 30 cycles at 94 °C for 30 s, annealing at 57 °C, 62 °C, and 59 °C (for Multiplex-1, -2 and -3, respectively), extension at 72 °C for 60 s, and final extension at 60 °C for 30 min.

PCR products were analysed using an Applied Biosystems 3130xl Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Allele sizes were called with GeneMapper 4.1[®] (Applied Biosystems, Foster City, USA). Each individual genotype and mother-offspring pairs were checked for errors, and the analyses were repeated in cases of uncertain or non-amplified peaks. Alleles were binned into size classes manually and by automated allele-binning software (TANDEM, Matschiner & Salzburger 2009) to correct for errors.

2.4 Population genetics analysis

The basic parameters for population genetics were calculated for the Rösenbeck populations. Numbers of alleles (A), effective number of alleles (A_e), observed heterozygosity (H_o), and Hardy-Weinberg expected heterozygosity (H_e) of planted and native adults and progenies were computed using GENALEX 6.5 (Peakall & Smouse 2012). Allelic richness (A_R) was calculated using the rarefaction method in HP-RARE (Kalinowski 2005) to account for different sample sizes. Unbiased multilocus estimates of inbreeding coefficients within population (F_{IS}) were made using INEST 2.1 (Chybicki & Burczyk 2009). The parameters were set to 200 000 for number of cycles, 2000 for thinning, and 20 000 for burn-in, in order to jointly estimate inbreeding coefficients and null allele rates based on Bayesian approaches. GENALEX 6.5 was also used to calculate pairwise genetic differentiations (F_{ST}) and their significance using 9999 permutations. MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004) was used to test each locus for stutter bands and large allele dropouts.

2.5 Parentage analysis

Two different approaches to parentage analysis were applied in the study: categorical and neighbourhood model approaches. The categorical approach implemented in CERVUS software (Kalinowski et al. 2007) was used to directly identify most likely parents and estimate dispersal distances and directions of pollen and seeds. The neighbourhood model implemented in NMπ (Chybicki 2018) was applied to estimate mating patterns when accounting for individuals' spatial positions and phenotypes.

Categorical method

Two types of analyses were conducted for parentage assignments. Parent pair analysis assigned candidate parent pairs to seedlings, and paternity analysis assigned candidate fathers to seeds with known mothers. In order to increase the confidence about parent identities, the assignments were made based on Delta (Δ), which takes the difference in LOD scores

between the two most likely parents (Marshall et al. 1998). However, single parents were assigned based on their LOD scores given by parent pair analysis. The critical values of Δ were calculated at strict (95 %) and relaxed (80 %) confidence levels during the simulations. The error rate was considered as 1 %, and selfing was included due to ash hermaphrodites. To increase the accuracy of the critical LOD and Δ values, 100 000 offspring were simulated. Confidently assigned parentages were checked and confirmed with gender observations. For Rösenbeck, the percentage of sampled potential parents was set to 85 % for parent pair analysis due to LDD potential of ash. The same parameter was lowered to 75 % for paternity analysis, because 20 % of the seeds were sampled from the trees located at the margins. The same parameter was set to 70 % for Tuse Næs.

The Neighbourhood model

The estimated model parameters included mating system parameters, including self-fertilization rate, seed and pollen immigration rates, and dispersal kernel parameters, and genotyping error rates per locus. Genders of the candidate parents were incorporated into the model as follows: 1 denoted for females, 0 denoted for males, and 0.5 showed hermaphrodites or unknown genders. Model parameter estimations and best model selection followed a stepwise approach: (1) marker specific error rates, selfing rates, and seed and pollen immigration rates were computed, and (2) seed and pollen dispersal kernel parameters were estimated (null model). For Rösenbeck, four models were applied to estimate mean dispersal distances and directionalities based on exponential-power kernel. Akaike Information Criterions (AICs, Akaike 1973) and relative weights of the models were used to select the best model (see Methods in Paper 1 for details).

For Valby Hegn, the standardized phenotypic variables (flowering intensity, fruit set, height, basal area, and PDS) were included into the model (full model) as covariates of individual reproductive success. Therefore, the best-fitting model included the phenotypic variables that influence reproductive success significantly ($\alpha = 0.05$). The best model was selected based on backward elimination approach by comparing the likelihoods of the competing models according to Chybicki (2018). Based on the best model, the cumulative probability distributions of dispersal distances were estimated using NM+ software (Chybicki & Burczyk 2010). The minimum threshold probability was 0.8 for assigning the parentages (see Methods in Paper 2 for further details).

2.6 Randomization tests

Randomization tests were applied in the Valby Hegn study to determine whether the assigned parents constitute a 'healthier' group in all mature trees. In this respect, the mean health (PDS) of the parents were calculated and compared with the general mean of the adult population. The likelihood of the observed parent (either mother or father) drawn from the candidate parent population of a given gender, regardless of its PDS, was tested. Two-tailed p-values were calculated based on 999 permutations for the significances of differences.

2.7 Linear and circular associations

Gender, ash dieback (ADB) susceptibility and reproductive fitness

A general linear analysis of variance model was applied to test whether gender differences among clones in Tuse Næs clonal field trial were significant. A linear regression model was computed also to explain the effects of gender on ADB susceptibility (PDS) and ADB susceptibility on reproductive success. The analyses were performed using 'tidyverse' package (Version 1.1.1; Wickham 2017) implemented in R software environment (R Core Team 2017).

The effect of ADB susceptibility on reproductive success was analysed with 31 clones classified as either M or F. The clones with gender scores up to 20 % were considered predominantly male (M), whereas the clones with gender scores of more than 80 % were regarded as predominantly female (F). The remaining clones were considered hermaphrodites (H). The applied linear regression model was $E\ (Y_i) = \beta_0 + \beta\ PDS_i$, where PDS_i was the susceptibility of the clones to ADB. The relationship between ADB susceptibility and reproductive fitness were assessed at four levels to isolate the effect of survival, since there were differences in number of living ramets per clone: (1) total seed set per clone (sum across all ramets), (2) total number of paternity per clone, (3) average seed set per living ramet per clone, and (4) average number of paternity per living ramet per clone.

Dispersal patterns and wind patterns

The effects of wind direction and speed on the realised dispersals in Rösenbeck were assessed using publicly available meteorological data from German National Meteorological Service (ftp://ftp-cdc.dwd.de/pub/CDC/observations_germany/climate/hourly/). The hourly wind data from Haaren station (ID No. 15120) and Arolsen-Volkhardinghausen station (ID No. 197) were used for pollination and seed dispersal periods, respectively. The data were further processed for the time period between 10 am–6 pm for each day between the 10th and

25th of April for the pollination period in 2014. The data between October 2008 and February 2012 were used for the seed dispersal period. The hours with precipitation (data from Brilon-Thülen station, ID No. 6264) were subtracted from the wind data to eliminate relative humidity. Both dispersal and wind data were analysed in 18 angle classes each with 20°. ORIANA software (v4.02, Kovach Computing Services, Pentraeth, Wales, UK) was used to study dispersal and wind patterns in the study site. Circular correlations were calculated to explain the relationship between dispersal and wind directions, while linear correlations were estimated to understand the relationship between LDDs and wind speeds (see Methods in Paper 1 for more details).

3   Summary of the results

3.1   Paper 1: Gene flow of common ash (*Fraxinus excelsior* L.) in a fragmented landscape

The first paper focuses on seed and pollen dispersal patterns in a fragmented landscape of Rösenbeck in Germany. In the study site, ash trees exist in small forest patches and as alley trees. Here, we hypothesized that fragmentation, nearby alley trees and local wind conditions influence mating patterns of ash. The objectives are to investigate genetic connectivity among ash trees and the effect of wind patterns on dispersal patterns.

Genetic diversity and differentiation

The results showed that there is high genetic diversity within the study population at the Rösenbeck site (Table 1). The mean H_e calculated for 13 SSRs was higher within native adults (0.49) and natural regeneration (0.48) compared to that within cultivated alley trees (0.45). Mean inbreeding coefficients for both adults and offspring were low: F_{IS} of the native adults (0.006) was lower than that of the seedlings (0.021) but higher than the seeds (0.002).

Table 1 Genetic diversity parameters for adults and progenies.

	N	**A**	**A_R**	**A_e**	**H_o**	**H_e**	**F_{IS}**	**NR**
Cultivated	58	4.692	4.690	2.172	0.520	0.453	0.012	0.159
Native	210	8.462	6.610	3.576	0.485	0.488	0.006	0.109
Seedlings	247	8.308	6.470	3.223	0.457	0.483	0.021	0.136
Seeds	500	9.460	6.190	3.288	0.464	0.472	0.002	0.115

N, number of individuals; A, mean number of alleles; A_R, allelic richness corrected for sample size; A_e, effective number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, sample mean inbreeding coefficient (Avg Fi); NR, mean null allele rates.

Microsatellite analysis showed that some of the cultivated alley trees had identical genotypes, suggesting that they might have been vegetatively propagated in the past. Of 58 cultivated trees, 36 had one single genotype whereas 15 had another. The pairwise F_{ST} values revealed higher genetic differentiation between cultivated and native trees (0.053; Table 2), supporting the potential clonal origin of the alley trees.

Gene flow and dispersal distances

Based on the two approaches applied for parentage analysis, 55–64 % of the seeds and 75–98 % of the seedlings sampled in the native forest fragment were parented by the trees within the same fragment. However, there exists a substantial amount of pollen immigration (26–45 %) from outside of the study site. The amount of pollen from the cultivated trees into the native forest fragment was low (2 %).

The two approaches differed in terms of the realised dispersal ranges of seeds and pollen. According to CERVUS, the realised dispersal distances for seeds ranged from 2 m to 473 m and for pollen from 0 m to 939 m. However, NMπ estimated much shorter distances for both seeds (2–90 m) and pollen (2–340 m) with a great potential dispersal distance for pollen, up to 4.5 km.

Table 2 F_{ST} pairwise genetic differentiations (below diagonal) and significances (above diagonal) for adults and progenies.

	Cultivated	Native	Seedlings	Seeds
Cultivated	0	***	***	***
Native	0.053	0	***	***
Seedlings	0.062	0.004	0	***
Seeds	0.059	0.005	0.006	0

p-values were determined using 9999 permutations; ***$p < 0.001$

Dispersal patterns versus wind patterns

Both pollen and seeds were dispersed in all directions corresponding to the local wind directions. There was no positive correlation between pollen dispersal distance and wind speed, but the correlation between seed dispersal distance and wind speed was significant ($r = 0.715$, $p < 0.001$; Table 3), suggesting that strong wind conditions facilitate LDD of seeds.

Table 3 Linear correlations between dispersal distances (DD) and wind speed (WS) in the same angle classes.

Dispersal Period	Mean DD and WS		Cumulative DD and WS	
	Pearson's r	p-value	Pearson's r	p-value
Pollen	-0.374	0.0047	-0.332	0.0009
Seed	0.212	0.0004	0.715	0.0073

3.2 Paper 2: Seed and pollen dispersal and reproductive success in ash forests affected by ash dieback: implications for conservation and restoration

The second paper focuses on gene flow and reproductive success of ash in a large, continuous forest of Valby Hegn in Denmark. Ash trees in the forest are severely affected by ash dieback, and this might have a negative impact on reproductive success. Here, our objectives are to estimate (1) dispersal distances of seeds and pollen, particularly from planted trees to surrounding older stands and (2) the effect of ash dieback on reproductive success.

Seed and pollen dispersals

Based on the best model predicted under the neighbourhood model, 24 % of the seeds and 40 % of the pollen were immigrants, both dispersal kernels followed a fat-tailed distribution (bs = 0.672; bp = 0.229), and the mean distances of seed and pollen were 67 m and 347 m, respectively. The predicted LDD of seeds was up to 300 m, whereas that of pollen was as far as 3 km. The amount of seed and pollen dispersals from the FP202 planting to the surrounding forest was high (respectively 39 % and 31 %), but this amount decreased with increased distance along the transect lines. Planted trees sired a low number of seeds (7 %) since the seeds were sampled from the trees located 100–200 m far from the border of the FP202 planting.

Reproductive success and health

Ash dieback symptoms (PDS) as well as fruit set, flower intensity and size significantly affected female and/or male reproductive success in the study population (Table 4). The mean PDS of both mothers (25 %) and fathers (21 %) were much lower relative to the mean PDS of their source populations (respectively 57 % and 51 %). The randomization tests revealed that the differences between groups were highly significant ($p = 0.001$).

Table 4 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; *$p < 0.05$

3.3 Paper 3: The effect of ash dieback susceptibility on female and male reproductive success: good news for European ash forests

The third paper estimates the effect of ash dieback susceptibility on male and female reproductive success in a clonal trial at Tuse Næs in Denmark. The trial was established in 1998 with 39 ash clones in randomized complete blocks. Here, we hypothesize that healthy trees are more successful parents compared to unhealthy trees.

Gender assessments

The differences among genders of the clones were statistically significant (F = 48.4, $p <$ 0.001). The average gender score per clone changed between 0 % and 92 %. Sixteen clones, with gender scores > 80 %, formed a distinct group and thus were classified as predominantly female. Fifteen clones with gender scores < 20 % were grouped as predominantly male, although the variation was continuous to some extent (see Figure 1 in Paper 3).

Paternity assignments

Paternity analysis confidently assigned candidate fathers to 51 % of the analysed seedlings, with high exclusion probability (0.9999). The number of successful pollinations showed variation among male clones. A single clone (Clone no. 18) was assigned as a candidate father for 46 (32 %) seedlings, whereas 11 clones were not assigned at all as candidate fathers. The variation in pollination per clone changed between 0 and 2.9 per ramet, when correcting for the number of living trees per clone (see Table 1 in Paper 3).

ADB susceptibility and reproductive fitness

Linear regression analysis showed that gender has no effect on the level of ADB susceptibility (t = -0.25, $p >$ 0.05). On the other hand, ADB susceptibility had a clear negative effect on seed set, and crown damage (percent damage score; PDS) explained a high amount of the observed variation (R^2 = 0.87 and 0.52) among females. On the male side, PDS explained less variation (R^2 = 0.50–0.35), and the correlations were significant only because of one male (Clone no. 18), which was the most effective candidate father as well as the least susceptible male genotype (see Table 2 and Figure 2 in Paper 3).

4 Conclusions

High genetic diversity within the population in Rösenbeck supports the previous findings in ash populations in Europe reporting high genetic diversity along with low genetic differentiation due to gene flow (Hebel et al. 2006; Heuertz et al. 2001; Morand et al. 2002; Sutherland et al. 2010; Tollefsrud et al. 2016). Gene flow is an efficient landscape process in ash, both in the fragmented landscape of Rösenbeck and the closed forest of Valby Hegn. In both sites, the dispersal of seeds and pollen was mainly composed of two components: frequent short-distance and rare long-distance dispersal. The majority of the realized dispersals occurred within 100–140 m, which can be regarded as an effective barrier to gene flow within a forest. However, ash pollen can potentially reach distances up to 3–4.5 km, suggesting that gene conservation stands in ash should be planned very carefully. This appeared to be the case in the Tuse Næs clonal field trial, since only half of the seedlings were confidently assigned to one of the 39 clones as the candidate father. The capacity for ash pollen and seeds to travel great distances (kilometres) was previously reported in a fragmented population of ash in Scotland (Bacles & Ennos 2008; Bacles et al. 2006), suggesting that fragmented ash populations in future landscapes may keep connecting through LDDs.

The mating patterns of ash are highly influenced by spatial positions and phenotypic characteristics of individuals, as well as atmospheric conditions in the study sites. In Rösenbeck, gene flow from the cultivated roadside trees to the nearby old-growth forest was limited (2 %). In Valby Hegn, on the other hand, the contribution of the planted trees originating from the FP202 clonal seed orchard was substantially high (31–39 %), and the number of the gametes from these planted trees decreased with increasing distance. Wind direction and speed played an important role in shaping the extent and patterns of gene flow in Rösenbeck, because both pollen and seeds were dispersed in all directions in correlation with local wind directions, and strong wind facilitated long-distance seed dispersal. This shows that local wind data can be useful when planning for new plantings. The phenotypic traits measured in Valby Hegn, including fruit set, flower intensity, size, and crown dieback affected individual reproductive success. As expected, fruit set and flower intensity were positively related to female and male reproductive success, respectively. Surprisingly, tree size was negatively related to male reproductive success. However, size may not be a suitable

measurement for reproductive fitness, because it is highly influenced by site conditions (Younginger et al. 2017).

Ash dieback negatively affected both male and female reproductive success in Valby Hegn and Tuse Næs, although the effect was less pronounced on males due to the siring ability of some unhealthy males. This suggests that sexual selection for lower levels of susceptibility may be slower on male side. The parents of the progenies formed a relatively healthy group in both populations, suggesting that next generation ash trees through natural seed fall will probably be more tolerant than their parental generation. Therefore, planted stands with healthy ash trees can potentially enrich surrounding forests through seed and pollen dispersals, which is promising for the future health of European ash genetic resources under dieback threat.

5 Future perspectives

Both the adaptation of populations and the extent of adaptation depend on phenotypic variation, strength of selection, fecundity, interspecific competition, and biotic interactions (Aitken et al. 2008). High genetic variation within ash populations, along with the LDD of ash seeds and pollen, is promising for the species' adaptive potential and encouraging for its existence in European ecosystems. However, future climatic conditions may become extreme or fluctuate, and novel disease outbreaks may occur due to newly emerging pathogens. For example, emerald ash borer (*Agrilus planipennis*) has been devastating ash populations in North America and Russia, and is currently spreading westward from Russia (Orlova-Bienkowskaja 2014). The extent of its impact on ash in continental Europe cannot yet be predicted. Therefore, the species' disease resistance mechanisms need to be better understood. Current genetic markers (Harper et al. 2016; Sollars et al. 2016) should be tested in ash populations in terms of their selection power for tolerant genetic material. Confirmed markers can be used to screen next generations for disease tolerance. However, additional transcriptome analyses may be required to identify candidate genes for disease resistance, since different subgroups of ash within Europe may respond to ash dieback differently (Downie 2017).

In the meantime, the process of recovery from ash dieback can be enhanced by plantings with seeds from ash genotypes selected and tested for disease tolerance, since healthy trees are expected to spread their seeds and pollen into their surroundings. Sexual selection, combined with high mortality from seedling to mature trees, may gradually produce more tolerant trees in subsequent generations, wherever gene flow can connect surviving trees across landscapes. This may prevent genetic drift resulting from decreased population sizes and reduced genetic variation due to ash dieback. Nonetheless, the true recovery of the species will depend on the speed and efficiency of the process.

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Paper 1

Gene flow of common ash (*Fraxinus excelsior* L.) in a fragmented landscape

Gene flow of common ash (*Fraxinus excelsior* L.) in a fragmented landscape

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Abstract

Gene flow dynamics of common ash (*Fraxinus excelsior* L.) is affected by several human activities in Central Europe, including habitat fragmentation, agroforestry expansion, controlled and uncontrolled transfer of reproductive material, and a recently introduced emerging infectious disease, ash dieback, caused by *Hymenoscyphus fraxineus*. Habitat fragmentation may alter genetic connectivity and effective population size, leading to loss of genetic diversity and increased inbreeding in ash populations. Gene flow from cultivated trees in landscapes close to their native counterparts may also influence the adaptability of future generations. The devastating effects of ash dieback have already been observed in both natural and managed populations in continental Europe. However, potential long-term effects of genetic bottlenecks depend on gene flow across fragmented landscapes. For this reason, we studied the genetic connectivity of ash trees in an isolated forest patch of a fragmented landscape in Rösenbeck, Germany. We applied two approaches to parentage analysis to estimate gene flow patterns at the study site. We specifically investigated the presence of background pollination at the landscape level and the degree of genetic isolation between native and cultivated trees. Local meteorological data was utilized to understand the effect of wind on the pollen and seed dispersal patterns. Gender information of the adult trees was considered for calculating the dispersal distances. We found that the majority of the studied seeds (55–64 %) and seedlings (75–98 %) in the forest patch were fathered and mothered by

the trees within the same patch. However, we determined a considerable amount of pollen flow (26–45 %) from outside of the study site, representing background pollination at the landscape level. Limited pollen flow was observed from neighbouring cultivated trees (2 %). Both pollen and seeds were dispersed in all directions in accordance with the local wind directions. Whereas there was no positive correlation between pollen dispersal distance and wind speed, the correlation between seed dispersal distance and wind speed was significant (0.71, $p < 0.001$), indicating that strong wind favours long-distance dispersal of ash seeds. Finally, we discussed the implications of establishing gene conservation stands and the use of enrichment planting in the face of ash dieback.

