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ORIGINAL ARTICLE

Presence of natural genetic resistance in *Fraxinus excelsior* (Oleraceae) to *Chalara fraxinea* (Ascomycota): an emerging infectious disease

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Fraxinus excelsior, common ash native to Europe, is threatened by a recently identified pathogenic fungus *Chalara fraxinea*, which causes extensive damage on ash trees across Europe. In Denmark, most stands are severely affected leaving many trees with dead crowns. However, single trees show notably fewer symptoms. In this study, the impact of the emerging infectious disease on native Danish ash trees is assessed by estimating presence of inherent resistance in natural populations. Disease symptoms were assessed from 2007 to 2009 at two different sites with grafted ramets of 39 selected clones representing native *F. excelsior* trees. A strong genetic variation in susceptibility to *C. fraxinea* infections was observed. No genetic or geographic structure can explain the differences, but strong genetic correlations to leaf senescence were observed. The results suggest that a small fraction of trees in the Danish population of ash possess substantial resistance against the damage. Though this fraction is probably too low to avoid population collapse in most natural or managed ash forests, the observed presence of putative resistance against the emerging infectious disease in natural stands is likely to be of evolutionary importance. This provides prospects of future maintenance of the species through natural or artificial selection in favour of remaining healthy individuals.
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Introduction

Plant populations are occasionally affected by emerging infectious diseases (EIDs) that pose threats to food stability, economy, biodiversity and conservation of plant species (Stukenbrock and McDonald, 2008). EIDs are caused by pathogens that have changed geographical distribution, undergone a host shift or an evolutionary change leading to increased virulence (Anderson *et al*., 2004; Woolhouse *et al*., 2005). The underlying elements of such changes are many and some can be assigned to human activities, such as intensive land use with a high representation of monocultures, reduction of biodiversity, increasing globalization as well as general climate change. As a consequence the emergence and effects of diseases in agricultural crops have been studied widely (Stukenbrock and McDonald, 2008). Many crops are specifically susceptible to such new or altered pathogens because of their narrow genetic background. It is generally assumed that adaptation of a wild species to new pathogens is dependent on the genetic variation within that species (Burdon, 2001). Although there is generally a higher level of genetic variation in wild plant populations, these are also occasionally affected by EIDs. Although the origin and emergence of these pathogens are often inexplicable, the consequences are conspicuous and devastating. Several examples have been observed with tree species over the past 100 years, of which the most well-known example is the Dutch elm disease that decimated the European populations of elm in the twentieth century (Brasier, 1990; Brasier, 2001).

The European common ash, *Fraxinus excelsior* L., is currently threatened by an EID causing extensive dieback throughout East, North and Central Europe. The symptoms were first observed in Poland in 1992 (Przybyl, 2002), and have since been recorded spreading in a stepwise pattern through Europe. The disease is expected to influence most European populations of *F. excelsior* within the near future. Reports have also confirmed *F. angustifolia* and other *F. excelsior* subspecies as susceptible taxa (Kirisits *et al*., 2009b). In Denmark, symptoms were first observed in 2003 and became common in 2005. The disease now affects populations of *F. excelsior* in all parts of the country (Skovsgaard *et al*., 2009). The symptoms include severe dieback of the crown due to occlusion of branches, wilting, bark necroses on stems and trunks and discolouration of the wood (Bakys *et al*., 2009b; Kowalski and Holdenrieder, 2009a; Skovsgaard *et al*., 2009). The lesions are formed around dead side twigs or around leaf scars, suggesting leaves as a possible point of infection.

The cause of the dieback has been identified as the pathogenic fungus, *Chalara fraxinea* Kowalski, (2006). The

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fungus has been isolated and confirmed by molecular markers from symptomatic trees in affected stands throughout Europe (Kowalski, 2006; Bakys *et al*., 2009a; Chandelier *et al*., 2009; Ioos *et al*., 2009; Johansson *et al*., 2010). The epidemiology of the fungus was further elucidated when the teleomorph was identified. Initially morphological characteristics and internal transcribed spacer sequence data suggested that *C. fraxinea* was identical to the known ascomycete *Hymenoscyphus albidus* (Roberge ex Desm.) W. Phillips (Kowalski and Holdenrieder, 2009b). However, recent results have shown that the teleomorph is indeed a previously undescribed species; *H. pseudoalbidus* (Queloz *et al*., 2010).

The Danish ash stands are currently greatly affected by the disease. Disease symptoms were initially more severe in younger stands; however, stands of all ages are now affected. It is also a common observation that substantial phenotypic variation in the degree of damage is observed in natural populations in which apparently healthy individuals stand alongside severely affected trees.

On the basis of observations over 3 years of trees replicated by grafting, we present the first assessment of genetic variation in *F. excelsior* with respect to differences in susceptibility to ash dieback caused by *C. fraxinea*. Assessments of the ramets reveal information on the degree of genetic control behind the variation in susceptibility. A strong genetic base behind phenotypic variation in resistance may confer the species resilience against the effects of the novel EID. In contrast, phenotypic variation not or only weakly based on genetics will have no effect on resistance in later generations. Variation in phenological traits is reported, because risk of infection may depend on factors such as synchronization between fungus and host.

We focus on two clonal field trials that are heavily affected by the disease. These field trials were established in 1998 and comprise on average 50 ramets each of 39 trees selected from 14 Danish locations. On the basis of this material, we quantify the damage caused by natural infections of each tree replicated by ramets and determine whether there are differences in symptom occurrence and development. We discuss the potential value of genetic variation in natural populations in response to EIDs.

