Forestry An International Journal of Forest Research

Institute of Chartered Foresters

Forestry 2018; **00**, 1–10, doi:10.1093/forestry/cpx059

Evidence from mortality dating of *Fraxinus excelsior* indicates ash dieback (*Hymenoscyphus fraxineus*) was active in England in 2004–2005

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Received 8 September 2017

Ash (*Fraxinus excelsior* L.) planted at six sites over the past 20 years was investigated. Three geographically isolated sites (Northumberland, Leicestershire and Devon) were compared with three sites in established areas of ash dieback in East Anglia, and the causal pathogen, *Hymenoscyphus fraxineus*, confirmed at all. Dieback severity, the frequency of stem basal lesions and pathogen apothecia, were quantified at all sites but despite high disease levels, tree mortality was low. Some trees had typical *H. fraxineus* stem cankers but had apparently died between 2001 and 2011, before the earliest UK records of *H. fraxineus*. Ring counts established beyond doubt the year of death and canker initiation in 27 dead trees. Cankers on the same trees were then tested for *H. fraxineus* using PCR-based detection, with pathogen presence confirmed as early as 2004/05 in some. This places *H. fraxineus* in England much earlier than previously thought, even pre-dating its documented arrival in neighbouring European countries. The advanced disease levels at some sites plus confirmation of *H. fraxineus* in old stem cankers, suggests that planting of infected *H. fraxineus* tree stock in England could date back to the early 1990s, with affected trees dying in the mid-2000s. Additionally, it raises questions about the origins of the infected plants and uncertainties about plant trade pathways.

Introduction

The fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (basionym: *Chalara fraxinea*, synonym: *H. pseudoalbidus*) is now acknowledged as the cause of serious dieback (Kowalski and Holdenrieder, 2009a,b) which threatens populations of European ash (*Fraxinus excelsior* L.) and narrow-leaved ash (*F. angustifolia* Vahl) across much of Europe (Keßler *et al.*, 2012). The first indication of the current epidemic in Europe dates from observations in the early 1990s of severe dieback of ash in Poland (Przybyl, 2002) and Lithuania (Juodvalkis and Vasiliauskas, 2002). However, at that time frost and drought were implicated as the cause of the symptoms and a biotic causal agent was not identified and named until about a decade later (Kowalski, 2001, 2006). It is now clear that *H. fraxineus* is an introduced pathogen which appears to originate from East Asia, with reports of the fungus from China, Japan, Korea and Russia (Zhao *et al.*, 2013; Baral and Bemmann, 2014; Han *et al.*, 2014; Zheng and Zhuang, 2014).

In its native range associated with Asian ash species (e.g. *F. mandshurica* Rupr. in Japan) *H. fraxineus* is reported to cause little or no damage (Zhao *et al.*, 2013; Drenkhan *et al.*, 2017). However, the behaviour of the fungus on native European ash species (*F. excelsior* and *F. angustifolia*) is very different (Keßler *et al.*, 2012). Initially disease symptoms on trees include foliage necrosis but infection then progresses from affected leaves and rachises (petioles) into shoot or stem tissue, killing the cambium and phloem and forming characteristic diamond-shaped lesions which can completely girdle affected branches and stems. To complete its life cycle *H. fraxineus* then sporulates by producing fruit bodies (apothecia) during summer months formed mainly on the rachises of fallen leaves infected the year before but also occasionally on infected dead shoots, stems and root collars of young ash trees in ground contact (Gross *et al.*, 2012; Kirisits *et al.*, 2012). However, apothecia can only be produced if both mating types (*MAT1-1* and *MAT1-2*) of the fungus are present and sexual reproduction occurs (Gross *et al.*, 2012). Once apothecia are formed in any number the disease can start to build-up to epidemic phase as wind-dispersed ascospores are released (Kowalski and Holdenrieder, 2009b; Timmermann *et al.*, 2011), leading to multiple infections on the foliage of nearby ash trees as dispersal of the spores can occur over hundreds of metres (Chandelier *et al.*, 2014).