Introduction

Pollen and seed dispersal patterns are important to determine the consequences of ecological and biological threats on plant populations, such as landscape changes, climate change and newly emerging pathogens. Habitat fragmentation can reduce genetic connectivity of plant populations [1] by reducing habitat size and increasing spatial isolation. The reduced population sizes over generations can decrease genetic variation within populations while increasing genetic differentiation among populations due to increased random genetic drift, inbreeding and restricted gene flow [2]. However, depending on the differences in species' responses, habitat fragmentation can also facilitate gene flow among fragmented populations [3–6]. It is therefore important to improve the understanding of gene flow patterns in both fragmented and non-fragmented forest ecosystems [7].

Natural forests are often replaced with managed large-scale monocultures, in order to meet the demand for wood and other forest products [8]. The global area of planted forests increased from 167.5 to 277.9 million ha between 1990 and 2015, and the increase was the second most rapid in Europe (37 %) [9]. By 2015, the planted forest area in Germany increased to 5.2 million ha with an annual increase of 2800 ha from 1990 to 2000 [9]. Such forest activities often involve the introduction of new genetic variants, and gene flow from plantations can influence the genetic diversity of native populations. If the gene flow from plantations is pronounced, it may result in loss of genetic diversity that reflects adaptation to unique ecological conditions and therefore call for specific gene conservation efforts [10, 11]. On the other hand, allochthonous gene flow from plantations might prevent genetic erosion of small native populations by restoring genetic variation and reducing inbreeding depression, leading to a recovery of fitness or genetic rescue [12, 13]. However, the actual degree of gene flow from plantations to old growth forests is unknown for most plantation programs.

Emerging infectious diseases (EIDs) are another potential cause behind loss of genetic diversity and local genetic patterns. Increasing in number worldwide [14], EIDs are typically introduced by humans to new ecosystems where they have not coevolved with their new host. They can threaten biodiversity through biomass loss and/or extinction of host species [15]. There are several examples of EIDs that have led to dramatic mortality in previously widespread tree species, but trees with natural resistance are often found in low frequency in natural populations [16]. Offspring of such rare survivors could found future populations. Accurate estimation of pollen and seed dispersal patterns in natural populations is therefore fundamental to predict the response of forest tree populations and ecosystems to such threats, since gene dispersal capacity will determine how fast high-fitness genes can increase in frequency across landscapes, and thereby the recovery potential of the species. In the absence of genetic connectivity, mating among related trees in genetic clusters around surviving trees can lead to inbreeding. Gene conservation strategies, in general, aim at creating high population connectivity among local gene pools while limiting unwanted gene flow [1, 10, 17]. In the case of EIDs this is complicated by the need for dispersal of resistance genes without losing important patterns of local adaptation.

Dieback and gene flow in ash

Common ash (*Fraxinus excelsior*, hereinafter ash) is a keystone species in natural plant communities in European forests [18]. It is distributed across Europe, from the Atlantic coast in the west to continental Russia almost up to the River Volga in the east, from Norway in the north to the northern parts of Spain, Italy, Greece, and as far as Iran in the south [19]. Ash is a valuable tree species in terms of its ecological characteristics, wood quality and high economic value [20]. However, the species is highly threatened by a pathogenic fungus, *Hymenoscyphus fraxineus* (synonym *H. pseudoalbidus*, anamorph *Chalara fraxinea*), which causes dieback [21–23]. The disease symptoms were observed in Poland in the early 1990s [24] but have since spread throughout Europe, causing massive tree mortality and threatening the species' existence in many forest ecosystems [25–27]. Dieback symptoms were first observed in Northern Germany in 2002, and since then increased damage has been reported throughout the country, resulting in ecological and economic impacts on forests, open landscapes, nurseries and urban plantings [28]. Previous studies have identified high genetic variation in disease susceptibility levels, indicating the species' genetic potential to recover through natural and artificial selection [29–31]. However, the speed of pathogen spread is expected to be much faster than the spread of resistance at the population level, because the

frequency of tolerant trees is low [27] and ash trees rarely flower before 10 years old. As a result, the speed of population recovery will depend on the survival rate, the reproductive fitness of surviving trees and the extent of pollen- and seed-mediated gene flow.

Estimation of gene flow based on parentage analysis

Categorical approach to parentage analysis [32] assigns candidate parents to their offspring at certain confidence levels using genetic information and thereby makes it possible to determine the distances and directions of realized pollen and seed dispersal events. The neighbourhood model [33–35], on the other hand, estimates mating model parameters, directly accounting for spatial distribution of individuals as well as their phenotypes, and infers genealogies after fitting the parameters. Parentage analysis allows detailed reconstruction of ongoing mating patterns. This may be used to precisely locate individuals or their groups with low versus high reproductive success. The neighbourhood model, however, allows accounting for confounding effects of dispersal and fecundity, and provides a predictive tool (the forward dispersal kernel [36]) for seed and pollen dispersal. Such a model can be particularly useful in the assessment of gene flow potential of a species. Therefore, using a combination of these approaches can provide more information on gene flow estimations.

Pollen and seed dispersal distances and directions can be calculated once paternal (pollen) and maternal (ovule-seed) contributions are identified. With seeds sampled on specific mother trees, pollination distances and directions can easily be calculated. However, in the case of seedlings, additional information regarding the gender of the both candidate parents is required. A prior assumption of the closest parent being the maternal parent is potentially misleading and can underestimate the seed dispersal distances [37]. Uniparentally inherited cytoplasmic markers can help revealing seed and pollen parents [38]. In the case of angiosperms, however, polymorphism levels at regional scales are often low with a single haplotype dominating in most regions [39]. It is therefore valuable to use repetitive macroscopic observation of ash flowers to identify gender.

The process of propagule dispersal is complex, and is influenced by both biotic and abiotic factors. For wind-mediated dispersal, wind conditions are obviously expected to play a substantial role in determining the dispersal directions and distances from parent trees. For example, dry, windy weather conditions promote long-distance dispersal (LDD) of seeds [40–43]. Likewise, clear warm days with low relative humidity, especially from midday to late afternoon (10 am-6 pm), are most favourable for pollen release [44–46]. Parentage

assignment methods allow tracking of pollen and seed dispersal events in terms of distance and directionality [47]. Additional meteorological data could help explain the potential range of pollen and seed dispersals within particular landscapes, regions or continents [48], and enable us to better understand to what extent these factors affect the dispersal patterns.

Objectives of the present study

The aim of the present study is to investigate genetic connectivity among ash trees in a fragmented landscape in Rösenbeck, Germany. A combination of categorical parentage and the neighbourhood model approaches was applied to estimate mating parameters and to describe dispersal patterns in our study site. Specifically, we studied the degree of reproductive isolation of a 2 ha natural population from cultivated trees planted within a few hundred meters. Gender information was used to calculate pollen and seed dispersal distances and directions. Actual wind and precipitation data were utilized to assess the effect of wind on the dispersal patterns. The outcomes are discussed in relation to the genetic management of ash, including its conservation and reproductive material use, especially under the current epidemic ash decline.

Materials and methods
Study species reproductive ecology

Both pollen and seed dispersal are mediated by wind in *F. excelsior*. Large numbers of female flowers are produced and pollinated in spring, before leaf flush. The pollination season in Northern and Central Europe lasts from April to May [49]. Seeds mature in September-October and the majority of the seeds are dispersed between October and February [50]. A fraction of the seeds can remain on the trees until the following summer [51]. The dispersal phenology corresponds to a deep seed dormancy that is not released until seeds have experienced both warm and cold stratification. The majority of the seeds, therefore, do not germinate until the second year after maturation [52].

The species presents a polygamous reproductive system where the individuals are phenotypically classified in a continuum from purely male to purely female, with a wide range of hermaphroditic intermediates [53, 54], but it is regarded as functionally dioecious [55]. Although hermaphrodite flowers are self-fertile, their paternal success is much lower than that of males at the seed set stage [56]. Earlier release of male pollen outcompetes hermaphrodite pollen in cross-pollinations, suggesting hermaphrodite flowers function

predominantly as female [57]. High daily concentrations of ash pollen (> 100 pollen grain/m^3) can remain in the air between 10–20 days [58].

Study site, demography of ash and sampling design

The location of the study site and the sampling scheme are shown in Fig 1. The study site is located about 1 km northwest of Rösenbeck (51°24'36" N 8°40'12" E), Brilon, North Rhine-Westphalia, Germany (Fig 1a). The fragmented landscape is composed of agricultural lands, forest patches and a highway spanning the middle. The oldest available map (1816–1850) of Brilon (www.tim-online.nrw.de/tim-online/initParams.do) revealed the historical fragmentation of the region, indicating that the landscape fragmentation must be at least 200 years old. In a radius of 5 km around the study site, ash mainly exists as single trees in different stands, mostly mixed with beech, and only exists seldom in more or less small pure stands. Thus the estimated abundance of ash in the area is less than 5 % (communicated with Martin Rogge from Arnsberg Forest, Landesbetrieb Wald und Holz Nordrhein-Westfalen).

The cultivated alley trees stretch for 1 km along both sides of the highway (Fig 1b). Apical and terminal buds were sampled from all mature, reproductive trees in the landscape (for about 500 m from the boundaries of the native stand) in January 2014 (Fig 1b). Among 268 adult trees sampled, 58 of them were alley trees. According to our estimations, the age of the native trees ranged from 20 to 80, with a few additional older ones, about 100–120 years old. The deciduous tree symbols placed on the historical map, covering the time period of 1936 and 1945 may indicate the time of establishment of the native forest, supporting our age estimations. Offspring were sampled in the privately owned native forest (2 ha) (Fig 1c). Leaves were sampled from 247 seedlings in July in the same year along 3 main transects, representing different parts of the forest (Fig 1c). A total of 500 seeds were collected on 10 mother trees (50 seeds per mother) in October 2014. Two of the mother trees are marginal; one of them is located in the southernmost edge of the forest while the other one is located in the land owner's factory yard (Fig 1c).
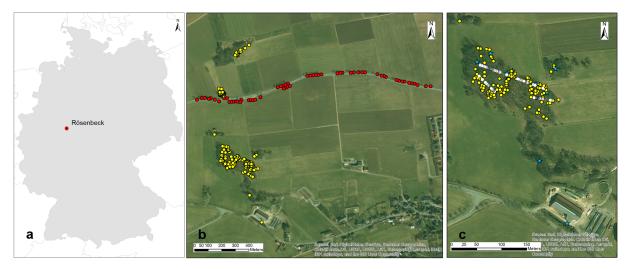
Fig 1. Map of the study site and sampling scheme.

(a) Location of the study site in Germany. (b) Sampling of the adult trees (cultivated trees along the road are shown as red circles and native trees in the landscape are marked as yellow circles). (c) Sampling of the offspring (mother trees of the seeds were illustrated as blue stars and the seedlings collected along 3 transects were marked as white circles). The maps were created using ArcMap™ 10.4.1, Copyright © ESRI.

Field data collection

The geographical position of each adult tree was recorded during the sampling period using Garmin GPSmap® 62s. The position of each seedling on the transect line relative to the start point was recorded, and the heights of the seedlings were measured. Visual observation of the adult tree flowers was conducted in two subsequent years in 2014 and 2015 when both cultivated and native trees were flowering at the same time. The flowers were observed between 11th–17th of April in 2014 and 21st–22nd of April in 2015 because flowering started earlier in 2014 relative to 2015. The trees were recorded as male, female or hermaphrodite according to their flower types. In case of the absence of flowering, any additional observations informative for gender identification, such as presence of dry seeds and seed stalks from the previous year and flower galls [59], were recorded. Seed set was observed in 2014 and 2015, in August.

DNA extraction and microsatellite analysis

Leaves and buds were stored at -20 ˚C until they were used for DNA extraction. Seeds were collected and stored at 4 °C in labelled bags. Prior to embryo extraction, randomly picked seeds were embedded in water for 1–2 days after pericarp tissues were removed. After

the rehydration, seeds were sliced into two halves with a razor blade and embryos were carefully picked up to avoid any contamination with maternal tissues. Total DNA was extracted from leaf, bud and embryo tissues using DNeasy® 96 Plant Kit (Cat No. 69181) according to the manufacturer's protocol (Qiagen, Hilden, Germany).

Microsatellite analyses were performed using 13 primers. FEMSATL8, FEMSATL11 and FEMSATL19 are frequently used primers developed for *F. excelsior* [60]. ASH2429 [61], FRESTSSR279, FRESTSSR308, FRESTSSR427, FRESTSSR528 [62] and Fp12378, Fp18437, Fp20456, Fp21064, Fp104136 [63] are EST-SSRs developed for *F. excelsior* or other Fraxinus species. The primers were labelled with two different fluorescent dyes (6[FAM]: FEMSATL8, FEMSATL19, ASH2429, FRESTSSR308 and Fp18437, Fp21064, Fp104136; 6[HEX]: FEMSATL11, FRESTSSR279, FRESTSSR427, FRESTSSR528 and Fp12378, Fp20456), and were combined in 4 Multiplexes (M) (M1: FEMSATL11, Fp18437 and Fp21064; M2: FEMSATL19, FRESTSSR427 and Fp12378; M3: FEMSATL8 and ASH2429, M4: FRESTSSR279, FRESTSSR308, FRESTSSR528, Fp20456 and Fp104136). Amplifications were performed in 14 µl reactions. Each reaction contained 1.5 µl of 1× Reaction Buffer: 0.8 M Tris-HCl (pH 9.0), 0.2 M $(NH_4)_2SO_4$, 0.2 % w/v Tween-20 (Solis BioDyne, Tartu, Estonia), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 µl 1 U of Taq DNA polymerase (HOT FIREPol® DNA Polymerase, Solis BioDyne, Tartu, Estonia), and 0.3 µM forward and reverse primer, and ~ 10 ng genomic DNA. A combination of hot start and touchdown procedure was applied for PCR conditions. Thermal cycling conditions were as follows: 95°C for 15 min followed by 10 cycles of 94°C for 1 min, annealing temperature step-downs each cycle of 1°C (from 60°C to 50°C) for 1 min, 72°C for 1 min. The annealing temperature for the final 25 cycles was 50°C for 1 min with the denaturation and extension steps as above. The final extension step was at 72°C for 20 min. PCR products were analysed using Applied Biosystems 3130xl Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Allele size calling was carried out with GeneMapper 4.1® (Applied Biosystems, Foster City, USA) using the software developer's default settings for the analysis parameters, except for the parameters for allele peak quality. Signal levels for minimum homozygous and heterozygous peak height were set to 200 RFU and 100 RFU, respectively. Minimum peak height ratio was set to 0.5 for heterozygote balance. Each individual genotype was checked for errors, and the analyses were repeated for missing or uncertain peaks. Mother-offspring mismatches in all loci were checked and repeated when necessary to avoid scoring PCR artefacts because of null alleles. Manual allele binning was conducted based on the

microsatellite repeat units and the cumulative allele size distributions were plotted in Excel for each locus. Manually binned alleles were compared to the ones generated by TANDEM, an automated allele binning software [64], to detect potential allele binning errors.

Population genetics analysis

Genetic diversity parameters including number of alleles (A), effective number of alleles (A_e), observed heterozygosity (H_o), Hardy-Weinberg expected heterozygosity (H_e) of cultivated and native adults and offspring were calculated using GENALEX 6.5 [65]. Allelic richness (A_R) was computed using rarefaction method in HP-RARE [66] to avoid bias due to different sampling size. INEST 2.1 software [67] was used for unbiased multilocus estimates of inbreeding coefficients within population (F_{IS}). The software jointly estimated inbreeding coefficients and null allele rates according to the Bayesian approach at the parameter settings of 200 000, 2000 and 20 000 for the number of cycles, thinning and burnin, respectively. Pairwise genetic differentiations (F_{ST}) and their significance were determined based on 9999 permutations using GENALEX 6.5 [65]. Each locus was checked for presence of stuttering and large allele dropout using MICRO-CHECKER 2.2.3 [68].

Parentage analysis

The assessment of gene flow in our study site was carried out with two approaches. The categorical approach to parentage assignment [69] was applied for direct identification of the most likely parents of the offspring (seeds and seedlings), and to estimate pollen and seed dispersal distances and directions from the parents to the location of the propagule deposition. The spatially explicit neighbourhood model approach [33–35] was additionally applied to provide more accurate estimates for the proportion of seed and pollen immigration and to further assess the patterns of parentages when accounting for spatial distributions of the individuals in our study site.

Categorical approach

Preliminary microsatellite analysis revealed the presence of identical genotypes among the cultivated trees, indicating their vegetative propagation in the past. Among 58 cultivated trees, 36 of them presented one single genotype (Genotype 1) while 15 of them presented another (Genotype 2) (S1 Table). Only one representative genotype from each group was kept for further analyses to prevent interferences with the parentage assignments due to identical

log-likelihood (LOD) scores. A total of 9 different genotypes (Genotype 1 + Genotype 2 + 7 unique genotypes) represented the cultivated tree gene pool in the parentage analyses.

Parentage assignments were performed using CERVUS 3.0.7 [70]. Parent pair analysis assigned candidate parent pairs to the seedlings, while paternity analysis assigned candidate fathers to the seeds with known mothers. Parentage assignments were made based on Delta (Δ), the difference in LOD scores between two most likely parents, in order to increase the certainty of the identity of true parents where multiple parents had positive LOD scores [69]. Single parent assignments were performed based on their LOD scores computed in the parent pair analysis. Prior to the assignments, simulations were run to calculate the critical values of LOD and Δ at strict (95 %) and relaxed (80 %) confidence levels. Because there were no missing data in the genotype data set, the minimum number of loci was set to 13. The default value of 1 % was kept as the proportion of error rate in the simulations. Selfing was incorporated into the simulations owing to the self-fertilization ability of ash hermaphrodites. The number of simulated offspring was set to 100 000 to increase the reliability of the critical LOD values. In spite of the complete sampling scheme of the study, the parameter for percentage of sampled potential parents was set to 85 % for the parent pair analysis due to the significant potential of long-distance gene flow of ash in fragmented landscapes. The same parameter was lowered to 75 % for the paternity analysis since 20 % of the seeds were collected from the mother trees in the margin of the forest patch.

After the parentage analyses, each confident assignment was subjected to further elimination using the gender information of each adult tree acquired from field observations in 2014 and 2015, in order to improve the reliability of the assignments. The assignments were excluded from further calculations when there was a discrepancy between the genders of the two assigned parents or no information about the genders of the assigned parents. This was only the case for 37 (out of 332) paternity, and 11 (out of 121) parent pair assignments. The confidently assigned single parent was only accepted as a seed parent when it was female and having no mismatching loci with the seedling. This was the case for 23 (out of 67) of the assigned single parents.