Materials and methods

Field trials

A set of 40 *F. excelsior* clones was applied to test the genetic background of observable tree-to-tree variation in exhibition of symptoms of novel ash decline. The 40 clones represent a selection of mature trees (ortets) with good health and stem form selected during a period from 1934 to 1997, that is, before ash dieback was reported in Denmark. The trees originate from 14 populations all of putative Danish origin (Table 1). Each of the 40 trees was grafted onto rootstocks in 1998 at two sites (Tuse Næs, N55° 45 57.99 E11° 42 47.48, northern Sealand and Tapsøre, N55° 24.237 E9° 27.459, south-eastern Jutland), producing on average 50 ramets per ortet (range 49–52). A subset of the ramets was checked for clonal identity with microsatellite markers (cf. description of DNA analysis below) to ensure that no mistakes were introduced at this stage. During this process, ramets from 2 of the 40 ortets were determined to be identical (no. 31 = no. 32), and the total number of tested clones was therefore 39.

The ramets were grown at two parallel test sites, each including approximately 25 ramets (graftings) of each of the now 39 *F. excelsior* ortets. The field trials are designed with randomized complete blocks and single-tree plots. In some cases, however, the blocks are not fully complete. The average number of replications was 26 at Tuse Næs and 27 at Tapsøre. The first assessment of symptoms of dieback was performed in 2007. Owing to mortality or unsuccessful grafting, only 1604 of the original 2016 ramets were available at the time of the first assessment, but the representation was nevertheless fairly balanced (35–46 living ramets per ortet). We have no systematic observation of symptoms at the two sites before 2007.

DNA genotyping

To exclude any structural patterns in the selected genets and to ensure they all were chosen from a homogenous gene pool, we analysed four neutral DNA markers (microsatellites). Leaf material was collected on 12 June from three ramets from each of the clones at the Tuse Næs site. Furthermore, all ramets in the old clonal collections (1930–40 selections) were sampled on 10 September 2008 (Nielsen *et al*., 2009). Two leaves were collected from each tested tree. The material was stored at −20 °C until DNA extraction. On an average, 35–40 mg leaf tissue per individual was frozen in liquid nitrogen and ground on a bead mill without any previous preparation. DNA extraction was carried out with the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol for frozen material.

The DNA extractions were kept undiluted for the PCR. The chosen microsatellite loci were FEMSATL11, FEMSATL12, FEMSATL16 and FEMSATL19 (Lefort *et al*., 1999). We used the modified primers for FEMSATL12 (Gerard *et al*., 2006).

PCR reactions were carried out using the Qiagen Multiplex PCR kit according to the manufacturer's instructions. The reaction volumes were scaled down to 15 µl. PCR amplifications were completed on Applied Biosystems thermo cyclers (models 9700 and 2700, Foster City, CA, USA) under the following conditions: an initial denaturation step of 15 min at 95 °C, 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 90 s and extension at 72 °C for 60 s, and a final extension step at 60 °C for 30 min. Each amplified product was diluted with 30 µl H_2O and visualized with an ABI3130xl sequencer from Applied Biosystems.

Population structure

Signs of population structure were studied to determine if clones represent one homogenous gene pool with no geographical clustering. This was carried out by analysing the four simple sequence repeat markers in the 39 ortets. Structure based on the Bayesian clustering approach was applied estimating likelihood of admixture q_k assuming $k = 1$–6 clusters based on MCMC algorithm as implemented in the Structure 2.3.1 software (http://pritch.bsd.uchicago.edu/structure.html) (Hubisz *et al*., 2009). We estimated the likely number of clusters

Table 1 Ortets applied in the analyses. Scions were collected from Danish stands and given populations

Clone nos.	*Population of origin*	*Selection year*	*Tuse Næs*		*Tapsøre*	
			No. established	*No. measured*	*No. established*	*No. measured*
01–10; 21–26	Haderslev, Dep. 36b	1997	23–24	16–22	26–27	18–24
11–17; 27–29	Gråsten, Dep. 85b	1997	23–24	15–20	26–28	21–24
18	Sorø II, Dep. 58b	1934	24	20	26	23
19	Stenderup, Dep. 10	1936	24	18	27	20
20	Frederiksgave, 74	1944	24	20	27	24
30	Stenderup, Prøvefalde	1938	23	19	27	21
31[a]	Stenderup, Dep.12	1944	23	16	26	22
32[a]	Stenderup, Dep.10	1944	24	19	26	21
33	Boller, Dep. 30	1944	24	19	26	25
34	Tåsinge. Dep. 5	1944	23	18	26	22
35	Sorø II, Dep. 63a	1944	23	15	27	23
36	Sorø II, Dep. 67a	1944	24	16	25	21
37	Sorø II, Dep. 82a	1944	24	22	26	23
38	Stasevang, Dep. 274	1944	23	21	27	20
39	Sorø II, Mølleskov D3	1945	24	16	28	20
40	Sorø II, Mølleskov E3	1944	23	14	27	22
	Total number of graftings		944	735	1067	881

Abbreviation: Dep., Department.
[a]Genotyping of all clones revealed that nos. 31 and 32 had the same genotype. We assume the mistake goes back to the time of scion collections in 1944 (it cannot be determined whether this is the tree from Dep. 12 or Dep.10 in Stenderup).