Hymenoscyphus fraxineus was first identified in February 2012 in southern England on nursery stock that had been imported from the Netherlands (Sansford, 2013). It was

subsequently confirmed in June 2012 on a landscaped site planted the previous year in Leicestershire (DR Rose and JF Webber, Forest Research, *pers. comm.*). Following this, the Forestry Commission (FC), Food and Environment Research Agency and other Government departments initiated an extensive surveillance programme, surveying stock in nurseries, new plantings and ash growing in the wider environment. As a result, more findings were made including a small number of trees on sites where ash had been planted up to five years earlier, providing evidence that some trees had been infected prior to planting out (Sansford, 2013). By October 2012 *H. fraxineus* infection had been found at many locations in the wider environment, particularly across East Anglia and Kent, with naturally regenerating saplings in established woodland and hedgerows showing symptoms. These wider environment findings indicated the early stage of an epidemic was underway, so a systematic survey of ash across the UK was undertaken in November 2012. It identified a total of 184 infected sites; 114 in the wider environment and 55 associated with recent plantings plus 15 nursery sites (Clark and Webber, 2017).

At the conclusion of 2012, the distribution of positive findings showed a concentration of wider environment infected sites along the eastern seaboard of Kent, East Anglia and sporadically in Lincolnshire and East Yorkshire northwards to Northumberland and Scotland (Freer-Smith and Webber, 2015). This suggested the possibility of disease establishment as a result of spore inoculum blown in the wind from continental Europe. Modelling undertaken to evaluate this concluded that there were at least 100 days between 2008 and 2011 on which environmental conditions including wind direction, rainfall and humidity, were conducive for dispersal of ascospores from infected ash in mainland Europe across the sea, thereby causing infection of ash predominantly in east and south-east England (PostBOX, 2012). However, there were also more isolated areas of infection with visible *H. fraxineus* symptoms concentrated within woodlands newly established since 2007, and some of the stands even had planting dates well before the disease had been recognized in Europe. With sites planted in the 1990s up to the mid-2000s, especially those located in more isolated areas, infection from windblown spores originating from outbreaks in neighbouring European countries appears very unlikely.

Therefore, although ash dieback disease may have established in the UK as result of airborne inoculum of *H. fraxineus* from mainland Europe, disease foci were also likely to have been initiated from infected imported stock in some parts of the country. We set out to explore the likelihood of disease spread from infected planting stock, and to establish how long ago this might have happened on sites which had a documented history of planting and subsequent management.

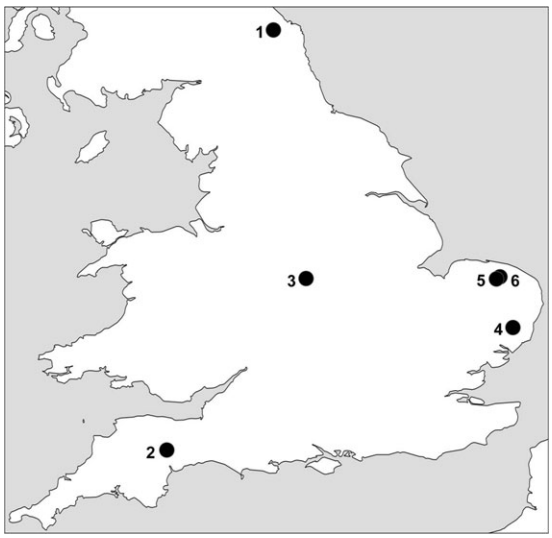

Figure 1 Locations of the six study sites in England. Site details are: (1) Wooler, Northumberland; (2) Bickleigh, Devon; (3) Swadlincote, Derbyshire/Leicestershire border; (4) Framlingham, Suffolk; (5, 6) two sites both near Reepham, Norfolk.