Point coordinates (x, y) of the seedlings relative to their respective transect lines were calculated using Euclidean distances. These coordinates were later transformed into the same geospatial coordinates with the adult trees to calculate the pollen and seed dispersal distances. To identify the directions of the dispersal events, azimuths (horizontal angle measured clockwise from the North) were calculated using QGIS 2.16 [71]. Pollen dispersal directions were calculated from father to mother tree while seed dispersal directions were calculated

from mother tree to seedling. A clonal genotype was assigned as a parent in 12 cases in total. In this case, mean pollination and seed dispersal distances and angles were taken into account.

The neighbourhood model approach

NMπ, a successor of NM+ software [72] was used to characterize mating/dispersal patterns in our study population based on the neighbourhood model. The estimated model parameters were frequency of self-fertilization, frequency of seed and pollen immigration from surrounding sources, mean distance of seed and pollen dispersal, shape of seed and pollen dispersal kernels, intensity (rate) of directionality (anisotropy) of seed and pollen dispersal and prevailing direction of seed and pollen dispersal. The software also estimated per locus genotyping error rates. The genders of the adult trees were incorporated into the model estimations according to their femaleness where 0 stands for male, 0.5 stands for hermaphrodite or unknown sex and 1 stands for female. A stepwise approach was followed during the estimations due to the sensitivity of the model estimations to initial input values. At first, genotyping error rates, frequency of immigrations and selfing rates were estimated. Using these parameters, four models were applied to estimate average dispersal distances and rate of directionalities based on exponential-power kernel. Simple exponential kernel (b = 1), fat-tailed (b < 1) and normal (b = 2) distributions were tested in these models. The model comparisons were made based on the Akaike Information Criterion (AIC) [73], and the best-fitting model was selected according to the calculated relative weights (e.g. [74]). The two most-likely genealogies inferred for each offspring in the best-fitting model were evaluated according to their associated posterior probabilities. The minimum threshold probability of 0.8 was taken into account to evaluate the seed and pollen dispersal distances in the study site. The dispersal potential in the study site was assessed based on the estimated fitted dispersal kernel function. The approximate 95 % confidence interval around dispersal kernel parameters, mean distance of seed and pollen dispersal and shape of seed and pollen dispersal kernels were estimated. Based on the best-fitting dispersal kernels, predictive cumulative probability distributions were computed using NM+ software.

Effect of wind on dispersal patterns

To assess the effects of wind direction and speed on the realized dispersal events, publicly available meteorological data was acquired from Climate Data Centre of German National Meteorological Service (ftp://ftp-cdc.dwd.de/pub/CDC/observations_germany/climate/hourly/) (S2 Table). Based on the flower

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observations in the study site in 2014, the time period within 10th–25th of April was considered as pollination period. Accordingly, hourly wind data in the same period in April 2014 was extracted from Haaren Station (ID No. 15120), located 26 km north of the study site. The data were further arranged by including only the time period between 10 am-6 pm for each day. Because long-term historical data was not available for Haaren Station, the next closest wind station (40 km southeast), Arolsen-Volkhardinghausen (ID No. 197), was chosen to extract the wind data for the seed dispersal periods between October 2008 and February 2012. The precipitation data from Brilon-Thülen station (ID No. 6264) (4 km to the study site) was used to remove the effect of high relative humidity by subtracting the hours with precipitation from the corresponding wind data. The dispersal and wind data were analysed in 18 angle classes each with 20° (18×20°= 360°). The patterns of pollen and seed dispersal direction and wind direction were assessed using ORIANA 4.02 (Kovach Computing Services, Pentraeth, Wales, UK) by constructing circular histograms, calculating mean directions, and computing circular correlations between dispersal directions and wind directions. Since the wind directions were also recorded as azimuth (0° North), there was no need for further conversion to make pairwise comparisons. To determine the effect of the frequency of strong winds on LDD, linear relationships between dispersal distances and wind speeds in the same angle classes were assessed using paired t-test for sample means. Pearson's correlation coefficients and their p-values were calculated using (1) the means of the dispersal distances and wind speeds, and (2) the cumulative dispersal distances and wind speeds in each angle class.

Results

Population genetics parameters

The A_R was higher in the native adult trees (6.61) than in the cultivated adults (4.69), and compared to the native adults, it was slightly lower in the seedlings (6.47) and in the seeds (6.19) (Table 1). The F_{IS} level of the cultivated adults (0.012) was slightly higher than that of the native adults (0.006) (Table 1). Compared to the native adults, the seedlings had higher F_{IS} (0.021) while the seeds had lower (0.002) (Table 1). All pairwise F_{ST} values of genetic differentiation were highly significant ($p < 0.001$), and showed that the offspring were differentiated from the cultivated roadside trees (0.062 and 0.059, respectively; Table 2), but not from the native adults (0.004 and 0.005, respectively; Table 2). There were no large allele dropout or stuttering detected in any loci. However, there were mother-offspring mismatches

detected, particularly in FEMSATL8 and Fp104136 loci. The estimated mean null allele rates across loci for the adults and offspring were between 0.11 and 0.16 (Table 1).

Table 1. Genetic diversity parameters for the adults and offspring.

	N	A	A_R	A_e	H_o	H_e	F_{IS}	NR
Cultivated	58	4.692	4.690	2.172	0.520	0.453	0.012	0.159
Native	210	8.462	6.610	3.576	0.485	0.488	0.006	0.109
Seedlings	247	8.308	6.470	3.223	0.457	0.483	0.021	0.136
Seeds	500	9.460	6.190	3.288	0.464	0.472	0.002	0.115

N, number of individuals; A, mean number of alleles; A_R, allelic richness corrected for sample size; A_e, effective number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, sample mean inbreeding coefficient (Avg Fi); NR, mean null allele rates.

Table 2. F_{ST} pairwise genetic differentiations (below diagonal) and their significance (above diagonal) for the adults and offspring.

	Cultivated	Native	Seedlings	Seeds
Cultivated	0	***	***	***
Native	0.053	0	***	***
Seedlings	0.062	0.004	0	***
Seeds	0.059	0.005	0.006	0

p-values were determined using 9999 permutations

*** *p* < 0.001

Parentage analysis

Parentage assignments and mating model estimations

Total exclusion probabilities for both parentage analyses were high (0.999) in CERVUS. Thirteen loci provided the mean heterozygosity of 0.49, and the mean numbers of alleles per locus were 10.3 for paternity and 9.5 for parent pair analyses. Using Δ, unique candidate fathers were confidently assigned to 332 (66.4 %) seeds, and among them 200 (62.1 %) were strictly confident. No mismatching loci were detected in 228 (70.8 %) of the assignments. Twenty paternal assignments were not statistically confident enough even though the candidate fathers had no mismatching loci with the mother and seed. In the case of the seedlings, the number of confidently assigned unique parent pairs was 121 (49 %). The level of confidence was strict for 41 (17 %) assignments. The overall number of trio loci with

no mismatch was 110 in the parent pair analysis. Sixty-seven (27 %) single parents were confidently assigned and among them 28 were strictly confident.

The paternity and parent pair analyses revealed 64.4 % and 74.9 % of gene flow from the native trees while 2 % and 1.2 % from the cultivated trees, respectively (Fig 2). Although the trio LOD and Δ scores were positive, 7.2 % of the paternity and 21.9 % of the parent pair assignments were not confident enough (Fig 2). Respectively, 26.4 % and 2 % of the paternity and parent pair assignments presented negative LOD scores, which are treated as putative immigrants (Fig 2). The overall selfing rate was very low (0.4 %).
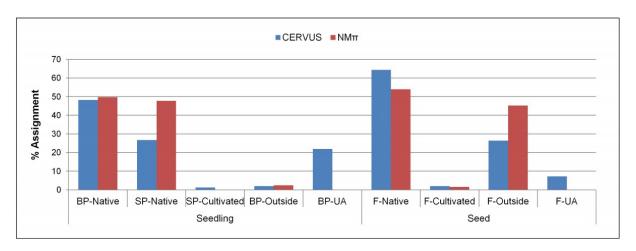
Fig 2. Breakdown of the parentage assignments (%).

BP, both parents; SP, single parent; UA, unassigned; F, father.

The AIC model comparisons of the applied neighbourhood models showed that the two-parameter exponential-power kernel with directionality (anisotropy) is the best model for the seed and pollen dispersal in the study site (S3 Table). The estimated mating model parameters and the standard errors using the best-fitting model were given in Table 3. According to the model estimations, pollen dispersal kernel follows a fat-tailed distribution ($b_p = 0.269$, $b < 1$) while seed dispersal kernel rather follows a normal distribution ($b_s = 2.085$, $b = 2$) (Table 3). The estimated frequency of seed and pollen immigrants were 3 % and 45 %, respectively, and the frequency of self-fertilization was 0 (Table 3).

Based on the best-fitting model, the parental profiles of the progenies were assessed according to the posterior probabilities of the two most-likely parentages inferred for each progeny (Fig 2). According to the parental profile, 123 (49.8 %) seedlings have both their parents inside of the sampled stand while 118 (47.8 %) seedlings have only mothers in the stand, and all of these parents are the native trees (Fig 2). On the other hand, 270 (54 %) seeds

were sired by the local native fathers while only 4 (1.6 %) of them were sired by the fathers from the cultivated alley trees (Fig 2).

Table 3. Mating model parameters estimated by the best-fitting neighbourhood model.

Parameter	s	ms	mp	1/ds	1/dp	bs	bp	ks	kp	as	ap
Estimate	0	0.032	0.455	0.026	0.002	2.085	0.269	0.934	1.099	0.155	0.076
Std. Error	-	0.015	0.020	0.002	0.001	0.437	0.065	0.125	0.116	0.021	0.015

s, frequency of self-fertilization; ms/mp, frequency of seed and pollen immigration from surrounding sources; ds/dp, mean distance of seed and pollen dispersal; bs/bp, shape of seed and pollen dispersal kernels; ks/kp, rate of directionality (anisotropy) of seed and pollen dispersal; as/ap, prevailing direction (azimuth) of seed and pollen dispersal (% 2π).

Realized and potential dispersal distances

The frequency distribution graphs of pollen and seed dispersal distances were constructed based on the dispersal events identified using two approaches to parentage analysis (Fig 3). A total of 405 (295 paternity + 110 parent pair) CERVUS- assigned and 282 NMπ- inferred pollen dispersal events were used to create the pollen dispersal histograms (Fig 3a). According to the CERVUS assignments, the pollination distances ranged from 0 to 939.26 m with a mean of 90.48 m (median = 43.86 m) while the estimated pollination distances by the neighbourhood model ranged from 2.24 m to 339.75 m with a mean of 40.47 m (median = 23.71 m) (Fig 3a). The seed dispersal histograms were constructed with 133 CERVUS- determined (110 parent pair and 23 single seed parent) and 129 NMπ- inferred seed dispersal events (Fig 3b). The range of seed dispersal distance detected using CERVUS was between 1.83 m and 473.53 m with the mean distance of 48.60 m (median = 30.77 m) (Fig 3b). NMπ- computed seed dispersal distances were between 1.83 m and 89.61 m with the mean distance of 30.37 m (median = 28.81 m) (Fig 3b). The majority of the pollen dispersal detected by CERVUS (79 %) and NMπ (94 %) occurred up to 100 m (Fig 3a), similar to the seed dispersal detected by CERVUS (95 %; Fig 3b). There was no seed dispersal detected beyond 100 m (Fig 3b) with NMπ while the CERVUS- identified seed dispersal beyond 100 m was 5.3 % (Fig 3b). The identified pollen dispersal beyond 100 m was 21 % with CERVUS and 6 % with NMπ (Fig 3a).
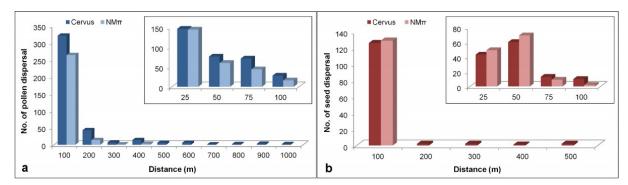
Fig 3. Frequency distributions of pollen (a) and seed (b) dispersal distances.

Lower left shows dispersal events in 100 m distance classes; Upper right shows close-up on short distance dispersal events up to 100 m.

The NMπ- estimated dispersal kernel parameters ds, bs, dp and bp, and the approximate 95 % confidence interval around them were given in Table 4. Based on the fitted kernel function, the estimated average distances of seed (ds) and pollen (dp) were 39 m and 571 m, respectively (Table 4). According to the cumulative probability distributions obtained for the maximum likely dispersal kernel parameters (Fig 4), 50 % of the seeds travel not further than 37 m and less than 1 % seeds travel further than 94 m. Compared to the seeds, 50 % of pollen disperses up to 266 m while 10 % pollen disperses further than 1 380 m, and 1 % pollen can reach distances as far as 4 478 m or more from the source plant.

Table 4. The approximate 95 % confidence interval around dispersal kernel parameters.

Parameter	Estimate	Q [2.5 %]	Q [97.5 %]
ds	39.1	33.9	46.2
bs	2.085	1.016	3.154
dp	571.0	201.6	Infinity
bp	0.269	0.110	0.428
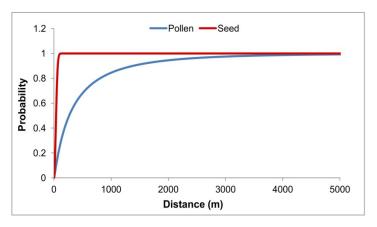
Fig 4. Cumulative probability distributions for the maximum likely dispersal kernel parameters.

Dispersal patterns versus wind patterns

Pearson's correlation coefficients (*r*) and their significances were calculated for linear associations between dispersal distances and wind speeds in the same angle classes, in the corresponding dispersal period (Table 5). Correlations were assessed by taking the mean and the cumulative dispersal distances and wind speeds, respectively (Table 5). Both correlations were found to be moderate and negative (-0.332 and -0.374, respectively) between pollen dispersal distance and wind speed. A positive but weaker correlation was calculated between seed dispersal distance and wind speed (0.212). However, the correlation was much stronger (0.715) when the cumulative distances and speeds were taken into account. All correlations were highly significant ($p < 0.01$).

Table 5. Linear correlations between dispersal distances (DD) and wind speed (WS) in the same angle classes.

| Dispersal Period	Mean DD and WS		Cumulative DD and WS	
	Pearson's *r*	*p*-value	Pearson's *r*	*p*-value
Pollen	-0.374	0.0047	-0.332	0.0009
Seed	0.212	0.0004	0.715	0.0073

The realized pollen and seed dispersals and wind patterns during the pollination and seed dispersal periods were graphed using circular histograms (Fig 5 and 6). The pollen dispersals occurred in all directions in accordance with the wind directions (Fig 5a, b). Mean pollination direction was 34.78° (Fig 5a) and mean wind direction was 48.71° (Fig 5b), both towards the northeast. The circular correlation between pollination and wind direction was calculated as 0.999 ($p < 0.05$). The estimated prevailing direction of pollen dispersal (ap) with the fitted model was 27.36° (ap = 0.076 in % 2π; Table 3), also towards the northeast.

Circular-linear histograms were constructed for seed dispersal and wind patterns for four dispersal periods between October 2008 and February 2012, by incorporating the linear components (dispersal distance and wind speed) into the angles (dispersal direction and wind direction, respectively) (Fig 6b). The seed dispersal events were identified in all angle classes except for 240° and 260°, the southwest directions (Fig 6a), while the wind blew in all directions (Fig 6b). The mean dispersal direction weighted by dispersal distance was calculated as 145.32° towards the southeast (Fig 6a). The mean wind direction, weighted by speed, was calculated as 217.88° towards the southwest (Fig 6b). The circular correlation between the seed dispersal direction and wind direction was 0.682 ($p < 0.05$). However, when

only direction was taken into account, the mean seed dispersal direction was towards the northeast (59.84°), which was similar to the neighbourhood model estimations for the mean prevailing seed dispersal direction (55.8° = 0.155 in % 2π; Table 3).
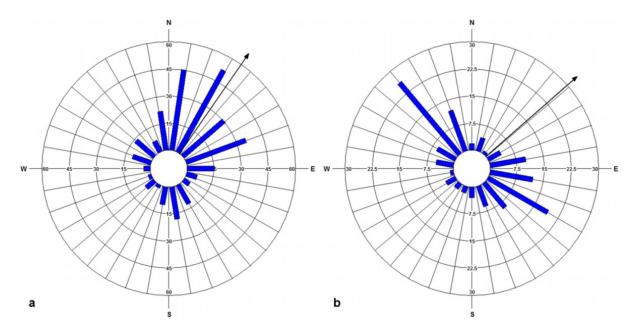
Fig 5. Circular histograms for pollen dispersal period in 20° angle classes.

Blue bars represent (a) mean pollen dispersal directions and (b) mean wind directions; Black arrows show mean directions.

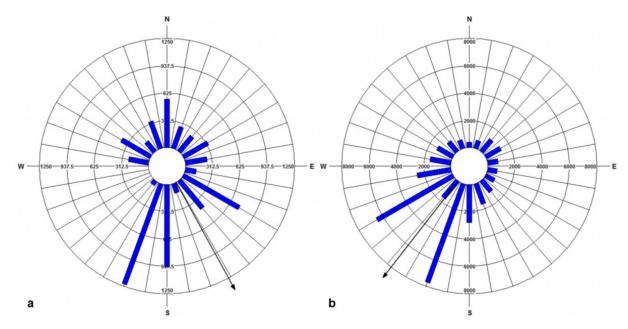**Fig 6.** Circular histograms of seed dispersal period in 20° angle classes.

Blue bars depict (a) mean seed dispersal directions weighted by dispersal distances and (b) mean wind directions weighted by wind speed; Black arrows show mean directions.

Discussion

Small and isolated populations may have limited genetic variation due to low genetic diversity, which may jeopardize their adaptive potential to various threats such as EIDs. However, common ash populations in central Europe often show high genetic diversity and low genetic differentiation due to LDD of ash seed and pollen, which may reduce the risk of small population size and increased isolation [75]. Similarly, we observed high genetic diversity in our study site in Rösenbeck. The mean H_e across the 3 genomic SSR loci was very high: 0.89 for the native adults and 0.87 for the seedlings and seeds, although lower, 0.72, for the cultivated adults. 10 EST-SSR loci showed much lower mean H_e, which was 0.37 for the cultivated and native adults and for the seedlings, and 0.35 for the seeds. Lower polymorphisms are well known in EST-SSRs because they are located within expressed genes and therefore are more conserved compared to genomic SSRs [76]. The mean H_e calculated for all 13 SSRs was higher in the native adults (0.49) and in the natural regeneration (0.48) compared to the cultivated trees (0.45) (Table 1). Using 6 neutral SSRs, high levels of gene diversities in *F. excelsior* adults (H_e = 0.767-0.818) and progenies (H_e = 0.777–0.805) have previously been reported in two sites in Ireland [77]. In the same study, planted adults were genetically not much differentiated from the native adults (F_{ST} = 0.013 in Clonee and F_{ST} = 0.014 in Kildalkey). Our pairwise F_{ST} values showed higher genetic differentiation between cultivated and native trees (0.053; Table 2). This result is in accordance with the potential clonal origin of the cultivated trees.

High levels of homozygosity have been previously reported in *F. excelsior* populations, reflecting a likely mix of Wahlund effect, null alleles, inbreeding and large allele dropout [4, 38, 78–80]. Recently, a lower level of mean F_{IS} (0.067) in ash populations in Northern Ireland has been reported [38]. Thomasset et al. [77] has also shown low levels of mean F_{IS} in two native ash populations (F_{IS} = 0.053 and 0.057), but higher mean F_{IS} in their progenies (F_{IS} = 0.131 and 0.122). In our study, the mean inbreeding coefficients for the adults and the offspring were much lower. The F_{IS} of the native adults was 0.006, lower than that of the seedlings (F_{IS} = 0.021) but higher than that of the seeds (F_{IS} = 0.002) (Table 1). The higher F_{IS} level of the seedlings may result from the sampling effect, which was along the three transect lines covering the different parts of the sampling site. Based on the same Bayesian procedure we used, Tollefsrud et al. [75] also reported very low inbreeding levels in 42 natural ash populations, even at the northern range margins, and attributed the differences in F_{IS} values among studies to the differences in SSR loci and methods used.