(K^*), which is calculated using ΔK, a statistic based on the successive shift of log probability of data between ascending values of K (Evanno *et al*., 2005). The analysis was based on 20 runs for $K = 1$–6.

Assessment of phenotypic damage

Damage was recorded for all living ramets at both sites in June 2007, 2008 and 2009. Each ramet was classified into one of five classes depending on the level of damage to the crown. Class 0 comprised ramets with no symptoms; class 1 included ramets with few insignificant damages and <10% loss of crown foliage; class 2 was assigned to ramets with >10%, but <50% damage; and class 3 was assigned to ramets that had lost >50% of the crown. Individuals that had died from infection were scored as class 4 (100% damage). In the data analysis, the classes were converted to per cent damage score (PDS) corresponding to the median per cent of the class' range; class 0, 0%; class 1, 5%; class 2, 35%; class 3; 75% and class 4 100%.

The *C. fraxinea* symptoms included occurrence of stem necroses and/or dead branches and twigs associated with a purple-brown colouring. The symptoms were easy recognizable in the field. However, we did not isolate the fungus systematically from each of the more than 1600 assessed ramets, and it cannot be excluded that part of the dieback of the crown can be attributed to other factors than *C. fraxinea* infection. During two assessments—Tapsøre, 2008 and 2009—we performed a careful assessment of stem necroses (a typical *C. fraxinea* symptom) on all ramets to determine whether this trait correlates with the phenotypic damage class that was based on assessment of the damage of the crown. In this study, the presence of necroses was classified as either: absent (score = 0), signs of early stage of necrosis development observed as abnormal coloured patches on the stem (score = 1), few and/or scatted necroses observed as necrotic bark associated with dead branches or twigs (score = 2) or substantial areas on the stem with necrotic bark, or fewer but severe necrotic areas on the stem (score = 3).

Additional phenotypic characterization of the tested clones

To determine whether phenological traits influenced the health of the ramets, height, flushing and leaf senescence were measured. The height and diameter (at 1.3 m) of all living ramets were measured in winter 2007/2008. At one site—Tuse Næs—phenology of the 39 clones was assessed during the growing season of 2009. The time of flushing (budburst) was scored 6 May, whereas leaf shed was scored on 12 October. The flushing time was scored according to a 1–9 scale: (1): winter state, (2): bud scales open, leaves visible, (3): beginning shoot growth, leaves folded, (4): more shoot growth, leaves half-folded, (5): shoot growth, leaves open and glossy, (6): double leaf growth, still glossy, (7): increased leaf width, beginning to dim, (8): leaves dim on oldest leaf whorl, still glossy in top of shoot, (9): all leaves dim. The leaf shed was scored on a 0–4 scale according to leaf colour, in which 0 was given to ramets with dark green leaves and 4 was given to ramets with completely yellow and fading leaves.

Statistical analysis of phenotypic data

The variables were analysed using the following linear model:

$$Y_{ij} = \mu + c_i + b_j + \varepsilon_{ij}$$

where Y_{ij} is the value of the trait in question, μ the grand mean, c_i the random effect of the clone i (that is, effect of different genotypes), b_j the fixed effect of block j in the trial and ε_{ij} the residual of plot ij that is assumed to follow a normal distribution $N\ (0,\sigma_e^2)$. Deviations from the assumptions were inspected by traditional graphic plots (Snedecor and Cochran, 1987).

The significance of clone effects was tested using a likelihood ratio test. For a model A with the genetic effect

included and a restricted model B without the genetic effect, the applied test statistic was $D_{A\to B}=2$ ($\log A_L-\log B_L$), assuming the probability distribution of $D_{A\to B}$ approximately χ^2_{RA-RB}, where RA is the number of parameters for model A and RB the number of parameters for the restricted model B (Gilmour *et al*., 2002). To avoid the effect of multiple testing the *P*-values for each of 10 variance components for clones were corrected by the sequential table-wide Bonferroni method (Holm, 1979).

To elucidate the implications for potential adaptive response, the total phenotypic variance and covariance was separated into variance and covariance components because of clones (σ^2_G) and effects of environment (σ^2_E) and the broad-sense heritability ($H^2_{bs}=\sigma^2_G/(\sigma^2_G+\sigma^2_E)$) was estimated for each trait. For susceptibility (PDS), we further calculated the genetic coefficient of variation (CV_G) as the square root of σ^2_G divided with the average PDS to assess the implication of genetic variation for adaptive potential (Houle, 1992). Phenotypic, genetic and environmental correlations between traits were estimated according to Falconer and Mackay (1996) based on variance and covariance components obtained from the software ASReml (Gilmour *et al*., 2002). Standard errors for the parameters were estimated from Taylor series approximations in the software ASReml (Gilmour *et al*., 2002). First-order autoregressions in row and column directions (Gilmour *et al*., 2002) were examined at both sites and proved significant ($P<0.001$) at Tapsøre. The error variance at this site was divided into an independent error variance and dependent error variance explained by first order autoregressions. H^2 and CV_G are estimated excluding the error variance because of heterogenous environment that is explained by row–row and column–column autoregressions. *t*-tests were applied to test phenotypic correlations between traits (simple Pearson's correlation coefficients).

The average performance in all analysed traits were estimated for each of the 39 studies clones as best linear unbiased predictions from ASReml, which apply the restricted maximum likelihood method (Gilmour *et al*., 2002).