Materials and methods

Site selection

In 2013, Forestry Commission England Plant Health surveyed ash stands planted from 1991 onwards for the presence of symptoms consistent with *H. fraxineus*. The survey identified a number of sites that were unlikely to have established from windborne inoculum but where disease appeared to have been present well before the first confirmed findings in 2012. Resources limited the number of sites that could be studied but two categories were defined: (1) Sites located in areas of established and widespread infection (in eastern England) and (2) sites geographically isolated from areas of established and widespread infection. Additional criteria for site selection were: each had a significant component of ash planted in blocks or groups; comparability in age and accessibility; a planting date before 2012; and a geographic distribution which gave national representation. All sites also contained a number of standing dead trees, many of which had cankers indicative of *H. fraxineus* infection prior to death. In all, six sites were selected and the presence of the pathogen confirmed in trees with current symptoms (see King and Webber (2016) for methods). The sites shown in Figure 1 comprised one in Devon, one on the Derbyshire/Leicestershire border, one in Northumberland and three in East Anglia where ash dieback symptoms were relatively widespread in 2012. Sites selected in East Anglia consisted of two neighbouring sites in Norfolk and one in Suffolk. Further details about each site including origin of the plants and year of planting are supplied in Tables 1 and 2.

To gather evidence of how long symptoms of ash dieback might have been visible on each site, all six sites were surveyed in detail in 2013. For each site outside East Anglia, a wider 1.5 km radius survey was also undertaken, to ascertain extent and distribution of symptoms in the wider environment. Efforts were made to look for factors that were indicators of longstanding disease including widespread dieback, tree mortality, presence of lesions at the tree collar often referred to as basal lesions (Husson *et al.*, 2012) and whether *H. fraxineus* apothecia were abundant in the litter layer under symptomatic trees.

When dead trees were located, evidence of *H. fraxineus* in the form of diamond-shaped cankers on the main stem was recorded (i.e. those likely to have been killed by ash dieback). Any dead trees with visible cankers were cut close to the base to reveal the stem cross-section and a count was made of the number of annual rings by eye, to establish an estimated year of death using the known planting date for each site.

Planting year and origin of tree stock

Evidence of the year of planting and the trees source was sought for each of the selected sites. Site owners, where available, were asked for relevant information, and Forestry Commission England's database was interrogated for further information on grant assistance for each site. Additionally, aerial photographs of each site from spring/summer 1999 were visually analysed

Table 1 *Fraxinus excelsior* sites in England evaluated for ash dieback with dating of tree mortality.

Location	Stand Area (ha)	Assessed compartment		Symptom Frequency[1] (in assessed compartment)		Assessment of dead trees		
		Plant origin	Year of planting[2]	Basal lesions	Dead trees	No. of trees assessed	Estimated years growth	Estimated year of death
Site 1 near Wooler, Northumberland	1.2	Nursery in Scotland	1999/2000	No data	Scarce	2 trees	3	2001/02
Site 2 near Bickleigh, Devon	28	Nursery in Belgium	1996/1997	Common	Scarce	1 tree	10	2004/05
						2 trees	11	2005/06
						1 tree	12	2006/07
						1 tree	15	2009/10
Site 3 near Swadlincote, Derbyshire / Leicestershire border	27	Nursery in England	2001/2002 (estimated)	Scarce	Scarce	3 trees	8	2007/08
						2 trees	9	2008/09
						1 tree	11	2010/11
						1 tree	12	2011/12
Site 4 near Framlingham, Suffolk	90	Nursery in England	1991/1992	Occasional	Scarce	1 tree	6	1995/96
Site 5 near Reepham, Norfolk	3	Nursery in England	1996/1997	Common	Occasional	4 trees	8	2002/03
						1 tree	9	2003/04
						1 tree	10	2004/05
						3 trees	11	2005/06
						1 tree	12	2006/07
Site 6 near Reepham, Norfolk	8	Nursery in England	1996/1997	Common	Occasional	4 trees	7	2001/02
						5 trees	8	2002/03
						1 tree	13	2007/08
						1 tree	15	2009/10

[1]Categories are quantified as: Scarce < 1 per cent, Occasional 1–10 per cent, Common 11–50 per cent.
[2]After the main planting year some replanting was undertaken the following year to replace plants that failed to establish, so planting and year of death may be +1 year.

for verification. Where very limited information was available (site 3), a live tree from the same compartment was felled and the ring-count information used to determine likely planting year (see below).