Categorical parentage approaches are powerful in detecting gene flow when microsatellite markers are highly polymorphic and sampling is efficient [81]. However, they may be inefficient to discriminate local and background pollen and seed sources if exclusion probabilities of genetic markers are not sufficient [82]. Unassigned parentages often occur due to both unsampled parents and sampled but not assigned local parents. Additionally, microsatellites are often prone to genotyping errors including null alleles [83] and mutations [84], and cryptic gene flow may lead to false-positive assignments [85]. This bias is expected to be lowered using mating models since they estimate gene dispersal parameters from probability models and can account for observed frequencies of multilocus genotypes in offspring, rather than from directly inferred parent-offspring pairs [86]. The empirical distributions of effective dispersal distances may also be biased by spatial distribution of individuals in a population [87]. The neighbourhood model can simultaneously estimate the rate of immigrants and mating parameters from given allele frequencies when accounting for the spatial arrangement of individuals and other available information on reproductive success [82]. For that reason, we applied two approaches to investigate seed and pollen dispersal patterns in our study.

The overall successes of the both parentage analyses using CERVUS were high in our study. Not only because using 13 loci with no missing allelic information provided us high exclusion probabilities (0.999), but also because the exhaustive sampling scheme of the adult trees played a role in increasing the chances of sampling the true parents. We also allowed mismatches (1 %) in the parentage analyses to increase the overall success rate [69]. We confidently assigned 66.4 % of the seeds to their unique fathers, 48.2 % of the seedlings to their unique parent pairs and an additional 27.9 % of the seedlings to their single parents (Fig 2). These assignment rates are high compared to the two most recent studies reporting on gene flow in ash using the same parentage analysis software. Taking Δ as a confidence measure and assuming 10 % of the potential parents sampled, Thomasset et al. [77] assigned 11.5-15 % of the seeds to their unique fathers at 80 % confidence level using 6 microsatellite loci. However, higher rates of sampling potential parents (95 %) with 7 loci resulted in higher assignment rates of the ash saplings to their parent pairs (44.1 %) in Northern Ireland, at least in one site [38]. Therefore, the success rate of the assignments is highly influenced by the number of adults sampled, marker loci used and confidence level chosen. However, CERVUS left respectively 33.6 % of the paternities and 23.9 % of the parent pairs unassigned (Fig 2) and the estimated mean null allele rate was 13 % overall. The neighbourhood model estimated 3.2 % and 45.5 % of seed and pollen immigration from outside of the study site, respectively

(Fig 2; Table 3). In case of seed immigration, this result is consistent with the CERVUS assignments which had negative LOD scores and were treated as putatively from outside (2 %; Fig 2). The neighbourhood model inferred that mothers of the remaining seedlings (21.9 %) unassigned by CERVUS were also sourced from the local native trees (Fig 2). The NMπ estimation for pollen immigration was higher (45.5 %) compared to the CERVUS estimation (33.6 %), which may indicate the presence of false-positive assignments among the assigned candidate fathers with 80 % confidence (Fig 2). On the other hand, the model used here is spatially explicit and assumes that male mating success decreases exponentially with increasing distance from mother and seedling [34, 35]. However, distance alone often explains a relatively moderate portion of the variation in mating patterns [37], and model predictions can be improved with additional parameters influencing individual mating success [72]. Using the neighbourhood model approach based on a 3000 m radius neighbourhood, Bacles et al. [4] reported higher pollen immigration (53 %) in a severely fragmented population of *F. excelsior* in Scotland. When including the reproductive success parameters such as plant size and flowering intensity to the neighbourhood model, Goto et al. [88] estimated 52.5 % of pollen and 31.5 % of seed immigration in *F. mandschurica* in a 10.5 ha plot in Japan. However, since the estimated immigration rates are sensitive to the defined neighbourhood size and the selection gradients influencing male mating success, model comparisons still remain as a challenge [72]. The results overall indicate that ash propagule, particularly pollen, has a great capacity for LDD. This is consistent with the complete sampling scheme of the study site and the demography of ash in a radius of 5 km around the study site. The cumulative probability distribution of pollen dispersal distances based on the best-fitting dispersal kernel (Fig 4) also provides a good support for the species' LDD potential.

Molecular markers provide enough information to assign parentage to offspring. However, reliable identification of pollen and seed dispersal distances requires additional information regarding the gender of the parental trees. This is relatively easy for the pollen dispersals as long as prior information about the genotypes of mother trees from which the seeds were collected is available. It presents a greater challenge to determine pollen and seed parents of established seedlings. Seed trapping can provide information about the genotype of maternal tissue [89, 90] that can only be informative for seeds. The number of dispersed seeds, their survival and recovery rates via seed tagging are also shown to be very low [91]. Bacles et al. [5] calculated seed dispersal distances among fragmented populations of *F. excelsior* based on a prior assumption of the nearest, or the single parent identified being the

maternal parent. However, this assumption may not always be true and therefore may cause an erroneous conclusion such that seed dispersal distances are always shorter than pollen dispersal distances [37]. Additionally, chloroplast haplotypes only provide limited help to identify the seed trees in ash [38] because their polymorphism levels at regional scales are very low, and a single haplotype dominates in most regions [39]. Two-year macroscopic observations of ash flowers in the study site made the gender identification of the adult trees possible in most of the cases: 89 % of assigned paternities and 91 % of the assigned parent pairs were confirmed by our field observations. We also accounted for the genders of the adult trees in the neighbourhood model estimations and the inferred parentages. This provided more accurate estimations of the dispersal patterns in the study site.

Plant improvement is based on the breeding of plants with desirable traits; however, gene flow from domesticated species cannot be regarded as universally beneficial [92]. In some cases, interpopulation crosses result in lowered fitness in the next generations that can be attributed to the disruption of local adaptation [93]. For this reason, pollen contamination via immigration of genes from domesticated populations to natural populations, and gene conservation reserves particularly, raises concern [10]. *F. excelsior* is one of the tree species silviculturally managed in the last 30–40 years in Europe, mostly due to its high quality wood [94]. It is also extensively used in urban landscape planting because of the aesthetic characteristics desired from urban trees such as autumn colour, attractive bark and flowers [95]. Planting density in ash varies across Europe from 500 to 10 000 trees per ha [20]. According to the National Forest Inventory, from 2002 to 2012, the area covered with ash in Germany increased by 17.4 % (250 000 ha), which represents 2.4 % of the total forest area [96]. Accordingly, measuring gene flow in the landscapes can help us infer the potential impacts of plantation practices. Using two approaches to parentage analysis, we estimated very limited gene flow from the cultivated trees to the native trees (1.2–2 %; Fig 2) in our study site. The majority of the gene flow occurred in the landscape among the native trees (Fig 2). This result may not be surprising due to the small size of the plantation compared to the native stand. In the two ash sites in Ireland, paternity assignments of the seeds revealed similar mating success of introduced and native trees and the offspring of native and planted trees were sired by native and planted trees [77]. In the same study, partial temporal isolation between planted and native trees better explained the structure of the pollen clouds. However, that was not an indication in our case. Flower observations were conducted twice (one per year) in our study site during the peak flowering time for both planted and native trees. In 2014, the year when the seeds were collected, we observed the flowers between the 11th and

17th of April. The German Weather Service also recorded first ash pollen release in the region on the 15th of April in 2014 (Station ID: 9247) (Available from: ftp://ftp-cdc.dwd.de/pub/CDC/observations_germany/phenology/annual_reporters/wild/historical/PH_ Jahresmelder_Wildwachsende_Pflanze_Esche_1951_2015_hist.txt). The small stand size of the cultivated trees compared to the native stand, their relative distance from the native mothers and the local wind direction may have affected the pollen clouds in our study site. On the other hand, Steinitz et al. [97] estimated higher gene flow levels from *Pinus halepensis* plantations to two conspecific native populations; the more isolated population had a lower gene flow level (22 %) than the less isolated one (49 %). The differences in seed and pollen dispersal results among different sites are highly dependent on phenological synchrony and relative fecundity of trees [33, 56], spatial proximity of populations and individuals [98], size of source and sink populations [99], topography of sites [100], wind direction and speed [101] as well as sampling scheme [32], intrinsic characteristics of plant diaspore such as size and shape [102] and on the method of choice [103].

Short distance dispersal (SDD) mainly shapes the local distribution of genotypes [1]. LDD of pollen and seed are regarded as critical in understanding many processes such as habitat fragmentation, genetic differentiation of populations, evolutionary responses to climate change and biological invasions [104]. The ability of trees for LDD is suggested to possibly promote their adaptive evolution in new environments through increasing genetic variation for fitness [48]. Seed dispersal is of special interest because a diploid seed carries twice the genetic material compared to a pollen grain [7], and especially because it is crucial for the ability of species to continuously colonize suitable sites in the mosaic of different microenvironments across managed and unmanaged forests and landscapes. In plants, pollen and seed dispersal typically follow a fat-tailed or leptokurtic distribution, indicating frequent nearest-neighbour mating along with rare LDD [105]. In our study, the estimated pollen dispersal kernel also followed a fat-tailed distribution ($b_p = 0.269$, $b < 1$; Table 3), with an average pollen dispersal distance of 571 m (Table 4). However, the seed dispersal distribution followed the Gaussian or a platykurtic kernel ($b_s = 2.085$, $b = 2$; Table 3), indicating that the mean seed density declined more rapidly beyond the mean dispersal distance of 39 m (Table 4). Based on the neighbourhood approach, Bacles et al. [4] reported a fat-tailed pollen dispersal kernel with a mean dispersal distance of 328 m in a severely fragmented population of *F. excelsior* in Scotland. In the same population, however, the predicted seed dispersal distribution was also fat-tailed, suggesting that the seed dispersal may also spread over tens of kilometres, and the authors concluded that deforested landscapes favours LDD of seed due to

the increased likelihood of winged-seed to be uplifted [5]. However, these results, including ours, were based on a single year and lack of inter-annual replications in the study sites and may present a limitation to the estimations because spatial and mating system parameters show significant temporal variation and the regeneration of natural stands generally takes years [106]. Based on CERVUS assignments, we detected 50 % of the effective pollen dispersed more than 44 m from the source trees, and 5 % beyond 467 m, up to 939 m (Fig 3a) whereas 50 % of the seeds were dispersed within 31 m from the source, and 5 % beyond 108 m, up to 473 m (Fig 3b). However, NMπ- computed distributions of the dispersal distances in the study site were both shorter, particularly for the seed dispersal distances (Fig 3a, b). The difference between the observed effective pollen dispersal distributions and the estimated dispersal kernels is due to their definitions: they represent backward and forward dispersal kernels, respectively, and thus different notions to describe the dispersal process [107]. On the other hand, LDD in different *F. excelsior* populations has been previously reported for both pollen [6,77] and seed [38] using parentage analysis approaches. The results overall suggest that gene flow in ash can occur in a wide range of distance, from tens or hundreds of meters to thousands. This is promising for the future existence of the species in our ecosystems considering ongoing habitat fragmentation, climate change and ash dieback. However, it also complicates the in situ gene conservation practices of the species.

Particularly for wind-mediated gene flow, available weather data could help to identify the potential patterns of pollen and seed dispersals in landscapes, and to better understand the meteorological factors driving pollen and seed emission and spread [48]. Using the previous knowledge of the weather conditions favouring propagule release and dispersal could also provide more accuracy in timing of these events. Among these, dry and windy weather conditions are stated to promote LDD of seeds [40–43]. Similarly, clear warm days with low relative humidity, especially from midday to late afternoon (10 am-6 pm), are also reported as most favourable for pollen release [44–46]. Our results showed that dry and windy weather conditions influenced the pollen and seed dispersal patterns in our study site. There was a significant positive correlation between the dispersal distances of the seeds and strong winds (0.715; $p < 0.01$; Table 5), indicating that frequent strong winds favoured LDD of seeds. Heydel et al. [101] pointed out a mainly positive relationship between horizontal wind speed and LDD in both forests and open landscapes, and showed the increase in the strength of the relationship with seed terminal velocity (V_{term}) during the fall. According to their results, turbulence is positively correlated to LDD for the species with Vterm $\geq$ 0.8 m/s and the effect of LDD changes from positive (Vterm = 3.2 m/s) to negative (Vterm $\leq$ 0.4 m/s). This is

consistent with our results when considering the medium V_{term} of *F. excelsior* (1.58 m/s). However, pollen dispersal distances were negatively correlated with the wind speeds (Table 5). This may not be surprising because maximum turbulence above the canopy often occurs in the afternoon when maximum pollen release also happens [108], and mechanical turbulence dominating at high wind speeds often results in downdrafts [109]. Other factors including propagule release height, vegetation type, topography, and landscape terrain are also previously mentioned as likely to influence LDD of plant propagules [101]. The ability of ash trees to maintain part of their fruits long after leaf shed may favour LDD of seeds that are only released during heavy autumn or winter storms. However, investigating the relative effects of these factors on dispersal events is beyond the scope of our study. In the study site, both pollen and seeds were dispersed in almost all directions in accordance with the actual wind directions (Figs 5 and 6). The mean direction of pollen dispersal had a good agreement with the mean wind direction, both towards the northeast (Fig 5). Similarly, the model estimations for the mean distributions of pollen and seed dispersal also indicated directionality, both towards the northeast (Table 3), in agreement with the prevailing wind direction in the study site. This may indicate unequal mating success of the males along with restricted dispersal in the study site, predominantly driven by the prevailing winds. Nonetheless, the mean direction of seed dispersal was towards the southeast while the mean wind direction was towards the southwest, when incorporating the dispersal distances and the wind speeds (Fig 6). This could be because of the unidentified LDD of seed in the angle classes towards the southwest direction, pulling the mean towards the southeast direction. Furthermore, the wind data used for the seed dispersal period was from a long-term data set, covering four dispersal seasons in between October and February. Nevertheless, overall findings were informative to understand the effect of dry and windy weather conditions on the propagule dispersals in our study site.

    H. fraxineus has spread across most of its distribution area in Europe within the last couple of decades, and is threatening *F. excelsior*'s existence and its associated ecosystem [19,110]. The emerald ash borer (EAB), *Agrilus planipennis* (Coleoptera, Buprestidae), on the other hand, has been predicted to cross the western border of Russia soon [111]. The fungus-weakened ash is expected to be more vulnerable to EAB attacks [112]. High standing genetic variation together with the ability for LDD of *F. excelsior* is promising for the species' existence in the European forest ecosystems and for its adaptive potential. Recent reports on the presence of genetic variation in dieback tolerance among ash trees [29,113] suggest the species' ability to recover from ongoing epidemic dieback [27]. However, the standing

genetic variation will greatly depend on the individuals' survival and reproduction, and the speed of population recovery will largely depend on the extent of pollen- and seed- mediated gene flow. Therefore, it presents importance to estimate gene flow patterns of ash populations in European landscapes based on a good experimental design and on combined analytical approaches to provide reliable information for the conservation management of ash.

Conclusion

Our results indicated an effective landscape process of gene flow in ash including the LDD of pollen and seed and the effect of the wind conditions on the dispersal patterns. In our study, the cultivated roadside trees in the landscape only provided limited gene flow into the old growth forest. However, our results still suggest that gene conservation stands should be planned carefully in ash, considering the LDD of ash propagule. A maximum of 100 m distance among trees may be recommendable for effective enrichment plantings in order to facilitate gene flow from healthy to unhealthy individuals. However, our results are based on a single small stand of *F. excelsior*, and the relative distributions of individuals, sampling design, the method of choice and local wind conditions seem to influence the estimated seed and pollen dispersal patterns in the study site. A combination of different analytical approaches to parentage analysis can provide a better resolution to gene flow studies. Available wind data from weather stations in proximity can be utilized for planning purposes.

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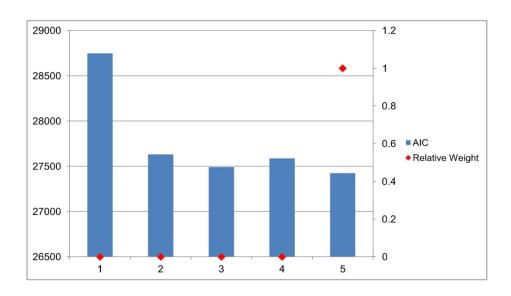
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Supporting Information

S3 Table. The Neighbourhood model comparisons.

	Log-L	n parameters	AIC	Delta AIC	Weight	Relative Weight
Null (ms, mp, errors)	-14362.8	11	28747.6	1322.9	5.444E-288	5.444E-288
Null + exponential kernels	-13803.11	13	27632.22	207.52	8.66173E-46	8.66173E-46
Null + exponential kernels + directionality	-13727.79	17	27489.58	64.88	8.15618E-15	8.15618E-15
Null + exponential-power kernels	-13779.13	15	27588.26	163.56	3.04367E-36	3.04367E-36
Null + exponential-power kernels + directionality	-13693.35	19	27424.7	0	1	1

Paper 2

Seed and pollen dispersal and reproductive success in ash forests affected by ash dieback: implications for conservation and restoration

Seed and pollen dispersal and reproductive success in ash (*Fraxinus excelsior* L.) forests affected by ash dieback: implications for conservation and restoration

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Abstract

The emerging infectious pathogen *Hymenoscyphus fraxineus* is devastating common ash (*Fraxinus excelsior* L.) populations across European forests. Artificial selection for tolerant ash trees has been initiated in several countries with the objective of establishing new tolerant ash forests by replacing damaged populations with tolerant plantings, or by enriching old ash forests with tolerant trees that can disperse their resistance genes via seeds and pollen into surrounding forests. The success of an enrichment strategy will depend on survival, reproduction, and seed and pollen dispersal of the planted trees as well as the presence and frequency of resistance genes, heritability of resistance, and how the variation levels of resistance translate into fitness. Here, we study gene flow and reproductive success in a Danish forest with high frequency of ash. We estimate the mean seed and pollen dispersal distances of ash as 67 m and 347 m, respectively. We also study a specific planted stand (seed

orchard offspring) and show that significant amount of seeds and pollen are dispersed from the stand into the surrounding forest, and the frequency of seed and pollen dispersal decreases with increasing distance from the planted stand. Using parentage analysis, we show that male and female trees with few symptoms of ash dieback are overrepresented as the parents of randomly sampled seedlings in the forest. We conclude that variation in reproductive success among the mature trees may create ongoing natural selection in favour of lowered susceptibility to ash dieback in European forests. We discuss our findings in the context of conservation of ash forests challenged by ash dieback.

Introduction

Common ash (*Fraxinus excelsior* L., hereinafter ash) is a valuable hardwood species of the European forest ecosystems and open landscapes, providing high-quality timber, landscape aesthetics, ecosystem services and biodiversity (FRAXIGEN 2005, Dobrowolska et al. 2011; Skovsgaard et al. 2017). Ash is important in both primary and secondary succession and crucial in forest communities since it often occurs in mixed stands together with the other broadleaved species such as beech, oak, sycamore and alder (Dobrowolska et al. 2011). Including 44 obligate and 62 highly associated species, a total of 953 species were identified as associated with *F. excelsior* (Mitchell et al. 2014). Ash has medicinal benefits due to its bark and leaf containing chemical compounds (Pliūra & Heuertz 2003) and leaves are nutritious as animal feed (FRAXIGEN 2005). Ash can grow well on many different site types, including dry, shallow calcareous soils and moist, fertile, non-acidic alluvial soils (Skovsgaard et al. 2017).