The stability of the response of the tested clones across environments was studied by estimating interaction between clones and localities in an analysis of variance according to the model:

$$Y_{ijk}=\mu+l_k+c_i+c_i\times l_k+b_j\,(l_k)+\varepsilon_{ijk}$$

where effects l_k is the fixed effect of locality, $c_i\times l_k$ the random interaction effect and remaining parameters as in model (1) above. The interaction effects were tested by a likelihood ratio test, and its relative importance quantified by $K=\sigma^2_{GxL}/\sigma^2_G$ (McKeand *et al*., 1997). From a biological point of view, the most interesting effect of genotype-by-environment interaction is that the relative ranking between clones can change in the presence of interaction in which case different clones may be favoured by natural selection for low susceptibility at different sites. We therefore looked further into the stability of clones across sites by estimating the genetic correlation between the two sites using a multivariate approach in which the same trait measured at two sites is considered as two different traits. The covariance between the traits will be the genetic covariance across sites (Falconer and Mackay, 1996), and high positive genetic correlation between sites will correspond to similar ranking at the two sites, whereas low (or negative) correlation will correspond to a situation in which the relative susceptibility of clones is site specific (Burdon, 1977).

The relationship between damage score and height, flushing time and leaf colouring was analysed based on linear regression analysis to provide a more detailed understanding of the relationship between these traits (likely to have been under stabilizing selection in natural populations), and the observed variation in damage level because of the EID.

Results

The 39 clones represented in the two trials were sampled from 14 geographical locations in Denmark (Table 1), but no population structure was detected when analysing the four simple sequence repeat markers in Structure analysis. The best fit in the model was obtained for $K=1$, also when analysing ΔK (data not shown). This supports that the 39 studied clones represent one single population, at least in relation to selectively neutral genes.

The symptoms of infection by *C. fraxinea* include formation of severe bark necroses. The presence of these wounds (assessed in Tapsøre in 2008 and 2009) was highly correlated with damage level. The correlation between presence of necroses and the PDS score was very high (in 2008 $r_G=0.96$ and in 2009 $r_G=0.94$). This indicates that PDS in the crown is a reliable approach to assess damages caused by *C. fraxinea*.

Highly significant genetic variation was observed in the degree of damage. This was the case for both sites, and for all 3 years of assessment (Table 2). In 2007, the average degree of damage varied between genotypes from 1% to 69% at Tuse Næs, and between 2 and 68% at Tapsøre (Figure 1). This variation corresponded to high genetic coefficients of variation, $CV_G=61\%$ at Tuse Næs and $CV_G=78\%$ at Tapsøre and broad-sense heritabilities of $H^2=0.39$ and $H^2=0.42$ for the two sites, respectively (Table 2). The observation of prominent genetic variation in susceptibility was repeated in 2008 and 2009 at both sites. The genetic coefficient of variation decreased as the damage from *C. fraxinea* increased, but the heritabilities remained almost unchanged at 0.40 at Tuse Næs and 0.49 at Tapsøre in 2009.

Interaction between clones and test sites in damage level was significant for all 3 years ($P_{PDS2007}<0.001$; $P_{PDS2008}<0.001$; $P_{PDS2007}=0.05$). However, the relative importance of the interaction was limited ($K=\sigma^2_{GxL}/\sigma^2_G=0.11$ (2007); 0.12 (2208) and 0.04 (2009)), and the ranking of the 39 clones in relation to damage caused by *C. fraxinea* remained very similar at the two sites in all 3 years. The genotypic correlation was thus very high, $r_G=0.90$, 0.95, 0.96 (Table 3), indicating that the genotype-by-environment interaction in the degree of susceptibility was mainly an effect of scale rather than rank differences among clones across sites. The repeated assessments showed a development of disease symptoms at both sites over the 3 years of observations. The general tendency at both sites is the degenerate progression of health during the assessed period 2007–2009 even though the level of damage was substantially different throughout the 3-year period (Figure 2). All 39 studied clones had at least one ramet with symptoms from 2007 and forward (Figure 3, for 2009). In 2007, we observed

Table 2 Variance components and broad sense heritability estimates (H^2) at Tuse Næs and Tapsøre

Tuse Næs							*Tapsøre*				
	PDS 07	*PDS 08*	*PDS 09*	*Height 07*	*Flushing 09*	*Senescence 09*		*PDS 07*	*PDS 08*	*PDS 09*	*Height 07*
σ^2_G	384	278	427	304	0.49	0.21	σ^2_G	349	537	505	1396
							Independent error variance (units)	477	460	536	4047
Residual variance	589	815	629	7 040	1.04	0.41	Residual error variance (heterogeneous environment)	125	200	190	20 764
σ^2_P	973	1093	1056	7344	1.53	0.62	σ^2_P	826	997	1041	5 443
Mean	32	32	55	313	4.1	1.7	Mean	24	41	56	423
CV_G	0.61	0.52	0.38	0.06	0.17	0.27	CV_G	0.78	0.57	0.39	0.09
H^2	0.39	0.25	0.40	0.04	0.32	0.34	H^2	0.42	0.54	0.49	0.26
s.e. (H^2)	0.06	0.05	0.06	0.02	0.06	0.06	s.e. (H^2)	0.13	0.07	0.10	0.06

Abbreviations: CV, coeffecient of variation, PDS, per cent damage score; σ^2_G, genetic variation; σ^2_P, phenotypic variation.
Auto-regressions in row and column directions were only significant at Tapsøre (see text).