Tree dating

At three of the sites a more rigorous ring-count analysis was undertaken (Table 2); samples were taken from 27 dead trees, consisting of basal discs and lengths of the main stem, each with one or more *H. fraxineus*-like cankers. All discs or stem sections were sealed individually in a plastic bag, labelled and returned to the laboratory for analysis. On receipt the samples were recorded and relabelled with deep-stapled metal tags; basal discs were then oven dried immediately, while stem sections were stored at 4°C pending molecular analyses as described in the next section.

For accurate counts of the annual growth rings in the 27 trees, basal discs were dried at 50°C over several days until they reached a constant weight, and then one surface on each disc was sanded to create a smooth, level surface so the growth rings could be counted. As the planting date of each tree was known, date of tree death could be calculated based on the ring counts. Annual growth rings were assessed only by presence; no quantification of increment size was attempted although reductions were usually apparent in the final 1–2 years of the tree's life

Once the stem sections with ash dieback cankers had been sampled for use in PCR detection of *H. fraxineus* (see below), each section was cross-cut at the widest part of the canker, then dried and sanded in the same way as the basal discs. Each section was also photographed (Figure 2), and the callus growth around the edge of the canker was examined to establish the year in which phloem and cambium was first damaged and therefore how many years prior to tree death the canker symptoms first appeared.

Additional sampling and analysis

A single tree was selected a few metres away from the pocket of ash with extensive and severe symptoms of ash dieback at site 3. It had several well developed cankers at the base of the main stem which extended up to 1.5 m from the base. The extent of callusing around the canker margins suggested that the cankers had been initiated some years earlier. The tree was felled in January 2015 and approximately 1 m of the cankered stem returned to the laboratory for analysis and dating.

Prior to dating of the cankers, isolation of *H. fraxineus* was attempted from the necrotic phloem tissue of each discrete stem lesions using methods of EPPO (2013). Isolates obtained were confirmed as *H. fraxineus* based on colony morphology and the PCR diagnostic of Johansson *et al.* (2010).

Detection of Hymenoscyphus fraxineus in cankers

All stem canker samples from dead trees were bagged and stored separately at 4°C to prevent any cross-contamination. In addition, two 'healthy' stem sections (i.e. with no external lesions) were collected from site 5 to act as controls. An isolated room with no history of *H. fraxineus* diagnosis was used to process the samples. Prior to this, and to avoid any possibility of contamination with *H. fraxineus* fungal

Table 2 Dating and PCR testing to detect the ash dieback pathogen *Hymenoscyphus fraxineus* applied to 27 diseased *Fraxinus excelsior* (ash) samples collected from three sites in England.