The influence of ash dieback

Ash 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 the cambium, discoloration in bark and epicormic shooting are the typical symptoms of the disease (McKinney et al. 2014; Skovsgaard et al. 2017). The 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, the disease symptoms were first noticed in 2002 (Thomsen & Skovsgaard 2012) but the disease

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was already widespread already by 2005, particularly in young stands (Skovsgaard et al. 2010).

Ash dieback is currently regarded as one of the most serious health problems of the European forests (Marçais et al. 2017) and a threat to European biodiversity (Pautasso et al. 2013). There is overwhelming evidence of the severity of the problem across Europe. Based on surveys conducted in France and Belgium in 2010, Marçais et al. (2017) reported up to 35 % annual mortality in young ash stands (< 5 cm dbh) 5-6 years after the pathogen arrival. In the same study, the mortality in the older stands was lower (3-11 %) and decreased as tree diameter increased. In Lithuania, the mortality rate in three different 8-year old progeny trials reached 90 % in 2010, only five years after their establishments in 2005 (Pliūra et al. 2011). A dramatic decline from 2009 to 2012 was reported in the ash tree populations in an 8 ha old forest patch in Estonia, where 48 % of the overstory and 39 % of the subcanopy trees died (Lõhmus & Runnel 2014). In northwestern Germany, the mortality rates of the surveyed stands with various age changed between 2 % and 39 % in 2015 (Enderle et al. 2017). In south-eastern Germany, the monitoring of 22 ash stands between 2010 and 2014 revealed that the number of trees categorized as dying or dead was up to 95 % in young stands, up to 78 % in pole stands and about 30 % in matured forests (Lenz et al. 2016). On the other hand, Kessler et al. (2012) reported a slow progression (5 %) in mean disease intensity in mature ash trees in 14 monitoring plots in lower Austria between 2008 and 2010 (as cited in Kirisits & Freinschlag 2012). In 22 forest observation plots with the density of ash trees ≥ 50 % in Switzerland, 50 % of the trees (dbh ≥ 10 cm) without previous symptoms were affected by the fungus in 2015 (Queloz et al. 2017). The mortality rate in 8 monitoring plots in Norway escalated to 52 % during 2009–2015, and was especially high among the youngest trees (Børja et al. 2017). Davydenko & Meshkova (2017) reported a 69 % increase in the disease prevalence in 6 stands with the age of 28-80 years in different regions in Ukraine from 2011 to 2015. In a Swedish study site, 90 % of the monitored veteran trees were affected by ash dieback in 2015 in comparison to 62 % in 2009, and the observed mortality rate was 11 % (Bengtsson & Stenström 2017). In a Danish field trial planted in 2004, tree mortality increased from 5 % to 70 % between 2006 and 2013 (McKinney et al. 2014).

Clonal and progeny trials of ash in Europe have revealed genetic variation in dieback susceptibility with both narrow- and broad-sense heritabilities often reported above 0.4 (Cleary et al., 2014; Enderle et al. 2014; Kjaer et al. 2012; Lobo et al. 2014; Lobo et al. 2015; McKinney et al. 2011; Muñoz et al. 2016; Pliūra et al. 2011; Stener 2013), verifying that the disease resistance is partially under genetic control and inherited from parents to offspring. It

has therefore been suggested that genetic variation in resistance may prove to be a long-term solution to ash dieback based on natural selection assisted by artificial selection (McKinney et al. 2014). However, the frequency of trees with reasonable to high levels of genetic resistance is expected to be low (1–5 %) within natural populations, and the relationship between ash dieback resistance and realised fitness has so far not been studied beyond the mortality found in young trees (Kjær et al. 2012; McKinney et al. 2014). Lack of this information makes it difficult to assess the likelihood of the recovery of ash in competition with other species in mixed European forests. The present paper addresses the relationship between variation in ash dieback symptoms and reproductive fitness.

Artificial selection of tolerant ash genetic material has been initiated in several European countries, including Austria (Heinze et al. 2017), Belgium (Sioen et al. 2017), Switzerland (Queloz et al. 2017), Czech Republic (Rozsypálek et al. 2017), Germany (Enderle et al. 2017), Denmark (Kjær et al. 2017), Ireland (McCracken et al. 2017), Lithuania (Pliūra et al. 2017), Poland (Gil et al. 2017), Sweden (Cleary et al. 2017), Slovakia (Longauerová et al. 2017), Great Britain (Clark & Webber 2017) and the Netherlands (De Vries & Kopinga 2017). The objective is, in general, to develop new plantings with improved health that can replace existing unhealthy ash forests.

Future plantings with offspring from the tree selection programs may also enrich existing ash forests, if improved trees are planted adjacent to or intermixed with old ash forests. However, the efficiency of such an approach will depend on the extent to which resistance genes spread via pollen or seeds from improved plantings into old-growth surrounding forests.

In plants, the complex process of gene flow incorporates biotic and abiotic interactions affecting mating, propagule production, dispersal, establishment, survival and maturation (Burczyk et al. 2004). Phenological synchrony and relative fecundity of individuals (Adams and Birkes 1991; Bochenek & Eriksen 2011; Chybicki & Burczyk 2013), 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 & 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 the dispersal patterns of pollen and seeds.

Will sexual selection and gene flow increase the resilience of the ash forests in the face of ash dieback?

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 the ash forest suffering from ash dieback, including from planted stands to the older surrounding ash forest. Our hypotheses are: (i) the planted stands with tolerant trees can enrich the surrounding forest in proximity with resistance genes through pollen and seed dispersal; (ii) tolerant trees have higher reproductive success compared to damaged trees and therefore they contribute disproportionally more to the next generation recruitments. We applied a spatially explicit mating model to determine the mating parameters and the dispersal distances of pollen and seeds in our study site. 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. We discuss our results in relation to the conservation and use of ash genetic resources under the pressure of ash dieback.

Materials and methods

Study populations and sampling design

The study site is located in Valby Hegn, Helsinge, Denmark (56°03'17" N 12°13'52" E) (Figure 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 (Figure 1). The central part of the study site is a 1.0 ha planted ash stand from 1958, which is one of the first plantings in Denmark based on seed from one of the oldest ash seed orchards (FP202), established in 1947 and consisting of 8 clones (Nielsen et al. 2009). A second 0.4 ha planted stand with the same origin and age is located in the northern part of the forest (see Fig. 1). The older ash planting next to the FP202 seed orchard offspring (Figure 1) dates back to 1939–1945 and are of putative Danish origin, either from natural seeding or plantings with seeds from Danish ash forests (unpublished records, The Nature Agency, Ostrupgaard, DK-3230 Græsted).

In February 2014, bud and wood tissues were sampled from 469 reproductively mature trees in the stands (approximately 80 % of the total number of mature ash trees in the study area). The sampling of the mature trees in the Western planted seed orchard offspring

(hereinafter the 'FP202 planted stand') was exhaustive. Leaves were sampled from 399 seedlings in the following spring (May 2014) along two main transects stretching from the planted stand towards the older trees (Figure 1). Transect 1 (~ 210 m) was placed in east-west direction whereas Transect 2 (~ 180 m) was placed in north-south direction (Figure 1). Finally, seeds were collected from 11 mother trees in the older ash stands around the planted stand in October 2014 (Figure 1).
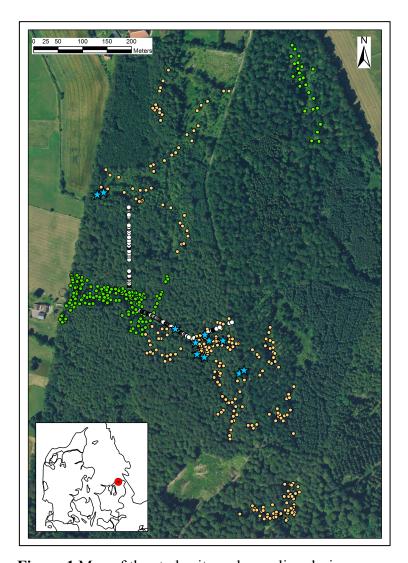
Figure 1 Map of the study site and sampling design.

Sampled adult trees (FP202 seed orchard offspring planted in 1958 are shown as green circles, while ash trees established before 1958 are marked as orange circles) and progenies (blue stars, mother trees of the seeds; 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.

Characterization of mature trees

For the mature trees, geographical coordinates, height and diameter at breast height (DBH) were recorded, and basal areas were calculated based on DBH assuming circular stems. Flowering was observed in May 2014: flower types (male, female or hermaphrodite) and flowering intensities based on a logarithmic scale, from 1 to 8, according to Kjær & Wellendorf (1997). Crown damage (ash dieback symptoms) was assessed in the summer 2014 as percent damage score (PDS) in 7 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 summer of 2014 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 the crowns. The positions of the sampled seedlings along the two transects were recorded.

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). Prior to DNA extraction, outer bark was removed and then cambium was cut into small pieces using a sterile scalpel. The extraction protocol for the wood samples included slight modifications: (1) AP1 + RNAse + Polyvinylpyrrolidone (up to 10 % end concentration) and (2) overnight incubation at 65°C. Fifty seeds per mother tree were randomly picked for DNA extractions. Seeds were embedded in water for 1–2 days for rehydration after their pericarp tissues were removed. They were then sliced into two halves with a razor blade and embryos were carefully picked up to avoid endosperm or seed coat. Leaf, bud and embryo samples were ground in 96-well plates with liquid nitrogen and 3 mm stainless steel beads in TissueLyser (Qiagen), whereas wood samples were processed one by one. 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 (Aggarwal et al. 2010), and Fp12378, Fp18437, Fp20456, Fp21064 and Fp104136 (Noakes et al. 2014). The details of the PCR conditions, allele size calling and allele binning followed Semizer-Cuming et al. (2017).

Data analysis

Mating patterns of ash in the study site were investigated based on the neighbourhood model approach (Adams & Birkes 1991) in order to utilise spatial information and demographic parameters in the analysis. The NMπ (Chybicki 2018) software was used to estimate mating system parameters and to reconstruct genealogies for progenies. The mating system parameters, including self-fertilization rate, seed and pollen immigration rates and dispersal kernel parameters were estimated, while accounting for the spatial positions of the individuals. The gender expressions ('femaleness') of the putative parents were included in the model where 1 denoted females, 0 described males and 0.5 indicated hermaphrodites and unknown genders. The standardized phenotypic variables including flowering intensity, fruit set, height, basal area and PDS of the putative parents were incorporated into the parentage model as covariates of male and/or female reproductive success. The effects of these phenotypic variables on reproductive success were estimated in terms of selection gradients assuming the model of multiplicative fitness components (Smouse et al. 1999; Chybicki 2018). In this model, the fitness of the *i*-th individual is proportional to the exponential function of presumed fitness covariates (measured phenotype characters), i.e. $\varphi_i = \exp\left(\sum_{k=1}^{K} \beta_k x_{ik}\right)$, where β_k is the effect of the *k*-th character on the fitness and x_{ik} is the value of the k-th character measure for the i-th individual. To model the dispersal process, we adopted the exponential-power kernel, which offers enough flexibility to fit different shapes and scales of the actual probability distribution of dispersal distances (Nathan et al. 2012; Bullock et al. 2017). A stepwise approach was applied for estimating the model parameters and selecting the best fitting model. At first, marker specific error rates, selfing rates and seed and pollen immigration rates were estimated. Using these estimated values, seed and pollen dispersal kernel parameters were computed (null model). The phenotypic variables were then incorporated into the model (full model) in order to assess the model performance with the selection gradients. A backward elimination approach was applied to select the best-fitting model, with significant phenotypic variables affecting male and/or female reproductive success. In this way we avoided data over-fitting while preserving the significant effects (Arnold 2010). 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 characterized by the highest *p*-value. The model comparisons were made according to the likelihood ratio test, based on the maximum values of the likelihood function for the competing models (Chybicki 2018). The procedure ended when any further elimination produced a significantly worse model (α =

0.05). In addition, the Akaike Information Criterion (AIC; Burnham and Anderson 2002) was used to compare model fit after penalizing model dimensionality.

Confidence intervals around parameter estimates were computed using the normal approximation with standard errors estimated based on the Hessian matrix of the log-likelihood function for the best model. Predictive cumulative probability distributions of seed and pollen dispersal distances were computed using NM+ (Chybicki & Burczyk 2010) under the best model according to the backward elimination procedure. The 50th, 95th and 99th percentiles were selected to characterize the dispersal potential of the species.

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 best inferred genealogy for each progeny was considered as true when the best genealogy was at least 4 times more likely than the second best genealogy, or the posterior probability of the inferred genealogy was 0.8 or more. The best genealogies for the seedlings were used to determine the realized contribution of the parents to the gamete pool, specifically pollen and seed dispersal from the planted seed orchard offspring to the seedlings distributed along the transect lines in the neighbouring forest (cf. Figure 1).

The average health (PDS) of the parents of the progenies (seeds and seedlings) was calculated and compared to that of the mature trees in order to study if the identified parents represent the healthier group within the mature trees. We applied a randomization approach 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 value. The significances of the differences were determined based on 999 permutations using two-tailed *p*-values.

Results

The model comparisons showed that the full model incorporating all selection gradients performed better (AIC = 37406.1, $p < 0.001$) when compared to the null model without any selection gradients (AIC = 37798.9) (Table 1). The backward elimination of the selection gradients starting from the full model (S2Table, Supporting Information) indicated that the model without tree height and female basal area performed best (AIC = 37402.5; Table 1).

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. Genes from the FP202 planting could be observed in the surrounding forest. The maternal profile of the seedlings along the

two transects (Figure 1) showed that 39 % of the seedlings that were successfully assigned parentage originated from the seeds dispersed from the trees in the planted FP202 stand, while 61 % of them came from the other trees in the area. The paternal contribution from the FP202 planting to the seedlings with assigned parentage was quite similar, 31 %, whereas 69 % of the seedlings were sired by the other trees in the area. These are the average figures for the two transects, and there was a clear pattern where the contribution from the trees in the planted FP202 stand decreased rapidly with the distance from the stand, as would be expected (Figure 2). A total of 7 % of the seeds collected from the trees 100–200 m east from the border of the FP202 planting (see Figure 1) were sired by the trees in the FP202 planting.

Table 1 Model comparison parameters for the null, full and the best model.

Model	LogL	npar	AIC	*p*-val (LR)	FS_g	PDS_g	H_g	BA_g	FI_b	PDS_b	H_b	BA_b
Null	-18880.5	19	37798.9	-	0	0	0	0	0	0	0	0
Full	-18676.1	27	37406.1	2.43E-83*	1	1	1	1	1	1	1	1
Best	-18677.2	24	37402.5	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; 1/0, included/excluded selection gradient; *, *p*-value of the LR test for the difference in fit between the null full ad the null 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.
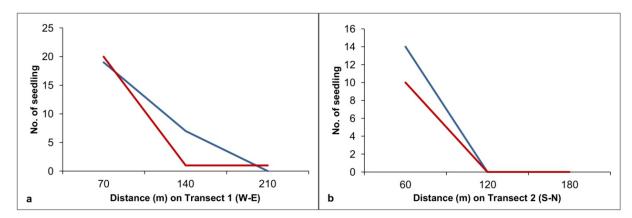
Figure 2 Gamete dispersal curves along Transects 1 (a) and 2 (b) from planted to natural population. Blue lines show seedlings where both parents are from the planted stand; red lines indicate that one of the parents is from the planted stand.

The mating model parameters and the standard errors computed based on the best model are given in Table 2. The estimated dispersal kernel parameters and the approximate 95 % confidence interval around them are given in Table 3. 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-fertilization (Table 2). Both seed and pollen dispersal kernels followed a fat-tailed distribution (bs = 0.672, bp = 0.229; Table 2 and 3). The mean dispersal distances of seed (ds) and pollen (dp) were estimated as 67.2 m and 346.7 m, respectively (Table 2 and 3).

Table 2 Mating model parameters based on the best model.

Parameter	s	ms	mp	1/ds	1/dp	bs	bp
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-fertilization; ms, probability of seed immigration; mp, probability of pollen immigration, ds, mean distance of seed dispersal (m); dp, mean distance of pollen dispersal (m); bs, shape of seed dispersal kernel; bp, shape of pollen dispersal kernel.

Table 3 Dispersal kernel parameters estimated at 95 % confidence interval (Q).

Parameter	Estimate	Q (2.5 %)	Q (97.5 %)
ds	66.6	54.1	86.7
bs	0.672	0.407	0.938
dp	346.7	154.1	ND
bp	0.229	0.101	0.357

ds, mean of the seed dispersal kernel (m); bs, shape parameter of the seed dispersal kernel; dp, mean of the pollen dispersal kernel (m); bp, shape parameter of the pollen dispersal kernel; ND, not determined.

The cumulative probability distributions of seed and pollen dispersals based on the best model (Figure 3) 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.
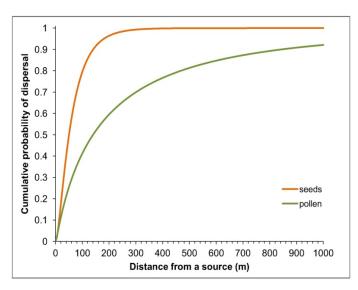
Figure 3 Cumulative probability distributions of seed and pollen dispersals.

Based on the best model predicted for the study population, the estimates for the relationship between the phenotypic characters and female and male reproductive success are provided in Table 4: observed fruit set is predicted to be positively related to realised female reproductive success (0.482), and similarly, flower intensity is also positively related to realised male reproductive success (1.246). 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 ($p < 0.05$).

Table 4 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

The estimated negative effect of crown damage on the reproductive success means that healthy trees have a higher likelihood of becoming parents for the next generation. In our study, the mean crown damage level (PDS) of the assigned mothers was only 25 % compared

to a general damage level of 57 % among the assessed females in the population. The difference was similarly large for male trees, where the assigned fathers showed and average damage level (PDS) of 21 %, again much lower than the general damage level (PDS) of 51% among males in the population. Based on the randomization test, the differences between the groups were highly significant ($p < 0.001$).

Discussion

Understanding complex mating processes and accurately estimating gene flow is important for genetic conservation and management of tree populations. Mating patterns and reproductive success in a population is expected to be influenced by several factors such as spatial positions, gender demographics, phenological synchrony and fecundity (Adams & Birkes 1991; Bochenek & Eriksen 2011; Klein et al. 2006). Several phenotypic characters influence mating success and their effects on female and male reproductive success can be estimated and used to support parentage reconstruction by the neighbourhood model (Chybicki & Burczyk 2013; Chybicki 2018). Our results on the neighbourhood model comparisons indicated that the mating model with all phenotypic selection gradients (full model) performed significantly better than the null model without any phenotypic information (ΔAIC = 392.8, $p < 0.001$; Table 1). Based on the neighbourhood model, Goto et al. (2006) also reported that the two-component models integrating phenotypic variables best explained the patterns of pollen flow in *F. mandshurica* in northern Japan (ΔAIC = 30.3-32.3). On the other hand, backward elimination of the phenotypic variables gave rise to a better model when compared to the full model (ΔAIC = 3.6; Table 1). Since our procedure relied on eliminating the selection gradients that did not differ significantly from zero, we believe the best-fitting model represents the optimum predictive model given the available information (i.e. data).