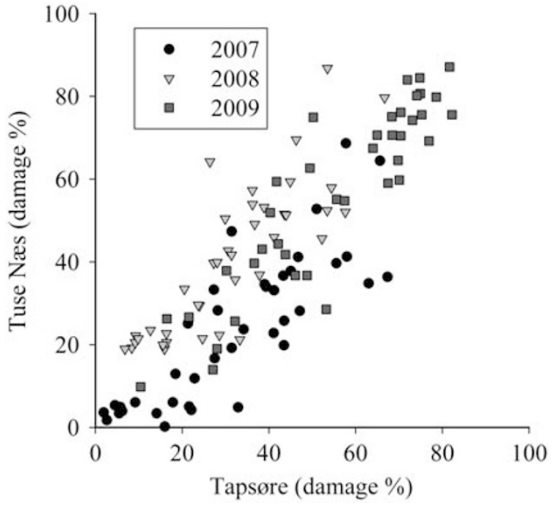

Figure 1 Correlation in clone performance (average per cent damage score) at the two test sites in the 3 years measured.

two peaks: nine clones (22.5%) with average PDS < 10%, and nine clones (22.5%) with PDS between 30 and 40%, whereas four clones (10%) had average PDS > 50% (Figure 4). The distribution changed during 2008 and 2009 towards an increasing number of clones with severe damage (PDS > 50%), and the tendency of a bi-modal distribution of clones became weaker (Figure 4). A few clones maintained a low level of damage, and average health of the least affected clone 35 remained unchanged from PDS = 12.9% in 2008 to PDS = 10.1% in 2009, whereas the average damage score of all clones increased from PDS = 36.1 to 55.9% from 2008 to 2009 (Figures 2 and 4).

A moderate negative genetic correlation was observed between damage level and height. On the basis of phenotypic values of the trees at both sites, small clones were in general more affected than fast-growing clones ($r_p = -0.23$ in 2009). The genetic correlation (r_g) was -0.22 and -0.24 at the two sites in 2009 (Table 3). The correlation was also negative within clones ($r_E = -0.29$ and -0.27, Table 3), that is, smaller ramets for a given clone were more damaged than larger suggesting either larger trees being less susceptible to the disease, or that growth is inhibited by disease. An observed negative phenotypic correlation between height and damage was thus found to have both a genetic and an environmental effect of equal magnitude.

Flushing and leaf shed traits were only scored in 2009 and only at the Tuse Næs trial. The genetic variation for both phenological traits were significant, and with H^2 of 0.32 and 0.34 (Table 2). A negative correlation was observed between these traits and damage level based on phenotypes of the trees (phenotypic correlation $r_p = -0.26$ (flushing) and $r_p = -0.35$ (leaf shed), that is damaged trees in general flushed and shed leaves later relative to healthier trees. However, the background of these correlations was found to be very different when separated into genetic and non-genetic components. There was a moderate-to-weak correlation between flushing and health at the genetic level ($r_G = -0.34$, Table 3), suggesting that clones with early flushing in general are relatively less susceptible. The correlation within clones (non-genetic origin) was comparable to the genetic correlation ($r_E = -0.26$, Table 3). A stronger, and high negative genetic correlation was observed between health and leaf shed and the correlation at the genetic level was substantially higher than the correlation within clones ($r_G = -0.73$, $r_E = -0.18$, Table 3). The genetic analysis thus identifies a strong association where clones that retain their leaves longer in the fall in general have a higher susceptibility to *C. fraxinea* infections (Figure 5).

Discussion

Genetic variation among clones in susceptibility

The structure analysis based on few but highly polymorphic markers revealed one uniform population with no sub-structuring that could have explained differences in susceptibility among the evaluated clones.

This study reveals strong genetic variation among trees from the Danish ash population in symptom development due to the EID. The 39 clones tested in the field trials responded very differently in terms of maintaining crown health and exhibiting level of symptoms following natural infections and spread of *C. fraxinea* that occurred on the sites during the 3 years of observation. The results show that a few clones are less susceptible to *C. fraxinea* than most. One of the

Table 3 Genetic, r_G (above diagonals) and environmental, r_E (below diagonals) correlations between traits at Tapsøre and Tuse Næs

	Tapsøre				*Tuse Næs*						*Across sites*
	PDS 07	*PDS 08*	*PDS 09*	*Height*	*PDS 07*	*PDS 08*	*PDS 09*	*Height*	*Flushing*	*Senescence*	*PDS*
PDS 07	1	0.95 *0.02*	0.91 *0.04*	−0.48 *0.16*	1	0.97 *0.02*	0.95 *0.03*	−0.28 *0.24*	−0.22 *0.17*	−0.78 *0.08*	0.90 *0.04*
PDS 08	0.36 *0.03*	1	0.94 *0.02*	−0.34 *0.18*	0.47 *0.03*	1	0.92 *0.04*	−0.34 *0.24*	−0.19 *0.18*	−0.71 *0.10*	0.95 *0.04*
PDS 09	0.25 *0.03*	0.55 *0.02*	1	−0.22 *0.19*	0.24 *0.04*	0.28 *0.04*	1	−0.24 *0.24*	−0.34 *0.16*	−0.73 *0.09*	0.96 *0.03*
Height	−0.52 *0.03*	−0.44 *0.03*	−0.29 *0.03*	1	−0.24 *0.04*	−0.28 *0.04*	−0.27 *0.04*	1	0.12 *0.04*	−0.03 *0.05*	
Flushing					−0.03 *0.05*	−0.03 *0.05*	−0.26 *0.05*	0.14 *0.04*	1	0.01 *0.07*	
Senescence					−0.05 *0.05*	−0.16 *0.05*	−0.18 *0.05*	−0.07 *0.05*	0.07 *0.18*	1	

Abbreviation: PDS, per cent damage score.
Standard errors are shown in italics.