Site number	Sample code	Tree height (m)	Symptoms on main stem (& height above ground level)	Planting year	Age at planting	Years of growth	Year of tree death	Years of growth around canker	Year infection-causing canker initiated	Outcome of RT-PCR-based detection of *H. fraxineus*
3	4	2	Five cankers, between 0.1–1.8 m	2001	2[1]	13	2012/13	2	2010/11	Inconclusive
3	22	3.5	Stem 1: 6 cankers 40 cm–2 m. Stem 2: 5 cankers	2001	2[1]	14	2013/14	3	2010/11	Inconclusive
3	23	2.5	Seven cankers, between 0.3–1.8 m	2001	2[1]	13	2012/13	2	2010/11	Inconclusive
4	3	1.6	Multiple small cankers on stem and branches	1991	2	15	2004/05	0	2004/05	**Positive**
5	1	1.6	Large canker at 0.5 m and above. Epicormics at base	1996	2	14	2008/09	1	2007/08	Inconclusive
5	2	2.6	Five large cankers, between 0.2–0.8 m, plus basal lesions	1996	2	13	2007/08	1	2006/07	Negative
5	5	2.5	Two cankers, between 0.5–1.2 m	1996	2	14	2008/09	0	2008/09	**Positive**
5	6	2	Seven cankers, between 0.3–1.2 m	1996	2	12	2006/07	1	2005/06	Negative
5	7	2.2	Two coalesced cankers at 0.4 m	1996	2	14	2008/09	2	2006/07	**Positive**[2]
5	8	2.3	One small canker at 1.0 m	1996	2	13	2007/08	1(?)	2006/07	Negative
5	9	2.4	Eight cankers between 0.2–1.0 m	1996	2	17	2011/12	1	2010/11	Negative
5	10	3.5	Multiple cankers between 0.2–2.5 m	1996	2	17	2011/12	1(?)	2010/11	**Positive**
5	11	4.5	Five cankers, between 1.0–3.5 m	1996	2	15	2009/10	2	2007/08	Inconclusive
5	12	3	Numerous cankers between 1.0–2.7 m	1996	2	14	2008/09	1	2007/08	Negative
5	13	4.2	Four cankers between 1.5–3.0 m	1996	2	14	2008/09	2	2006/07	Negative
5	14	2.4	Numerous coalesced cankers between 0–1.2 m	1996	2	15	2009/10	1	2008/09	**Positive**[2]
5	15	5	Multiple cankers between 0.5–4.0 m	1996	2	17	2011/12	1	2010/11	Negative
5	16	2.5	Five cankers between 0.5–1.5 m	1996	2	14	2008/09	1	2007/08	Negative
5	17	2.5	Six cankers between 0.3–2.0 m	1996	2	14	2008/09	1	2007/08	Inconclusive
5	18	3.4	Numerous cankers between 0–2.0 m	1996	2	14	2008/09	1	2007/08	Inconclusive
5	19	4.7	Six cankers between 1.3–3.5 m	1996	2	14	2008/09	1	2007/08	Inconclusive
5	20	2.5	Three large cankers between 0.3–0.8 m	1996	2	14	2008/09	2	2006/07	**Positive**
5	21	3	Multiple cankers between 0–2.0 m	1996	2	14	2008/09	2	2006/07	Negative
5	24	3.2		1996	2	15(14–18)		1	2008/09	Inconclusive

Continued

Table 2 *Continued*

Site number	Sample code	Tree height (m)	Symptoms on main stem (& height above ground level)	Planting year	Age at planting	Years of growth	Year of tree death	Years of growth around canker	Year infection-causing canker initiated	Outcome of RT-PCR-based detection of *H. fraxineus*
			Multiple cankers between 0.5–3.0 m				2009/10 (2008–13)			
5	31	2	One canker at 0.7 m	1996	2	13	2007/08	2	2005/06	Negative
5	32	2.5	Three cankers above 1.2 m	1996	2	13	2007/08	1	2006/07	Negative
5	33	2	Three cankers between 0.5–1.5 m	1996	2	14	2008/09	1(?)	2007/08	Negative
5	34		Healthy stem material	–		–	–			Negative
5	35		Healthy stem material	–		–	–			Negative

[1]Planting year estimated.
[2]Presence of *Hymenoscyphus fraxineus* confirmed for these samples by both RT-PCR and end-point PCR and ITS sequence analysis.

material or DNA, the room was thoroughly disinfected with both 70 per cent ethanol (v/v) and 10 per cent sodium hypochlorite solution (v/v; bleach). All the stem sections were also rinsed with 70 per cent ethanol for 1 min, followed by bleach solution for 1 min, and finished by a wash in sterile distilled water for 1 min, after which they were air-dried at room temperature. A mallet and chisel (wiped with bleach and flame dried) were used to split logs longitudinally, and a sliver of underlying xylem tissue beneath the canker was excised using a similarly cleaned scalpel. Each xylem sample was then transferred to a sterile tube and stored at −20°C, until subsequent DNA extraction using a DNAmite kit (Microzone Ltd, UK) into a volume of 300 µl. For subsequent use in real-time PCR (RT-PCR) testing, 100 µl of the DNA extract was purified using a OneStep™ PCR Inhibitor Removal Kit (Zymo Research, USA).