Our findings suggest that plantations can modify the gene pool of existing forests, not only by replacing them but also by exchanging genes. Depending on the objectives and the specific conditions, this may be considered as genetic pollution or genetic enrichment. The potential negative implications for gene conservation when genes from commercial ash plantings mix into old growth forests include risk of lost genetic diversity and patterns of local adaptation as previously discussed in Semizer-Cuming et al. (2017). It is therefore important to balance the improved and unimproved trees in landscapes, where native populations can serve as a locally adapted, unimproved gene pool. 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. These native ash populations can be important to ensure that rare alleles, which can be important for future adaptation to new biological challenges, are maintained outside the tree improvement programs that are based on strong selection in favour of improved resistance. Our results suggest that genes from the planted trees can spread readily over several hundred meters into the existing ash populations. As expected, the frequency of gametes originating from the planted trees decrease with increased distance from the plantation (Figure 2). This is especially the case for the seeds, while pollen can influence a large area around enrichment plantings. Based on our results, 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 have full genetic origin from the enrichment plantation, while a larger zone will contain seedlings with one of the parents originating from the enrichment trees. If parentage in the enrichment planting provides substantially improved resistance to the disease, this can allow continued strong selection in favour of such offspring among the hundreds of thousands of ash seedlings in the forest floor.

Thomasset et al. (2014) raised the conservation concern that dispersal of genes from planted into native populations may decrease local adaption. It is therefore important that future planted European ash forests are adapted to the 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 the 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 from in many different eco-regions.

Gene flow may also be important in promoting adaptation to new abiotic stress caused by climate change (Aitken et al. 2008). The existing ash forests may also benefit from the enrichment with new genetic origins from the areas adapted to a climate that is close to the predicted future climate. Our estimates of the dispersal kernels with a fat-tailed distribution (bs and bp < 1; Table 2) suggest that seed and pollen dispersal in ash are composed of frequent short distance dispersals combined with rare long distance dispersals (LDDs). This result is in correspondence with the previous studies on *F. excelsior* (Bacles et al. 2005; 2006; Bacles & Ennos 2008; Beatty et al. 2015; Heuertz et al. 2003; Semizer-Cuming et al. 2017).

The capacity of ash for LDDs is encouraging in terms of the species' adaptive potential to persisting new environments. Gene flow from populations translocated from milder to colder environments may thus provide a source of pre-adapted alleles into conservation populations once they reach reproductive maturity (Aitken et al. 2008).

Pollen and seed dispersal distances are also important to the risk of fragmentation of ash populations in future landscapes as a consequence of ash dieback. LDD of plant propagules, especially of seeds, is vital for species' colonization at suitable sites in managed and unmanaged forests and open landscapes (Cain et al. 2000; Kramer et al. 2008). Our study showed that the distance of 100–140 m could be an effective barrier for seed dispersal within a forest. Therefore, based on the estimated seed dispersal kernel, we should not expect effective long distance seed dispersal that may lead to gene exchange between forest fragments. On the other hand, pollen showed a great potential to travel very long distances as indicated by the 95 % and 99 % quantiles of the pollen dispersal kernel of 1.3 km and 3 km, respectively. Similarly, in a severely fragmented *F. excelsior* population in Scotland, the mean pollen dispersal distance was 328 m based on the neighbourhood model (Bacles et al. 2005) and reached up to 1.9 km based on the maximum-likelihood (ML) paternity analysis (Bacles & Ennos 2008). In the same population, 8 % of the effective seed dispersal was originated from the dispersals between 100 m and 3 km (Bacles et al. 2006). In the fragmented landscape of Rösenbeck in Germany, the estimated mean dispersal distance of *F. excelsior* pollen was even greater (571 m) based on the neighbourhood approach (Semizer-Cuming et al. 2017). With respect to our study, the same model estimated a shorter average dispersal distance of pollen (197 m) 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 dispersals in ash occurs in short distances, rare LDDs over hundreds and thousands of meters, in particular of pollen, may still keep fragmented ash populations connected with each other in future landscapes.

The realized effect of enrichment plantings in the long time span depends on the fitness of the introduced trees. In the first generation, this will be determined by the fitness of the introduced trees relative to the fitness of the existing trees, and in the second generation, by the fitness of the offspring (seedlings) of the introduced trees and cross-pollinations relative to the fitness of the offspring of the existing trees. Our results showed that fruit set, flowering intensity, health and size (basal area) all contribute to the individuals' reproductive success (Table 4). Total seed production was also correlated with maternal resources in *Plantago coronopus*, a widespread short-lived herb with dimorphic seeds (Braza et al. 2010).

Bateman's principle (Bateman 1948) states that female reproductive success is limited by the amount of resources available to produce progeny whereas male reproductive success is limited by the number of matings achieved and increases with more mating opportunities (Burd et al. 1994). In the study population, more reproductively successful males flowered more intensively and thus produced more pollen. Similar to our results, Goto et al. (2006) reported that flowering intensity affected male reproductive success of Japanese Manchurian ash and males with low flowering intensity contributed little to the pollination. In a clonal seed orchard of Douglas fir (*Pseudotsuga menziesii*) in France, male reproductive success increased with male flowering intensity as well as proximity and phenology overlap with mother trees (Burczyk & Prat 1997). Among six floral morphological characters, flower production positively influenced male fertility in insect pollinated wild radish (*Raphanus raphanistrum*)(Morgan & Conner 2001). On the other hand, morphological features correlated with flower number were found poor predictors of male reproductive success in a population of *Chamaelirium luteum* in Orange County in North Carolina, USA (Smouse et al. 1999).

The most novel finding in our study is probably that crown damage due to ash dieback reduces reproductive success; likely triggering a sexual selection in favour of improved resistance, but the intensity of the effect was stronger on females (-0.674) than on males (-0.238). This may have resulted from intrinsic differences between maternal and paternal investments since the cost of reproduction is usually higher for females than for males (Obeso 2002). If the resources are limited due to crown damage and the reproduction is much costlier for females, unhealthy females cannot produce enough seeds while some of their male counterparts can still produce enough pollen to reproduce. It has been previously shown that plants can occasionally enhance flowering under stress conditions as an emergency response in order to produce the next generation (Wada & Takeno 2010). The biotic stress caused by *H. fraxineus* may lead ash individuals to compromise between reproduction and defence response (Denancé et al. 2013) and this may happen differently in the two genders. This difference may slow down the process of natural selection acting upon dieback tolerance in subsequent generations.

We expected that larger trees would be reproductively more successful because they in general will be older and can invest more resources in reproduction. However, basal area was negatively related to male reproductive success and not significantly related to female reproductive success in our study. Goto et al. (2006) reported a positive effect of tree size (DBH) on the reproductive success of male Manchurian ash in Japan; however, the trees with greater than 40 cm of DBH contributed equally to the seed production. Tree fecundity was

positively influenced by tree size in pedunculate oak (*Quercus robur*) in the mountain ranges of central Spain (Moracho et al. 2018). Nonetheless, in a temperate forest of Japan, individual tree size did not have a clear positive relationship with seed development in a heterodichogamous maple (*Acer mono*), suggesting that seed development in this species is regulated more by pollination than by individual resources (Shibata et al. 2009). It makes sense that resources are allocated to survival and reproduction instead of wood production when increased growth stops offering competitive advantage to individuals (Becker et al. 2000). Size may also be a poor predictor of reproductive success under extreme or competitive neighbourhood conditions (Aarssen 2015). The findings in the present study support that direct measures of fitness are important to supplement the simple measure of biomass growth in order to obtain an appropriate surrogate for fitness, as concluded by Younginger et al. (2017).

Estimating overall fitness is challenging under natural conditions, especially when it is difficult to track the fate of an individual's offspring to reproductive maturity (Campbell et al. 2017). The fitness of the offspring itself was not investigated in this study, but reconstructed genealogies allowed us to assess the health status of the trees that successfully contributed to the progeny generation. We observed a pronounced pattern where healthy trees where, were overrepresented as parents for the next generation. The assigned parents on average only showed half the level of crown damage compared of the general damage level in the study population. The implication is that we expect that the next generation will have reduced susceptibility to ash dieback, because the trait is known to be heritable as discussed in the introduction above. However, so far estimates of narrow-sense heritability have been based on studies of half sib families grown in plantations. Lobo et al. (2015) applied parent-offspring regression to verify that resistance are 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 correlation and estimated narrow sense heritability close to zero. Although our results clearly suggest that ongoing sexual selection takes place in in favour of healthy trees in the damaged ash forests, it is therefore still not possible to estimate how much this in itself can increase the resistance level of the next generation. We do expect that selection in favour of young trees with high level of resistance will continue as the seedlings grow older (zygotic selection), because high heritability with higher mortality among susceptible offspring compared to more resistant offspring has been documented from several trials based on relatively young trees (Kjær et al. 2012; Pliūra et al. 2011; Lobo et al. 2014; Muñoz et al. 2016). The fact healthy trees are

overrepresented in the regrowth is therefore very positive because the frequency of trees with very high level of resistance is low in the native ash forests.

To conclude, our results suggest that we can expect the next generation of ash trees from natural seed fall will have improved levels of tolerance compared to their parental generation due to the interplay between sexual and zygotic selection. The extensive gene flow, especially pollen flow, is expected to prevent small effective population sizes and therefore reduction in genetic variation and random genetic drift. This is encouraging for the future health of the species in the European forests, although we still cannot estimate if the speed of this predicted progress is natural resistance and 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 interventions may thus prove a valuable tool to ensure the future existence of the species in European forests and ecosystems.

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Supporting Information

S2Table. 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	*p*-value
0	M0	-18676.1	27	37406.1	1	1	1	1	1	1	1	1	NA
1	M1	-18704	26	37460.04	0	1	1	1	1	1	1	1	7.47E-14
1	M2	-18694.6	26	37441.14	1	0	1	1	1	1	1	1	1.16E-09
1	M3	-18676.1	26	37404.16	1	1	0	1	1	1	1	1	0.806496
1	M4	-18676.1	26	37404.12	1	1	1	0	1	1	1	1	0.887537
1	M5	-18727.4	26	37506.84	1	1	1	1	0	1	1	1	3.82E-24
1	M6	-18678.9	26	37409.7	1	1	1	1	1	0	1	1	0.01796
1	M7	-18677.1	26	37406.2	1	1	1	1	1	1	0	1	0.147299
1	M8	-18683.6	26	37419.1	1	1	1	1	1	1	1	0	0.000108
2	M9	-18705.5	25	37461.08	0	1	1	0	1	1	1	1	1.61E-14
2	M10	-18694.8	25	37439.58	1	0	1	0	1	1	1	1	9.33E-10
2	M11	-18676.2	25	37402.34	1	1	0	0	1	1	1	1	0.63904
2	M12	-18727.5	25	37504.9	1	1	1	0	0	1	1	1	3.75E-24
2	M13	-18678.9	25	37407.7	1	1	1	0	1	0	1	1	0.018167
2	M14	-18677.1	25	37404.22	1	1	1	0	1	1	0	1	0.147299
2	M15	-18683.6	25	37417.1	1	1	1	0	1	1	1	0	0.000109
3	M16	-18707.9	24	37463.88	0	1	0	0	1	1	1	1	1.57E-15
3	M17	-18697.1	24	37442.12	1	0	0	0	1	1	1	1	1.02E-10
3	M18	-18727.6	24	37503.18	1	1	0	0	0	1	1	1	3.63E-24
3	M19	-18679	24	37405.94	1	1	0	0	1	0	1	1	0.01796
3	M20	-18677.2	24	37402.48	1	1	0	0	1	1	0	1	0.143502
3	M21	-18683.7	24	37415.36	1	1	0	0	1	1	1	0	0.000106
4	M22	-18709	23	37464.04	0	1	0	0	1	1	0	1	1.56E-15
4	M23	-18698.4	23	37442.72	1	0	0	0	1	1	0	1	8.07E-11
4	M24	-18731.5	23	37508.96	1	1	0	0	0	1	0	1	2.11E-25
4	M25	-18681.1	23	37408.16	1	1	0	0	1	0	0	1	0.005584
4	M26	-18684.2	23	37414.48	1	1	0	0	1	1	0	0	0.000183

LogL, log-likelihood; df, degrees of freedom; AIC, Akaike Information Criterion; 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; 1/0, included/excluded; NA, not applicable.

Paper 3

The effect of ash dieback susceptibility on female and male reproductive success: good news for European ash forests

The effect of ash dieback susceptibility on female and male reproductive success: good news for European ash forests

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Abstract

The introduced invasive pathogen *Hymenoscyphus fraxineus* causes extensive damage on European ash trees (*Fraxinus excelsior*). Heritable variation in susceptibility to ash dieback (ADB) has been observed among ash trees in natural and planted populations, but it is not clear how variation in susceptibility influences the reproductive fitness of the trees. Here we hypothesize that healthier male and female trees contribute more gametes to the following generation compared to the unhealthy ones. We tested the hypothesis by studying gender, seed production and paternal success in a clonal field trial with 39 replicated clones. In the trial, disease development has been followed since 2007, and the susceptibility level of each clone is well characterized. This material allows us to explore the relationship between susceptibility and reproductive success (female and male). The clones revealed a clear gender dimorphism with an approximate 2:2:1 male/female/hermaphrodite ratio. Among the female clones, we observed a strong negative influence of ADB susceptibility on seed production, i.e., females with low levels of susceptibility produced substantially more seed compared to highly susceptible females. On the male side, we observed the same trend, but the picture was

more complicated. The male clone with the lowest level of susceptibility was by far the most effective pollen donor, but highly susceptible males also sired some offspring. The overall results represent good news for the potential recovery of ash forests. Not only will selection act in favour of ADB tolerance due to high levels of mortality of susceptible individuals from germination until maturity, but also sexual selection among mature ash trees at generation turnover will work in favour of healthy, highly reproducing trees and further reduce susceptibility.

Introduction

Forest tree species have to cope with various threats, such as emerging infectious diseases, climate change, habitat loss and fragmentation, which in combination can put them under severe pressure. In forest trees, the primary causes of emerging infectious diseases are fungal and fungal-like pathogens, whose numbers increased 13-fold only from 1995 to 2010 due to expanding networks of international trade and travel (Santini et al. 2013). EIDs present a real challenge for conservation by causing rapid and extensive reduction in numerical abundance and by changing the genetic composition of host populations (Altizer et al. 2003). The outcome of an epidemic in terms of host mortality depends not only on pathogen establishment, development and virulence but also on how a disease outbreak influences the reproductive success and ultimately the fitness of the host species.

Fraxinus excelsior L. or common ash (Oleaceae, hereinafter ash) is an important component of European forest ecosystems and a valuable timber species (Pliūra & Heuertz 2010). Ash has been decimated in continental Europe by an invasive fungal pathogen, *Hymenoscyphus fraxineus* (synonym *H. pseudoalbidus*, anamorph *Chlara fraxinea*) since the early 1990s (McKinney et al. 2014), when it was first observed in Poland (Przybyl 2002). Genetic studies suggest that the pathogen originates from East Asia (Zhao et al. 2013; Zheng & Zhuang 2014), where it is asymptomatically associated with Asian *Fraxinus* species (Cleary et al. 2016; Nielsen et al. 2017). High mortality due to the pathogen in the European ash stands of *Fraxinus excelsior* regardless of age (Bakys et al. 2009; Skovsgaard et al. 2010; Lenz et al. 2016) raises concern about the future existence of the species.

Previous research based on clonal ash stands such as in Denmark (McKinney et al. 2011), Sweden (Stener 2013; Cleary et al. 2014) and Germany (Enderle et al. 2014) has documented a high level of genetic variation in dieback susceptibility among ash individuals, giving rise to high broad-sense heritability estimates (e.g. H^2 = 0.42–0.54 in Tapsøre, Denmark, McKinney et al. 2011). Progeny trials have further revealed high levels of narrow

sense heritability in dieback tolerance (e.g. $h^2 = 0.40$–0.49 in Pliūra et al. 2011; $h^2 = 0.37$–0.52 in Kjær et al. 2012; $h^2 = 0.42$–0.53 in Lobo et al. 2014; $h^2 = 0.42$ in Muñoz et al. 2016), suggesting that tolerance is inherited from parents to offspring. While genetic variation in disease tolerance is known to affect individual survival (Lobo et al. 2014), much less is known on how the level of susceptibility of a given tree influences its reproductive success. However, a recent study found a clear overrepresentation of healthy trees among parents of seedlings on the forest floor of a Danish mixed forest (Semizer-Cuming et al. submitted). Sexual selection may therefore provide an important contribution to natural selection in favour of improved resistance. This issue is particularly important to clarify, as the frequency of tolerant ash trees in nature is low (McKinney et al. 2011).

Fitness is a measure of the ability to produce successful offspring. Plants that produce more seeds and fertilize more ovules than the population average therefore have higher fitness (Primack & Kang 1989). Female fitness can be estimated by counting the relative number of mature seeds assuming equal germination percent. On the other hand, the estimation of male fitness requires assignments based on paternity analysis using highly polymorphic codominant markers (Marshall et al. 1998). The aim of the present study is to test the hypothesis that healthier male and female trees have higher gamete contribution to the subsequent generation compared to the unhealthy ones. We study the variation in fertility in a Danish ash clonal trial with the clonal replication of 39 genotypes that have been characterized in terms of ADB susceptibility based on 10 years of measurements. The implications of the observed relationship between ADB tolerance and reproductive success are discussed in relation to the conservation and management of the species.

Material and methods

Study site and sampling

The study site is located in Tuse Næs (55° 45' 57.99" N 11° 42' 47.48" E) in Northern Sealand, Denmark. It is a clonal field trial, established in 1998 based on a randomized complete block design with 39 individual *F. excelsior* genotypes (39 clones). Each clone was grafted onto rootstocks with approximately 25 replications (ramets). Details of the trial and the origin of the 39 clones can be found in McKinney et al. (2011). In the trial, seeds were collected in October 2012 from the ramets of three open pollinated female clones, representing relatively healthy genotypes (Clone nos. 30, 33, 35) and located in the centre of the trial. Seeds were germinated and grown for two years in a green house. At the time of seed

collection in 2012, the number of ramets per clone had already been reduced to 4–21 due to high mortality caused by ADB.

Phenotypic assessments

All ramets of the 39 clones have been monitored in terms of crown damage since 2007. Crown damage was evaluated according to five-scale damage classes: Class 0 represented no crown damage, Class 1–3 indicated an increasing damage (Class 1: < 10 %, Class 2: 10–50 %, Class 3: > 50 %) and Class 4 meant 100 % crown damage (dead). For statistical analyses, crown damage scores were converted into percent damage scores (PDS) based on the median values of the class percent ranges (cf. McKinney et al. 2011). Broad-sense heritability for ash dieback (PDS) was estimated to be 0.4 in 2009 in Tuse Næs (McKinney et al 2011). Inflorescences of all flowering trees were observed in May 2015 and genders scored according to a 1–9 scale, corresponding to proportion of female flowers: (1) 0 %, (2) 12.5 %, (3) 25 %, (4) 37.5 %, (5) 50 %, (6) 62.5 %, (7) 75 %, (8) 87.5 % and (9) 100 %. Gender of one genotype (Clone no. 28) could not be determined due to the lack of flowers, and this clone was therefore excluded from further analyses concerning gender. All living trees were scored in terms of seed set in July 2015 (a year with abundant flowering) using a relative scale from 0 to 9, where 0 corresponded to no seed set and 9 to very heavy seed set. Tree heights were measured in February 2015.