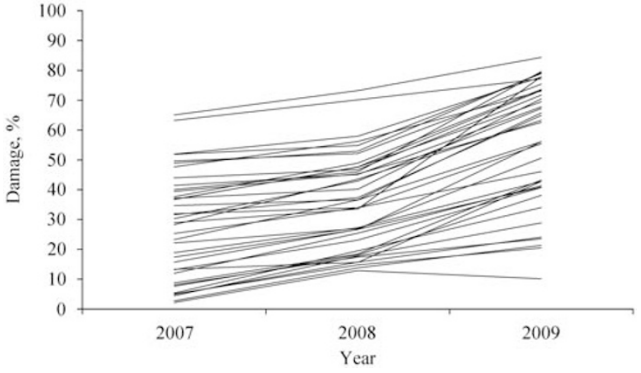

Figure 2 Development of disease symptoms 2007 to 2009. Average per cent damage for each clone at both sites is shown for each year.

39 tested clones maintained an average damage of 10% (with an insignificant effect on overall health of the tree) (Figure 2). Broad-sense heritability of 0.40–0.49 in 2009 suggests that almost half of the observable phenotypic variation in level of damage is because of genetic variation in susceptibility. The finding of strong genetic background in the observed variation is supported by genetic correlation of health scores between the two sites close to 1 leaving room for very limited genotype-by-environment interaction. Thus, the ranking between clones is basically unaffected by environment, that is, the same genetically determined resistance to the disease is working in both environments. The high genetic coefficient of variation suggests that the observed genetic diversity reflects high evolutionary potential (Houle, 1992). This is supported by preliminary results of studies of half-sib families that indicate susceptibility being inherited from mother to offspring based on high levels of additive genetic variation and high narrow-sense heritability (unpublished data).

In agricultural crops with low genetic diversity, it is well known that an increase in susceptibility to new or recurring diseases is often the consequence of specific selection of inbred lines grown in large monocultures (Burdon *et al*., 2006; Salvaudon *et al*., 2008). In contrast, in naturally occurring woody species the principle of protection against EIDs through high genetic diversity is less studied. Nevertheless, variation in susceptibility to new pathogens has been observed in species undergoing fungal infection epidemics. Differences in susceptibility to the pathogenic fungi *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* were observed between European *Ulmus* species, and between clones within species (Pinon *et al*., 2005; Solla *et al*., 2005). *U. glabra* and *U. laevis* are still quite common in Denmark (Nielsen and Kjaer, 2010a; Nielsen and Kjaer, 2010b), and the Danish landscape may also include *Ulmus* trees that have survived the outbreak of *O. novo-ulmi*. Similar observations of genetic variation in susceptibility towards the blight fungus *Cryphonetrica parasitica* that eliminated the American chestnut in the early 1900s were observed within the species (Bettite and Diller, 1954). In the case of canker disease in North American butternut trees caused by *Sirococcus clavigignenti-juglandacearum* variation in susceptibility has also been observed and presence of resistance in healthy individuals growing alongside diseased trees is supported by controlled inoculation experiments (Ostry and Moore, 2008).

Although variation in susceptibility to EIDs in the mentioned species has been observed, a quantitative estimate of resistant individuals in wild population has not been proposed. Our trials comprise 39 individuals of a Danish population of which only 1 individual (no. 35) remained unaffected with $<10\%$ crown damage despite severe infection pressure. Given that our sample is indeed representative for the Danish ash population, we expect that only a small proportion of the wild population will have inherent resistance against this devastating disease. In the coming years, further assessments of the trials will reveal whether resistance in the few healthy clones is stable and whether selection of remaining healthy trees in the landscape will constitute a good basis for a new breeding population.

Height, growth and damage level

Ash trees of all sizes are affected by the disease, however it has previously been reported that smaller trees are more severely affected (Skovsgaard *et al*., 2009). The negative correlation between size and health shown in this study also points towards a tendency that smaller trees are more affected by *C. fraxinea* infections. In our study, it should be noted that we deal with grafting of trees that in some cases are likely to have a physiological age of more than 150 years (for example, no. 18 selected as a mature tree in 1934). However, our observation of correlation between size and health within the clones (r_E) indicates that the correlation is not simply because of some clones being generally larger and more resilient than others. We expect no correlation between vigorous growth before disease and degree of susceptibility after the fungus occurred in Denmark, but it cannot be ruled