Real-time PCR analysis

All 29 DNA samples (27 from symptomatic material, 2 healthy controls) were tested for the presence of *H. fraxineus* using species-specific multiplex RT-PCR (EPPO, 2013; Table 2), with two technical replicates of each DNA extract tested. RT-PCR simultaneously targeted both *H. fraxineus* and host plant DNA using different primer/probe combinations (Ioos *et al.*, 2009; Ioos and Fourrier, 2011) and used a Roche Lightcycler 480 instrument (Roche Diagnostics, Basel, Switzerland). Data analysis used the LightCycler 480 (version 1.5.0.39) software with the absolute quantification second derivatives maximum method used to calculate the crossing point (Cp) values. Samples that produced Cp values of <40 for both technical replicates targeted to *H. fraxineus* DNA were considered positive for *H. fraxineus*. Samples for which only a single replicate produced a Cp value of <40 were considered inconclusive. Samples that produced Cp values of <40 for both technical replicates targeted to host plant DNA (but with neither replicate producing a signal for *H. fraxineus*) were considered negative. Both positive (*H. fraxineus* and 'healthy' plant template DNA of known Cp value) and negative (no-template) water controls were included in all testing.

Results

Site observations

Site 1: Near Wooler, Northumberland

This isolated *c.* 1.2 ha stand of almost pure ash was planted between 1999 and 2001. Owner records provided planting year and details of the supplying nursery; the latter confirmed these records and the age of stock supplied (Tables 1 and 2). The supplying nursery was in England but was known to occasionally supplement stock by purchasing trees from a wholesaler which routinely imported tree stock from mainland Europe. Planting positions were clearly visible in 1999 aerial photographs.

Initial investigation revealed one area of the stand with notably poorer growth. There was little mortality and the two trees that died in *ca.* 2001 (Table 1) were so decayed that cause of death was inconclusive. Ash dieback symptoms were most developed in the area of poorest growth and some naturally regenerating ash trees in this area also had limited symptoms, indicating recent infection. All trees were established in tree shelters so no assessment for basal lesions was made. Apothecia were found readily under the symptomatic trees with poorer growth and less frequently elsewhere.

Occasional shoot dieback was observed on some mature trees immediately adjacent to the planted stand suggesting recent disease spread. The 1.5 km radius survey around the site revealed sporadic symptoms in mature, young and naturally regenerating ash trees in contiguous woodland to the north of the site up to 1.2 km away. No symptoms were identified in the many hedgerow trees dividing fields to the east of the site, and no infection was detected on younger recently planted ash stands 800 m to the north.

Site 2: Near Bickleigh, Devon

The 28 ha site comprised pure blocks of various broadleaf species originally planted in 1996, with additional planting in 2008; planting year and plant origins were provided by the owner (Tables 1 and 2). Planting positions were clearly visible in 1999 aerial photographs.

Initial inspection suggested that a high percentage of the more recently (2008)-planted ash had symptoms consistent with *H. fraxineus* infection including stem/branch cankers and wilting, although apothecia were not easily found on fallen rachises under these trees (A. Whybrow and B. Jones, *pers. comm*). In contrast, the ash trees planted in 1996/1997, and also a single block that had been replanted to replace failed cherry trees, showed heavy dieback with many apothecia under