DNA genotyping

Leaves were collected from the 39 clones and from 285 2-year old seedlings, and stored at -20 °C until DNA extraction. DNA extractions were carried out with ~ 40 mg leaf tissues using the DNeasy® 96 Plant Kit (Cat no. 69181) according to the manufacturer's protocol (Qiagen, Hilden, Germany). Microsatellite analyses were performed with 9 selected primer pairs: FEMSATL11 and FEMSATL19 (Lefort et al. 1999), FEMSATL12 (Gerard et al. 2006), ASH2429 (Bai et al. 2011), FRESTSSR308, FRESTSSR427 and FRESTSSR528 (Aggarwal et al. 2011), and Fp18437 and Fp21064 (Noakes et al. 2014). The primers were labelled with four different fluorescent dyes (6-FAM: FEMSATL12, Fp18437 and Fp21064, VIC: FEMSATL11, FRESTSSR308 and FRESTSSR528, NED: FEMSATL19 and ASH2429, PET: FRESTSSR427) and combined in 3 multiplexes (Multiplex-1: FEMSATL11, FEMSATL12 and FEMSATL19; Multiplex-2: Fp18437, Fp21064 and FRESTSSR528; Multiplex-3: FRESTSSR308, FRESTSSR427 and ASH2429). PCR amplifications were carried out in 15 µl reactions using the Qiagen Multiplex PCR Kit (Cat no. 206143) according

to the manufacturer's instructions. PCR amplifications were performed under the following conditions: initial denaturation at 95 °C for 15 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C, 62 °C and 59 °C (for Multiplex-1, -2 and -3, respectively), extension at 72 °C for 60 s, and final extension at 60 °C for 30 min. PCR products were analysed on the ABI3130xl Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Each individual genotype and each mother-offspring pair were checked for errors, and the analyses were repeated in case of uncertain or non-amplified peaks. Alleles at each locus were binned into their size classes using a combination of manual and automated allele binning (Matschiner & Salzburger 2009) in order to detect potential binning errors.

Paternity analysis

Paternity analysis was carried out using CERVUS 3.0.7 (Kalinowski et al. 2007). The assignments were made based on Delta (Δ), the difference in LOD scores between the two most likely parents, to increase the certainty to identify the true parent when multiple parents had positive LOD scores (Marshall et al. 1998). The critical values of Δ were calculated at strict (95 %) and relaxed (80 %) confidence levels during the simulations. The minimum number of loci was set to 8, and error rate was kept at 0.01. Selfing was allowed in the model, as some ash clones were hermaphrodites. To increase the reliability of the critical LOD values, 100000 offspring were simulated. The percentage of sampled potential fathers was set to 70 %. Confident paternity assignments were further checked and confirmed with the gender score of each genotype.

Analysis of gender and the effect of ADB susceptibility on reproductive fitness

We applied a general linear analysis of variance model to test for significant differences in gender scores among the 38 clones (excluding Clone no. 28 with unassessed gender) using the ramets as replications. For the further analysis, clones with a gender score (proportion of female flowers) of up to 20 % were considered predominantly male (M), whereas clones with gender score more than 80 % were regarded as predominantly female (F). The remaining clones were considered hermaphrodites (H). In this regard, the numbers of predominantly male, predominantly female and hermaphroditic clones were 15, 16 and 7, respectively. The ratio does not necessarily reflect the situation in native populations, because the clones were specifically selected for the trial in 1998 with the objective of containing the equal number of males and females.

The effect of ADB susceptibility (observed as percent crown damage; PDS) on reproductive success (Y) was analysed based on a linear regression model. The relationship between male fertility and ADB susceptibility was analysed based on the data from the 15 predominantly male clones, while the relationship between female fertility and ADB susceptibility was analysed based on the data from the 16 predominantly female clones. We applied the linear regression model $E(Y_i) = \beta_0 + \beta\,PDS_i$, where PDS_i was the susceptibility of the clones to ADB. In order to isolate the effect of survival in the clonal trial (differences in number of living ramets per clone), we analysed the relationship between reproductive fitness and ADB susceptibility at four levels: (i) total seed set per clone (sum across all ramets), (ii) total number of observed paternities per clone, (iii) average seed set per living ramet of each clone, and (iv) average number of observed paternities per living ramet of each clone. Heights of the single trees were initially included in the models, but they showed no significant differences, but they showed no significant differences and were therefore excluded from the final analysis. Statistical analyses were conducted using the 'tidyverse' package (Version 1.1.1; Wickham 2017) implemented in R (R Core Team 2017).

Results

Gender assessment

The assessment of gender showed significant differences among clones (F = 48.4, $p <$ 0.001). The average gender scores ranged from purely male (Gender score = 0 % for Clone no. 11 and 20) to mostly female (Gender score = 92 % for Clone no. 22) (Table 1). The 16 predominantly female clones formed a distinct group with the gender score of > 80 % whereas the border between male and hermaphroditic clones was less clear (Figure 1). The predominantly males did not carry any seed, but several of the predominantly females had sired offspring (Table 1). The level of ADB susceptibility was not correlated with the gender score (t = -0.25, $p >$ 0.05; Figure 1).

Paternity assignments

Paternity was assigned with high exclusion probability (0.9999), and no mother-offspring mismatches were detected. Paternity analysis successfully assigned candidate fathers to 144 (51 %) of the analysed seedlings; hereof 102 (36 %) at strict confident level (95 %), and one was self-fertilized. The number of successful pollinations varied among males. Eleven clones were not yet assigned as candidate fathers whereas a single clone (Clone no. 18), the healthiest male clone in the trial, was assigned as a pollen donor for 46 offspring

(Table 1), which corresponds to 32 % of the total assignments. When correcting for the number of living ramets per clone, the variation in pollination per clone varied from 0 to 2.9 per ramet (Table 1).
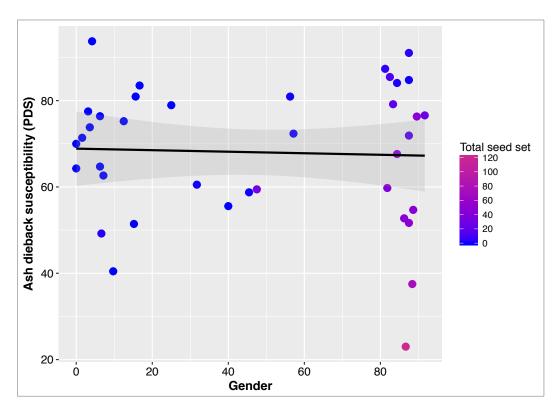
Figure 1 Gender of the 38 clones and their level of susceptibility to ADB (PDS) in 2015. Legend shows total seed set per clone in 2015.

The effect of ADB susceptibility on reproductive fitness

The negative effect of ADB susceptibility on seed set was highly significant, and the ADB damage score (PDS) alone explained a high proportion of the observed variation in total seed set and average seed set per living ramet (R^2 = 0.873 and 0.518) among the females (Table 2; Figure 2). PDS in general explained less variation (R^2 = 0.504–0.349) on the male side (Table 2), and the relationship was only significant due to a single male (Clone no. 18) that was by far the most effective pollen donor and also the male genotype with lowest susceptibility (Figure 2). The differences in the relationship between reproductive fitness and ADB susceptibility among the tested clones were significant at all four levels (Table 2).

Table 1 Gender score, seed set (SS), sired offspring (Pat), average percent ADB damage score (PDS), average PDS score for living ramets per clone (PDS LR), and number of living ramets (LR) of the 39 clones in the study site.

Genotype (Clone)	Gender[1]	Gender score (%)	Total SS 2015	Total Pat 2012	Average SS per LR 2015	Average Pat per LR 2012	PDS[2] 2012 (%)	PDS[2] 2015 (%)	PDS LR 2012 (%)	PDS LR 2015 (%)	LR 2012	LR 2015
1	M	16.67	0	8	0.00	0.80	87.50	83.50	75.00	45.00	10	6
2	M	15.63	0	0	0.00	0.00	89.06	80.94	75.00	37.50	7	6
3	H	45.45	1	18	0.09	1.29	56.50	58.75	32.86	16.36	14	11
4	M	6.25	0	0	0.00	0.00	83.33	76.39	75.00	46.88	12	8
5	H	57.14	1	0	0.13	0.00	86.76	72.35	75.00	38.13	10	8
6	M	12.50	0	2	0.00	0.13	82.95	75.23	75.00	50.45	15	11
7	H	40.00	0	0	0.00	0.00	79.44	55.56	71.54	33.33	13	12
8	H	25.00	0	3	0.00	0.30	86.84	78.95	75.00	42.86	10	7
9	M	3.13	0	0	0.00	0.00	87.50	77.50	75.00	50.00	10	9
10	M	9.72	0	2	0.00	0.09	72.05	40.45	70.91	27.37	22	19
11	M	0.00	0	1	0.00	0.08	84.72	70.00	75.00	39.00	12	10
12	M	6.62	3	1	0.18	0.06	75.26	49.21	72.35	43.24	17	17
13	H	47.50	32	20	3.20	1.43	41.11	59.44	24.29	27.00	14	10
14	M	1.56	0	2	0.00	0.17	78.33	71.39	67.50	35.63	12	8
15	M	6.25	0	3	0.00	0.19	76.67	64.72	72.19	35.91	16	11
16	H	56.25	1	0	0.17	0.00	86.25	80.94	69.38	37.50	8	6
17	M	3.57	0	0	0.00	0.00	60.00	73.82	24.44	36.43	9	7
18	M	15.18	0	46	0.00	2.88	37.14	51.43	17.50	27.14	16	14
19	M	7.14	0	3	0.00	0.27	56.76	62.65	33.18	29.44	11	9
20	M	0.00	0	9	0.00	0.64	59.52	64.29	39.29	31.82	14	11
21	F	84.38	44	0	4.89	0.00	61.58	67.63	33.64	31.67	11	9
22	F	91.67	26	3	3.71	0.27	60.26	76.58	31.36	36.43	11	7
23	F	81.82	42	2	3.82	0.15	52.86	59.76	23.85	23.18	13	11
24	F	84.38	2	1	0.40	0.14	87.95	84.09	62.14	30.00	7	5
25	F	87.50	4	2	0.80	0.25	89.47	91.05	75.00	66.00	8	5
26	F	86.25	44	0	3.14	0.00	56.82	52.73	47.22	25.71	18	14
27	F	81.25	5	1	0.83	0.17	89.74	87.37	67.50	60.00	6	6
28	NA	NA	0	4	0.00	0.67	92.11	93.68	75.00	60.00	6	3
29	F	82.50	13	1	2.17	0.11	87.38	85.48	52.22	33.33	9	6
30	F	89.58	45	1	6.43	0.08	48.95	76.32	23.46	32.14	13	7
31	F	83.33	20	2	1.67	0.11	86.25	79.17	72.63	37.50	19	12
33	F	88.39	71	1	4.73	0.06	37.50	37.50	21.88	16.67	16	15
34	F	87.50	48	3	3.69	0.20	40.28	51.67	28.33	33.08	15	13
35	F	86.67	122	0	7.63	0.00	8.33	23.00	7.94	15.94	17	16
36	F	88.64	46	2	4.18	0.15	48.44	54.69	36.54	34.09	13	11
37	F	87.50	1	0	0.13	0.00	84.32	84.77	71.25	58.13	12	8
38	F	87.50	15	1	1.36	0.07	77.86	71.90	69.00	46.36	15	11
39	H	31.73	1	1	0.07	0.06	69.74	60.53	59.12	31.43	17	14
40	M	4.17	0	1	0.00	0.20	93.75	93.75	66.00	66.00	5	5

[1]Gender: F, female; M, male; H, hermaphrodite; [2]Differences among clones in PDS were highly significant in both 2012 ($F = 10.8$, $p < 0.001$) and 2015 ($F = 3.8$; $p < 0.001$), unpublished data.

Table 2 Linear regressions for the effect of ADB susceptibility (PDS) on reproductive fitness.

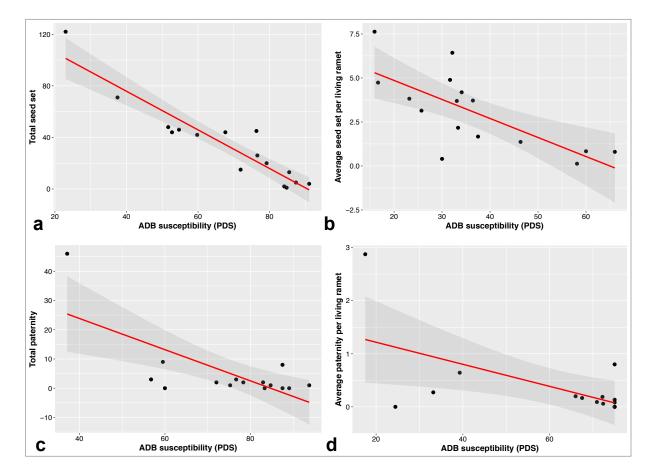|	ADB susceptibility (PDS)				
	Mean	**df**	**R²**	**t**	***p(>|t|)***
Total seed set	70.29	14	0.873	-9.816	1.17e-07***
Total paternity	68.70	13	0.504	-3.632	0.00304**
Average seed set per living ramet	54.35	14	0.518	-3.879	0.00167**
Average paternity per living ramet	37.66	13	0.349	-2.637	0.02050*

Figure 2 Relationship between ADB susceptibility (PDS) and (a) total seed set per clone; (b) average seed set per living ramet per clone; (c) total paternity per clone; (d) average paternity per living ramet per clone. Red lines show fitted regression lines within 95 % confidence interval.

Discussion

Our study showed that clones with low level of ADB had higher reproductive fitness, but the effect appeared to differ between both genders. A strong continuous negative relationship between ADB susceptibility and female reproductive success was observed, and the rank in the damage score of the clones therefore corresponded relatively close to the

opposite rank in seed set. However, the relationship between ADB susceptibility and male reproductive success was less straightforward, because some male clones with high damage scores had sired offspring (e.g., Clone no. 1 had sired 8 ovules; Table 1). The difference between the influence of ADB on female and male reproductive fitness probably reflects the differences between maternal and paternal investments in reproduction. Intersexual difference in reproductive investment is a known phenomenon in dioecious plant species, and the cost of reproduction is generally higher for females than males (Antos & Allen 1999; Cipollini et al. 1994; Korpelainen 1992; Obeso 2002). For example, in the desert populations of an evergreen dioecious shrub (*Simmondsia chinensis*), males allocate 10–15 % of their resources to reproductive tissues whereas females allocate 30–40 % of their resources to reproduction at 100 % seed set and female reproductive investment would be equal to that of males at ~ 30 % seed set (Wallace & Rundel 1979). In *Salix* species, females also allocate more resources to reproductive tissues than males and thus have higher reproductive costs in return (Ueno et al. 2007). Queenborough et al. (2007) studied intersex costs of reproduction in 16 tropical tree species of Myristicaceae in Amazonian lowland forests and concluded that female trees invested > 10 times more biomass than male trees in total reproduction. In ash, crown damage due to ADB probably limits the available resources for reproduction in both genders, but highly susceptible males may still afford to produce and release pollen before flushing unlike their highly susceptible female counterparts, which may not afford to produce mature seeds while restoring the crown with epicormic shoots during the growing season. The difference among clones in relative female fitness is likely to be even stronger than suggested by the results presented in Figure 1 and Table 1, because seed set was scored based on a relative scale from 0 to 9, where a tree with the crown full of seeds (score 9) most likely had more than 9 times more seed compared to a tree with very few seeds in the crown (score 1).

Only a small fraction of *Fraxinus excelsior* trees in natural populations can be expected to have a low level of susceptibility (1–5 %; McKinney et al. 2014), but the findings of the present study suggest that the frequency of individuals with low susceptibility are likely to increase in the following generations due to the higher reproductive fitness of their parents, especially on the maternal side. Several studies have shown high levels of narrow-sense heritability in the level of ADB susceptibility (as discussed above in the introduction)., and the superior reproductive success of the healthy females and males in our study is therefore promising for the future resilience of European ash forests. Nonetheless, the results indicate that selection for lower levels of susceptibility is slower on the male side, where also unhealthy males flower and produce pollen. Our results support the findings from a recent

study on ash regeneration in a Danish forest, where healthy trees were overrepresented as parents of seeds and young seedlings (Semizer-Cuming et al. submitted). It is indeed good news for European ash forests, because higher contribution from healthy trees at generation turnover followed by lower mortality among next generation of trees with low levels of susceptibility must gradually give rise to more tolerant ash trees in next generations. It is difficult to predict the expected overall progress due to lack of reliable estimates of *in situ* heritability relevant for the two selection stages, but the progress may be significant as strong selection can be involved in both stages, and effective gene flow is likely to connect surviving trees across large forest landscapes (Bacles and Ennos 2008; Bacles et al. 2005; 2006; Semizer-Cuming et al. 2017). Still, it remains to be revealed whether the process will be fast and efficient enough to ensure the true recovery of the species.

Acknowledgements

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Contributions to the scientific papers

My contributions to the three co-authored papers are described below.

For all three papers, I contributed to the design of the study in close cooperation with the co-authors.

For Paper 1 and 2, I was responsible for all the fieldwork. I took part in all the fieldwork, except for some field assessments in Denmark made by Lars Nørgaard Hansen while I was in Germany. I was responsible also for the entire DNA analyses, including the optimization of the techniques for both papers.

For Paper 3, I took part in the fieldwork in 2016, while the data on flowering and damage score for the previous years, as well as the seeds in 2012, were collected under the responsibility of the co-authors. I performed the genotyping and DNA analyses of the parents and progenies.

For all of the papers, I was responsible also for the overall analysis of the data, except for the model runs and analyses in the NMπ software. Igor Jerzy Chybicki provided guidance with the software for Paper 1 and performed the analyses for Paper 2.

For all three papers, I was further responsible for drafting the manuscripts based on the discussions with the co-authors and for finalising them according to their input.

Synopsis

Common ash (*Fraxinus excelsior* L.) is a keystone species, ecologically and economically important in European forests since it supports many associated species, and also produces valuable timber. The transformation of continuous forests to smaller forest fragments influences how ash trees exchange genes, and reduced gene flow may lead to a decrease or a loss of diversity and increased inbreeding. The patterns of genetic diversity are also influenced by plantation programs. Planted forests are often based on seeds of non-local origin, and sometimes from seed sources with narrow genetic variation. Seed and pollen from such plantings will mix with that of local trees, and the combined influence of fragmentation and planting programmes will, over generations, influence the genetic resources of ash trees and most likely their ability to respond to new challenges including climate change, new pests and pathogens. In recent years, the emerging infectious pathogen, *Hymenoscyphus fraxineus*, has caused severe ash dieback with high mortality throughout Europe. This may influence the genetic diversity of ash forests dramatically, because high mortality can create inbreeding and loss of diversity in the absence of effective gene flow, but also because the ability to resist the disease has been shown to be under strong genetic control. It is therefore important to understand how gene flow takes place across forests and landscapes in order to support sound management of endangered European ash trees and forests. The present thesis presents and discusses three studies that contribute new knowledge on how pollen and seeds are dispersed across forests and landscapes. The studies are based on field observations and DNA analyses using population genetics approaches.

Paper 1 addresses seed and pollen movement within – and into – a small, native forest fragment surrounded by agricultural land at Rösenbeck, Germany. The ash trees in the small forest are of putative local origin, while roadside trees close to the forest were planted. Categorical and neighbourhood model approaches were applied to estimate gene flow patterns at the study site. Local meteorological data was used to evaluate the effect of wind on the dispersal patterns of seed and pollen. The results showed that the forest fragment has maintained a high level of genetic diversity. Long distance dispersal (LDD) events of both seed and pollen, and the influence of local wind conditions on the dispersal patterns, were documented. The majority of the seeds (55–64 %) and seedlings (75–98 %) sampled in the native forest patch had both parents among mature trees within the same patch, and pollen flow from the cultivated road side trees was very limited (2 %). However, a considerable amount of the seeds and seedlings (26–45 %) must have been pollinated by trees located in

more remote parts of the landscape, suggesting substantial pollen immigration into the study site. The results show that fragmented ash forests are likely to receive substantial gene flow through pollen, but also that trees located within a maximum of 100 m distance will most likely be able to mate. This finding is important for planning of gene conservation stands. Available wind data from weather stations in proximity can support in the planning process.