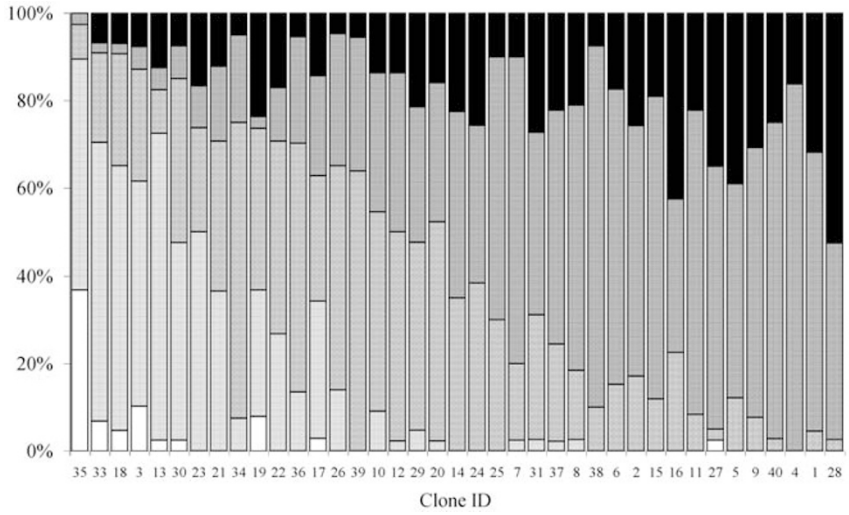

Figure 3 Distribution of ramets in health damage score classes for individual clones in 2009. Colour indication: white = 0% damage, light grey = 0–10% damage, medium grey = 10–50% damage, dark grey = 50–100% damage and black = 100% damage. Clones are ranked by their average damage score in 2009.

out. However, to our knowledge, relationship between vigorous growth before the disease and disease susceptibility has not been found yet.

The dieback symptoms were first reported in Denmark at least 3 years before the first measurement of the two trials (Thomsen and Skovsgaard, 2006). We cannot exclude that small trees show stunted growth because of infections, and it is also possible that larger trees induce stronger resistance than smaller trees simply because of better general vigour.

Finally, as the health measurements are based on the proportion of crown damage and the crowns of smaller trees are composed of lesser branches than larger trees, a given infection and subsequent wilt due to *C. fraxinea* infection may simply result in a higher relative proportion of dead branches in the crown in smaller trees compared with large trees. For these reasons, it is difficult to infer on the likelihood that the observed genetic variations in susceptibility is related to genetic variation in growth (under stabilized natural selection), but our findings do not point towards such a relationship.

Effect of phenology on susceptibility

An interesting result of this study was the strong correlation between health and leaf senescence ($r_G = -0.78$, Figure 5), indicating that clones less affected by *C. fraxinea* must be found among clones with early leaf shed. This relationship was relatively weak among ramets within clones (low $r_E = -0.18$), indicating that variation in leaf senescence could only be poorly explained by the degree of infection of a given tree independent of its genotype. The correlation is not likely to be an artefact because of delayed leaf shed of infected trees, because this would also be reflected on r_E. Rather, the late leaf shed may indeed be causally associated with the genetically determined resistance. The life cycle of *H. albidus* is probably dependent on the infection of ash leaves during the growth season (summer) at the time when the spores are released and infect growing leaves (Kirisits *et al*., 2009a). Hyphae are established in the shed leaves in which fruiting bodies are formed in the following season, releasing spores for infection of fresh leaves. If the aggressive *C. fraxinea* follows this cycle, trees must be infected through leaves through the node into the wood, with the result that early leaf shed may lead to fewer infections of shoots. We observed necroses around leaf scars and from small side branches and discolouration of bark and phloem that suggests that hyphae spread through this tissue. This is in concordance with previously reported symptoms (Bakys *et al*., 2009a; Halmschlager and Kirisits, 2008; Kirisits *et al*., 2009a; Kirisits *et al*., 2009b; Skovsgaard *et al*., 2009; Szabo, 2009). It is unknown how the *C. fraxinea* spores initially establish hyphae in leaves or wood, therefore it can be speculated that leaf phenology and physiology can influence the ability to penetrate to the living woody tissue successfully. Rapid leaf senescence could therefore shorten the infection period and risk of hyphal growth into the woody tissue.

The genetic correlation in health between the two sites in all 3 years of observations led us to speculate whether the less susceptible trees simply are able to escape disease through early leaf shed, or whether genetically activated resistance is induced to reduce or contain infections. No clones were entirely free from symptoms, suggesting that infection is probable, but perhaps less likely in early senescing clones. A major feature of the variation in susceptibility was the difference in dynamics from 2007 to 2009 in which most clones decreased rapidly in health following first symptoms, whereas a few clones maintained low level of damage even in infected trees. This suggests that the observed variation is not simply a result of reduced risk of infection in healthy clones, but it is likely that these clones are able to limit the spread of infections by mechanisms of partial resistance. Ongoing controlled inoculations and quantification of necroses between selected healthy and susceptible clones may reveal the cause of observed variation. The results suggest that susceptible individuals have a prolonged growth season, whereas senescence is induced