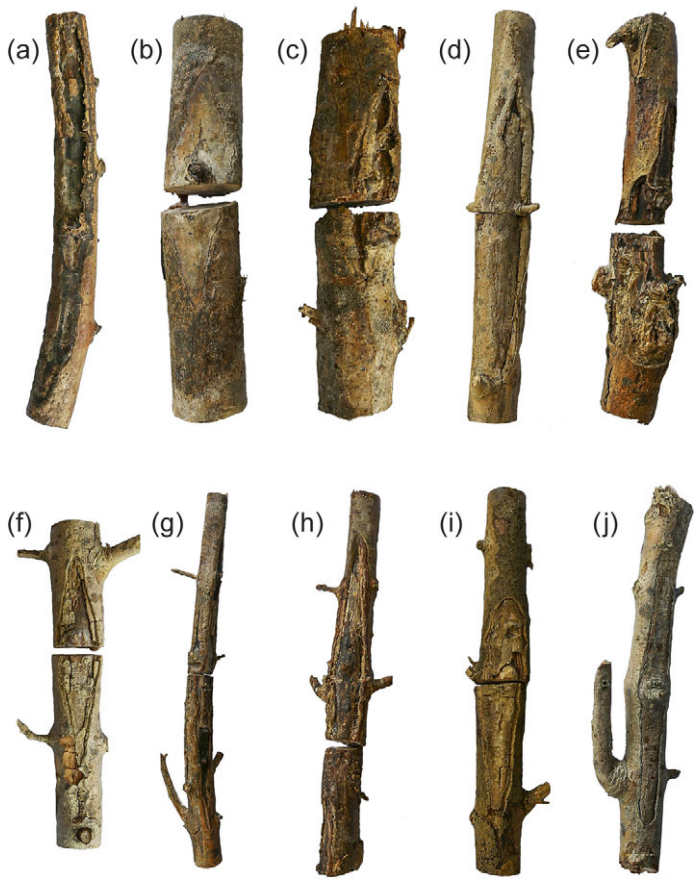

Figure 2 Canker symptoms likely to be caused by *Hymenoscyphus fraxineus* on dead ash trees planted in 1996 and detailed in Table 2. Cankers on trees that died in: (a) 2012/2013 (sample 4), (b) 2011/2012 (sample 10), (c) 2009/2010 (sample 11), (d) 2008/2009 (sample 13), (e) 2009/2010 (sample 14), (f) 2011/2012 (sample 15), (g) 2008/2009 (sample 17), (h) 2008/2009 (sample 20), (i) 2008/2009 (sample 21), (j) 2007/2008 (sample 32).

these trees. Basal lesions were also common on trees in the same block but seen only infrequently in adjoining areas of ash. Overall, mortality was low and estimated year of death was between 2004 and 2010 (Table 1).

The 1.5 km survey revealed sporadic symptoms in hedgerows and in an area of recently planted ash almost 1.5 km away, with the highest proportion of symptoms in trees to the north-west of the site. Symptoms were also occasionally identified in neighbouring mature ash woodlands. Another isolated site (but with more recent signs of infection) was found over 4.5 km away to the south east.

Site 3: Near Swadlincote, Derbyshire/Leicestershire border

This 27 ha broadleaf plantation (planted from 1996 to 2008) contained older stands of ash planted in small groups, and younger plantings of ash in blocks of varying size. Symptoms on this site had been reported to be worst on trees planted in 2008. However, closer inspection revealed that although some trees planted several years earlier had only recently become infected, there were other pockets of similarly aged ash with severe symptoms, including a small number of trees with basal lesions suggesting a longstanding disease presence. The most extensive dieback was concentrated in a group of approximately 20 ash trees, planted within a compartment of mixed broadleaved trees and adjacent to heavily affected areas of 2008-planted ash which were downhill. Mortality in this pocket was confined to seven trees which appeared to have died between 2007 and 2012 (Table 1). A further three trees sampled during a later visit were found to have died between 2012 and 2014 (Table 2).

Sporadic ash planted over a similar timescale were found with *H. fraxineus* infection almost to the edge of the 1.5 km survey radius. Most of the symptomatic trees were located to the east of the site, following the direction of prevailing winds.

Site 4: Near Framlingham, Suffolk

This extensive newly established 90 ha broadleaf wood was created between 1991 and 2002 on farmland which surrounded small pockets of long established ash woodland. The owner was able to confirm the planting years of compartments from site records. The supplying nursery was no longer in business, but anecdotal information suggested that it occasionally purchased stock from various sources including from mainland Europe. Established trees were clearly visible in 1999 aerial photographs; the ash had generally been planted in small pure blocks.

The amount of dieback across the site varied, but basal lesions were only occasionally observed. Although investigations initially concentrated on areas identified by Forestry Commission England aerial surveillance as those with the greatest levels of dieback, there was no single area where symptoms were more developed and therefore where the disease might have been present longer than elsewhere. Only a single dead tree with typical ash dieback symptoms was identified (Table 1). Apothecia were found readily on rachises across the large site, including under the mature canopy of one small ancient ash stand where they sometimes occurred on small woody material in the leaf litter rather than on rachises.