Paper 2 addresses the gene flow and reproductive success of ash forests based on a study site in Valby Hegn, Denmark. Compared to Rösenbeck, Valby Hegn represents a much larger forest complex, where single and planted compartments of ash trees are intermixed with mainly oak and beech trees. Unlike Rösenbeck, the ash trees in Valby Hegn were already severely influenced by ash dieback when the study was performed. The study site was further selected because it included a mature planting with trees from an old Danish-seed orchard ('FP202'). The setup of the FP202 planting made it possible to estimate seed and pollen flow from a planted seed source into surrounding older stands, which is of special interest, since it may provide a model for enriching existing ash forests with planted trees that are highly resistant to ash dieback. This is an option that can be considered to improve the fitness of future generations of existing native ash forests. The neighbourhood model was applied to estimate mating system parameters and genealogies, and to understand the relationship between individual reproductive success and the phenotypic traits including fruit set, flower intensity, size, and crown dieback (i.e. ash dieback symptoms). The results showed that a large amount of seeds (39 %) and pollen (31 %) were dispersed from the planted trees into the surrounding forest, although the frequency of the gametes from the planted trees decreased rapidly with increased distance. The mean seed and pollen dispersal distances were 67 m and 347 m, respectively. The findings suggest that seed dispersal beyond 100–140 m is rare within a forest, but LDDs through pollen over hundreds (sometimes thousands of meters) must be expected, allowing trees that survive ash dieback to mate. Planted trees with high levels of ash dieback resistance are therefore likely to spread their genes into fairly large neighbourhoods as pollen donors, while seeds from enrichment plantings probably will be able to colonise areas with 0–100 m from the plantings. A very interesting finding was that the seedlings randomly sampled in Valby Hegn predominantly originated from healthy parents. This was the case for both male and female parents. The findings suggest that enrichment plantings with seeds from tolerant ash genotypes can potentially improve the future health of the species in European forests affected by ash dieback.

Paper 3 directly addresses the effect of ash dieback susceptibility on male and female reproductive success. This study is based on a clonal field trial in Tuse Næs, Denmark, where

the ash dieback susceptibility of 39 clones has already been studied for several years. Gender, seed productivity and paternity success of the 39 clones were estimated in order to test the hypothesis that the clones with the lowest levels of susceptibility had the highest levels of reproductive success. The results revealed that females with low levels of susceptibility produced more seeds compared to highly susceptible females. On the male side, the pattern was the same, although less clear. The male clone with the lowest level of susceptibility (Clone no. 18) was by far the most effective pollen donor. However, highly susceptible males also produced some offspring, showing that selection for lower levels of susceptibility is slower on the male side. The overall result is that healthy ash trees are likely to contribute more to the next generation due to sexual selection in favour of less susceptible individuals. This supports the observations from Valby Hegn (Paper 2 as discussed above), that healthy trees are likely to become overrepresented when founding the next generation ash trees at generation turnover. This is good news for European ash forests, although the future of ash forests remains uncertain, and is a still major concern.

Zusammenfassung

Die Gemeine Esche (*Fraxinus excelsior* L.) ist eine ökologisch und ökonomisch wichtige Baumart in Europäischen Wäldern, da sie viele assoziierte Arten aufweist und wertvolles Holz liefert. Die Transformation zusammenhängender Wälder in kleinere Waldfragmente, umgeben von landwirtschaftlichen Flächen, beeinflusst den Austausch von Genen bei der Esche, wobei reduzierter Genfluss zu einer Abnahme oder dem Verlust von Biodiversität und erhöhter Inzucht führen kann. Die Muster genetischer Diversität werden außerdem von Pflanzprogrammen beeinflusst. Gepflanzte Wälder werden oft mit nicht-lokalen Samen und manchmal auch mit Samen begrenzter genetischer Variation begründet. Samen und Pollen von solchen Pflanzungen mischen sich mit denen lokaler Herkünfte und der kombinierte Einfluss von Fragmentierung und Pflanzung beeinflusst, über Generationen, die genetischen Ressourcen der Esche und sehr wahrscheinlich ihre Fähigkeit, auf neue Anforderungen einschließlich des Klimawandels, neuer Schädlinge und Pathogene zu reagieren. In den letzten Jahren hat der neu aufgetretene infektiöse Erreger *Hymenoscyphus fraxineus* ein starkes Eschentriebsterben mit hoher Mortalität in ganz Europa verursacht. Dies könnte einen dramatischen Einfluss auf die genetische Diversität von Eschenwäldern haben, da eine hohe Mortalität unter Abwesenheit von effektivem Genfluss zu Inzucht und dem Verlust genetischer Diversität führen kann und auch, weil gezeigt wurde, dass die Fähigkeit dem Eschentriebsterben zu widerstehen starker genetischer Kontrolle unterliegt. Daher ist es wichtig zu verstehen wie Genfluss zwischen Wäldern und Landschaften stattfindet, um ein solides Management gefährdeter Europäischer Eschen und Eschenwälder zu unterstützen. Vor diesem Hintergrund werden in dieser Arbeit drei Studien präsentiert und diskutiert, die neue Erkenntnisse über die Pollen- und Samenverbreitung zwischen Wäldern und Landschaften liefern. Die Studien basieren auf Feldbeobachtungen und DNA-Analysen unter Verwendung populationsgenetischer Verfahren.

Beitrag 1 behandelt die Samen- und Pollenbewegung innerhalb und in ein kleines, ursprüngliches Waldfragment in Rösenbeck (Deutschland), das von landwirtschaftlichen Flächen umgeben ist. Die Eschen in dem kleinen Waldstück sind vermutlich lokaler Herkunft, während Straßenbäume in der Nähe des Waldes gepflanzt wurden. Kategoriale und Nachbarschaftsmodelle wurden verwendet, um Genfluss-Muster im Untersuchungsgebiet zu schätzen. Lokale meteorologische Daten wurden genutzt, um den Effekt von Wind auf Verteilungsmuster von Samen und Pollen zu evaluieren. Die Ergebnisse zeigten, dass das Waldfragment eine hohe genetische Diversität bewahrt hat. Die Fernverbreitung (long-

distance dispersal (LDD)) von Samen und Pollen sowie der Einfluss lokaler Windverhältnisse auf die Verbreitungsmuster wurden dokumentiert. Ein Großteil der Samen (55–64 %) und Sämlinge (75–98 %), die in dem Waldfragment beprobt wurden, hatten beide Elternbäume innerhalb des Fragments und der Polleneintrag von den gepflanzten Straßenbäumen war sehr gering (2 %). Allerdings muss ein beträchtlicher Anteil der Samen und Sämlinge (26–45 %) von weiter entfernten Bäumen bestäubt worden sein, was einen erheblichen Polleneintrag in das Untersuchungsgebiet nahelegt. Die Ergebnisse zeigen, dass fragmentierte Eschenwälder wahrscheinlich einen beträchtlichen Genfluss durch Pollen erhalten, aber auch, dass Bäume innerhalb einer maximalen Distanz von 100 m sehr wahrscheinlich in der Lage sind, sich zu befruchten. Dieser Befund ist wichtig hinsichtlich der Planung von Generhaltungsbeständen. Dabei können verfügbare Winddaten von nahegelegenen Wetterstationen den Planungsprozess unterstützen.

Beitrag 2 behandelt Genfluss und Reproduktionserfolg von Eschenwäldern basierend auf einem Untersuchungsgebiet in Valby Hegn, Dänemark. Im Vergleich zu Rösenbeck repräsentiert Valby Hegn ein viel größeres Waldgebiet, in dem einzelne gepflanzte Abteilungen der Esche mit hauptsächlich Eiche und Buche gemischt sind. Im Gegensatz zu Rösenbeck waren die Eschen in Valby Hegn bereits stark vom Eschentriebsterben betroffen als die Studie durchgeführt wurde. Das Untersuchungsgebiet wurde außerdem ausgewählt, weil es eine voll entwickelte Pflanzung mit Bäumen einer alten Dänischen Samenplantage ('FP202') umfasste. Die Einrichtung der FP202-Pflanzung ermöglichte eine Schätzung des Samen- und Polleneintrags von einer gepflanzten Samenquelle in umliegende Bestände. Dies ist von besonderem Interesse, da es ein Modell-Beispiel für die Anreicherung von bereits existierenden Eschenwäldern mit Eschen, die eine hohe Resistenz gegenüber dem Eschentriebsterben aufweisen, darstellt. Dies wäre eine Option zur Steigerung der Fitness nächster Generationen in natürlichen Eschenwäldern. Das Nachbarschaftsmodell wurde zur Schätzung von Paarungssystem-Parametern, Abstammung und der Beziehung zwischen individuellem Reproduktionserfolg und phänotypischen Merkmalen wie Fruchtansatz, Blühintensität, Größe und Kronensterben (d.h. Symptome des Eschentriebsterbens) verwendet. Die Ergebnisse zeigten, dass große Anteile von Samen (39 %) und Pollen (31 %) von den gepflanzten Bäumen in den umgebenen Wald gelangten, wobei allerdings die Häufigkeit von Gameten der gepflanzten Bäume mit steigender Distanz schnell abnahm. Die mittleren Samen- und Pollenverbreitungsdistanzen betrugen 67 m bzw. 347 m. Diese Ergebnisse legen nahe, dass eine Samenverbreitung von mehr als 100–140 m selten innerhalb eines Waldes vorkommt, aber LDDs durch Pollen über hunderte (teilweise über tausende)

Meter erwartet werden kann, was Befruchtungen zwischen Bäumen, die das Eschentriebsterben überlebt haben, ermöglicht. Es ist daher wahrscheinlich, dass Bäume, die eine hohe Resistenz gegen das Eschentriebsterben aufweisen, als Pollenspender ihre Gene in eine recht große Nachbarschaft verteilen, während Samen von Anreicherungspflanzungen vermutlich in der Lage sind, Areale von 0–100 m entfernt von den Pflanzungen zu besiedeln. Bemerkenswert war, dass die stichprobenartig gesammelten Sämlinge in Valby Hegn überwiegend von gesunden Elternbäumen stammten. Dies traf sowohl auf weibliche als auch männliche Elternbäume zu. Dies legt nahe, dass Anreicherungspflanzungen mit Samen toleranter Eschengenotypen die Gesundheit von Europäischen Beständen, die vom Eschentriebsterben betroffen sind, potenziell verbessern kann.

Beitrag 3 behandelt direkt den Effekt der Anfälligkeit für das Eschentriebsterbens auf den Reproduktionserfolg von männlichen und weiblichen Bäumen. Diese Studie basiert auf einem Klonversuch in Tuse Næs in Dänemark, wo die Anfälligkeit für das Eschentriebsterben von 39 Klonen seit mehreren Jahren untersucht wurde. Geschlecht, Samenproduktion und Vaterschaftserfolg der 39 Klone wurden analysiert, um die Hypothese zu testen, dass die Klone mit der geringsten Anfälligkeit den höchsten Reproduktionserfolg aufwiesen. Die Studie zeigte, dass weibliche Bäume mit geringer Anfälligkeit mehr Samen produzierten als weibliche Bäume mit hoher Anfälligkeit. Dasselbe Muster zeigte sich auch bei männlichen Bäumen, wenn auch nicht so deutlich. Der männliche Klon mit der geringsten Anfälligkeit (Klon Nr. 18) war mit Abstand der effektivste Pollenspender, aber auch anfällige männliche Bäume produzierten einige Nachkommen, was zeigt, dass eine Selektion für geringe Anfälligkeit langsamer auf der männlichen Seite stattfindet. Insgesamt zeigten die Ergebnisse, dass gesunde Eschen aufgrund von sexueller Selektion zugunsten weniger anfälliger Individuen eine höhere Wahrscheinlichkeit aufweisen zur nächsten Generation beizutragen. Dies unterstützt die Beobachtungen in Valby Hegn (Beitrag 2 wie oben diskutiert), dass beim Generationswechsel gesunde Bäume vermutlich in der nächsten Generation überrepräsentiert sein werden. Dies sind gute Nachrichten für Europäische Eschenwälder, auch wenn die Zukunft der Eschenwälder ungewiss bleibt und noch immer Anlass zur Sorge gibt.

Dansk Sammendrag

Almindelig ask (*Fraxinus excelsior* L.) er en økologisk og økonomisk vigtigt træart i de europæiske skove, hvor arten både understøtter biologisk diversitet og fungerer som en vigtig tømmerart i skovbruget. Vores brug af skovene og det omkringliggende landskab påvirker imidlertid hvordan træerne kan udveksle gener indenfor og mellem skove. Tidligere sammenhængende skove er mange steder blevet fragmenteret til små skove omgivet af landbrugsjord. Reduceret genspredning øger risiko for tab af genetisk variation og øget indavl i de kommende generationer af asketræer. En anden faktor, som kan påvirke asketræernes genetiske variation, er plantning af træer baseret på frø fra forædlede frøkilder. Gener fra disse plantninger kan på sigt blande sig med de oprindelige bestande, og derved ændre disses oprindelige genetiske mønstre. I de senere år har sygdommen asketoptørre, som skyldes en indslæbt svamp (*Hymenoscyphus fraxineus*) fra Asien, desuden skabt stor dødelighed i europæiske askeskove. Sygdommen forventes at påvirke den genetiske variation, fordi høj dødelighed reducerer bestandstørrelser, men også på grund af genetisk variation i træernes modtagelighed for sygdommen. Genspredning i ask sker via vindspredning af pollen og frø. Det er derfor vigtigt at kende omfanget af pollen og frøspredning, og hvordan dette spiller sammen med den menneskelige påvirkning, for at kunne understøtte en bæredygtig forvaltning af de truede askeskove. Med det formål for øje, præsenterer denne afhandling resultaterne fra tre studier baseret på feltobservationer og DNA analyser, fortolket i en populationsgenetisk sammenhæng.

Det første studium (Paper 1) undersøger pollen og frøspredning i en mindre naturlig bestand af ask, som vokser omgivet af et landbrugslandskab i Rösenbeck, Tyskland. I nærheden af bestanden er en række asketræer af ikke lokal oprindelse plantet som vejtræer. I studiet benyttes to forskellige modeller ('Categorical' og 'neighbourhood model') til at estimere hvor langt – og i hvilken retning – frø og pollen bliver spredt i den lille skov. Resultaterne sammenlignes med lokale målinger af de fremherskende vindforhold på forskellige årstider. Studiet viste, at askebestanden har en høj genetisk diversitet, og at den løbende modtager en betydelig andel af pollen udefra (26–45%). Det er imidlertid kun en meget lille del (2%), som stammer fra de plantede vejtræer. Studiet viste også, at vindforholdene påvirker retning og længde af pollen og frøspredning, herunder lang distance spredningerne (long distance dispersal, LDD). Hovedparten af de undersøgte frø og frøplanter havde deres forældre i selve bevoksningen (henholdsvis 55–64% og 75–98%), peger studiet på, at må forventes en betydelig tilførsel af gener udefra - selv i isolerede bestande som

Rösenbeck. Et forhold, som er vigtigt at tage i betragtning, når man f.eks. udpeger og forvalter bestande med henblik på genbevaring. Men resultaterne viser også at træer, som vokser op til 100 meter fra hinanden, stadig kan bidrage til gensidig bestøvning. Data om vindforhold kan til en vis grad benyttes til at forudsige genspredningen.

Det andet studium (Paper 2) er metodemæssigt tæt beslægtet med studiet i Rösenbeck, men er foretaget i Valby Hegn, et gammelt skovområde i Nordsjælland. Askeskovene i Valby Hegn adskiller sig fra Rösenbeck på flere parametre. De fleste træer (også i de gamle bevoksninger) er oprindeligt plantede, og der er tale om et større sammenhængende område, hvor asketræer vokser som enkelttræer eller i mosaik af bevoksninger, omgivet af primært bøge og ege. Desuden findes i Valby Hegn en plantning med afkom fra en gammel dansk frøplantage ('FP202') i kanten af skoven. Studiet undersøger specifikt hvordan gener fra denne forædlede frøkilde spreder sig ind i de omkringliggende askebevoksninger. Endelig er bevoksningen i Valby Hegn, modsat Rösenbeck, voldsomt påvirket af asketoptørre. Udover at undersøge pollen og frøspredningen i en svækket aske skov, undersøger studiet derfor også i hvilket omfang det er de sundeste træer, som bidrager mest til den næste generation. Graden af spredning af frø og pollen fra det plantede FP202 frøplantage afkom er interessant, fordi det kan belyse i hvilket omfang fremtidige plantninger med afkom fra udvalgte, sunde asketræer, kan berige modstandskraften i omkringliggende, eksisterende askeskove gennem løbende spredning af pollen og frø. Studiet afslørede, at den plantede bevoksning fra FP202 i betydelig grad spreder både pollen og frø ind i nabobevoksningerne, selvom effekten aftager hurtigt med afstanden. Helt generelt peger analyserne på, at 50% af frø spredes mindre end 50 meter fra deres modertræer, mens 50% af bestøvningerne sker mellem træer indenfor 140 meters afstand. Konklusionen er, at askefrø sjældent spredes over 150 meter, men en lille andel af bestøvninger foregår sandsynligvis mellem træer adskilt med kilometers afstand (LDD). Berigelsesplantning med sundt afkom kan forventes, at påvirke de omkringliggende skove positivt, men ud over et bælte på 100 meter vil det primært være ved at bestøve eksisterende, sunde asketræer i de gamle bevoksninger. Et andet vigtigt resultat fra studiet var, at de sunde træer havde højere reproduktiv fitness end de svækkede træer. De undersøgte afkom havde således forældre, som i gennemsnit var markant sundere end gennemsnittet for hele bestanden af modne asketræer i det undersøgte område.

Det tredje studium (Paper 3) følger op på studiet fra Valby Hegn ved specifikt at undersøge sammenhængen mellem modtagelighed for asketoptørre og evnen til at producere afkom. Studiet er baseret på analyse af 39 kloner (genotyper), som vokser i en af Naturstyrelsens frøplantager på Tuse Næs nord for Holbæk. Hver klon var oprindelig

repræsenteret med ca. 25 podninger i plantningen, og forskellene mellem de 39 kloner i deres modtagelighed for asketoptørre er blevet fulgt siden 2007. Ud fra målinger af funktionelt køn (hanlig versus hunlig), frøsætning og bestøvningssucces (det sidste ved hjælp af DNA analyser af frø) kunne den reproduktive fitness estimeres og sammenlignes med klonernes modtagelighed for asketoptørre. Resultaterne var klare for det hunlige køn: Kloner med den laveste modtagelighed producerede flest frø. For det hanlige køn var billedet det samme, men mindre tydeligt. Den sundeste hanlige klon (Klon no. 18) var langt den mest effektive pollendonor blandt hanklonerne, men blandt de øvrige hanlige kloner var der ikke den samme tydelige sammenhæng mellem modtagelighed og bestøvningssucces. Samlet set viser studiet, at man kan forvente en betydelig forøget reproduktiv succes blandt træer med høj grad af naturlig resistens overfor asketoptørre. Samlet set er resultaterne opmuntrende for de europæiske askeskoves fremtid. Man må forvente, at asketræer, som overlever asketoptørre, vil være i stand til at bestøve hinanden. Og de sunde træer må forventes at sprede flere frø sammenlignet med skovenes modtagelige træer. Samtidig vil plantning af afkom fra udvalgte træer med særlig høj modstandskraft kunne berige de eksisterende askeskove gennem spredning af frø og især pollen. Om det vil være nok til at redde askens rolle i de europæiske skove, er derimod stadig et åbent spørgsmål.

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