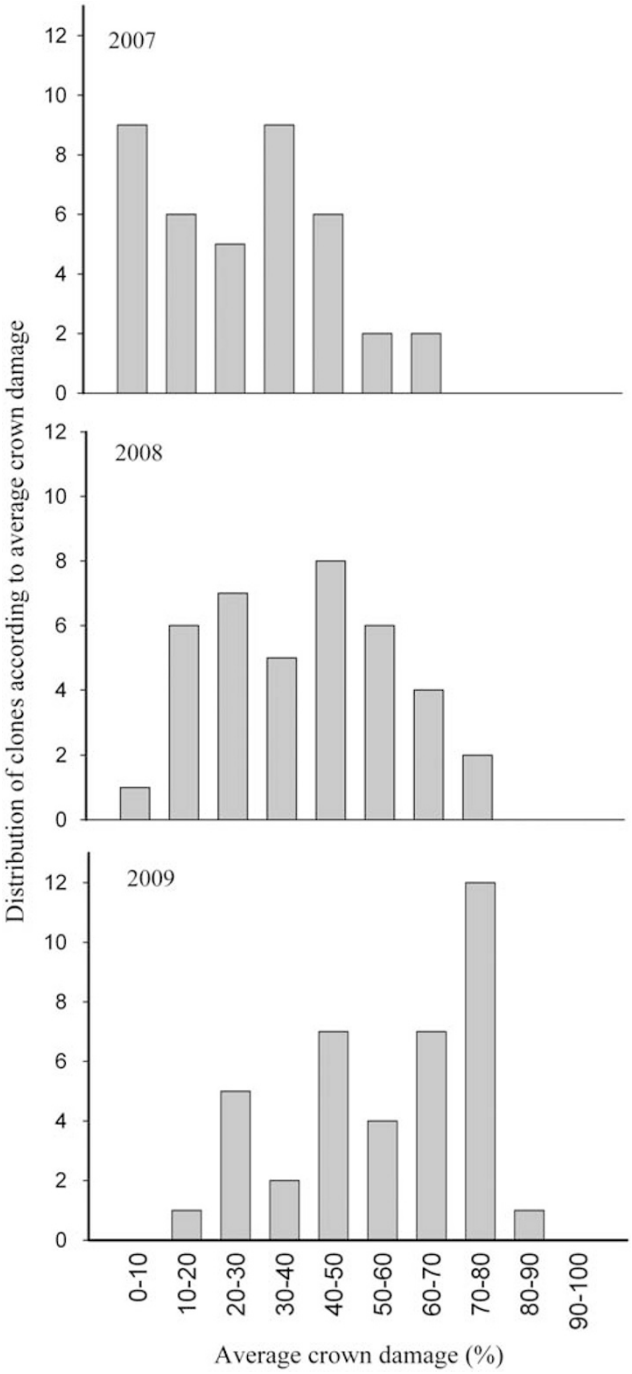

Figure 4 Distribution of clones according to crown damage assessed over 3 years.

earlier in the resistant clones. Susceptibility could be determined by the allocation of carbon into synthesis of secondary metabolites active in plant defence or into the growth metabolism of the trees. The observations may therefore illustrate the classical concept of trade-offs in plant defence between growth and defence (Herms and Mattson, 1992). However, we have no data to indicate whether more susceptible trees have greater vigour in disease-free conditions.

Interestingly, in the case of Dutch elm disease, seasonal variation and phenology seems also correlated to

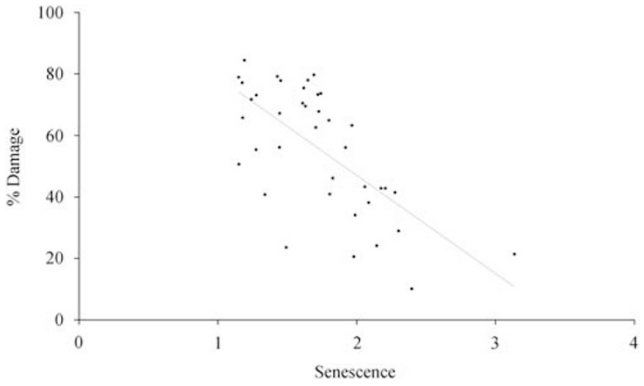

Figure 5 Correlation between average per cent damage for each clone at both sites and the average senescence measured as leaf shed at Tuse Næs.

susceptibility, but is related to vector–insect behaviour (Takai and Kondo, 1979; Ghelardini, 2007; Santini *et al*., 2005). There are no known vectors of *C. fraxinea*, which is believed to be spread by wind (Kowalski and Holdenrieder, 2009b).

Phenological traits are expected to be under stabilizing selection and do not necessarily constitute the direct cause of variation in susceptibility. However, gene(s) that control this variation in susceptibility may also be involved in resistance towards other pathogens and could have been maintained for this reason. It is also possible that the variation reflects previously neutral polymorphism that has now become of high fitness value since the outbreak of the EID. The wind-pollinated *F. excelsior* is likely to have maintained large effective populations numbers as gene flow in the species has been reported to have been substantial even between apparently fragmented populations (Bacles *et al*., 2005). The high gene flow and large effective population size have contributed to the high level of genetic variation in ash, which in response to the new EID renders a few individuals resistant.

Concluding remarks

The impact of the novel EID on *F. excelsior* populations in Europe is already of substantial magnitude and measures must be taken to conserve the species. Empirical studies, in field and in a disease-free environment, have been initiated using clones included in this study. Controlled inoculations are expected to elucidate whether variation in susceptibility is caused by phenology or resistance mechanisms—or a combination of both. Presence of natural genetic variation in susceptibility opens prospects for adaptation of the species to the new pathogen through natural selection for trees with higher fitness because of observed partial resistance in this study. This study suggests that genetic resistance is partial and the frequency of susceptible trees is high. Still, this leaves a small subset of the population with very high relative fitness for effective natural selection, as the disease seems to kill or severely damage trees. At this stage, it is difficult to infer the outcome of such a natural selection scenario, because the dynamics will depend on co-evolution between the host and the pathogen, and thereby the durability of resistance (Salvaudon *et al*., 2008). Further insight into the population

genetics and life history of the fungus in relation to the host is needed to predict the likely outcome of the ongoing spread of the disease.

Conflict of interest

The authors declare no conflict of interest.

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