A formal 1.5 km survey was not undertaken as this site was one of the many identified in East Anglia over the autumn/winter 2012 Forestry Commission surveys. However, other nearby sites were visited and confirmed to be infected as part of this in depth investigation. One dead tree was identified that had died between 2004 and 2005 (Table 2)

Site 5: Near Reepham, Norfolk

This small 3 ha site was planted in 1996, and was originally owned, established from the same stock and managed by the same estate as site 6. The two sites are approximately 400 m apart, separated by a disused railway, open farmland and fragments of woodland, including older ash stands and younger, more recently planted ash. The owner was able to supply details of planting year and the supplying nursery. The latter also confirmed the age of stock supplied (Tables 1 and 2) and confirmed that any *Fraxinus* stock supplied would have been grown on the nursery from seed. Planting positions were clearly visible in the 1999 aerial photographs.

Aerial surveillance of sites 5 and 6 revealed worse levels of dieback than elsewhere in the area, and the extent of dieback

suggested a high level of mortality. However, ground inspection revealed most trees were still alive. Dead trees were scattered across the site, and some larger individuals appeared to have been entirely girdled by *H. fraxineus* basal lesions. Apothecia and sclerotized rachises were also abundant across the site. Most of the dead trees were between 1 and 3 m in height, and initially 10 of the smallest dead trees with obvious cankers were dated as having died between 2002 and 2007 (Table 1). A further 23 trees sampled during a later visit were found to have died between 2006 and 2012 (Table 2).

As with site 4, a formal 1.5 km survey was never undertaken. However, inspections of adjacent ash stands revealed an absence of similarly well established symptoms, while nearby mature and ancient stands of ash did not have high levels of dieback and only a few apothecia were found underneath them.

Site 6: Near Reepham, Norfolk

This 8 ha site that was established at the same time as site 5 in 1996, and had similar site conditions. Details of the planting date and stock origins were the same as site 5. Subsequently the site had been sold and had not been thinned. There was limited mortality and most of the dead trees were 1–3 m in height and estimated year of death ranged from 2001 to 2010 (Table 1). Many near-dead, smaller trees had been girdled but still had epicormic shoots close to their root collars but largely hidden by the grass sward. Eleven of the smallest dead trees with typical ash dieback cankers were sampled (Table 1). Many others had basal cankers consistent with longstanding *H. fraxineus* infection, and when some were felled for investigation, it was clear that incremental growth had been reducing over a number of years after establishment, suggestive of long-term infection. Another indicator of a long established outbreak was abundant apothecia on both fallen rachises and small sections of woody material on the forest floor (Kirisits *et al.*, 2014).

Tree dating

Table 1 shows the data for the 36 dead trees that were initially identified for investigation at the six sites. Most of the planting stock had been obtained from UK nurseries and only the stock for the Devon site (site 2) had definitely come directly from a non-UK source. Nursery and/or estate records for sites 1, 2, 4, 5 and 6 confirmed that all plants were two years old when planted out and probably stock for site 3 would have been the same. Replacement stock was planted the year following establishment to make good any planting failures. Apart from site 3, all the replacement plants were known to be sourced from the same nurseries that supplied the original stock.

With the exception of site 1, all the sites had at least one tree that had died some years earlier with one or more diamond-shaped canker(s) on the stem consistent with the symptoms caused by *H. fraxineus*. Although the planting date of most of the cankered dead trees was likely to be the initial establishment year, it could not be ruled out that some of the trees might be one year younger (i.e. were replacement stock). However, based on the ring-count assessments made in the field and the known planting dates for each site, the earliest year of tree death was estimated at 1995/96 (site 4) and the most recent in 2011/12 (site 3), with 19 out of the total of 36 sampled trees estimated to have died by 2004/05.

When a more detailed tree dating analysis was undertaken using a further 27 dead trees taken from sites 3, 4 and 5 (Table 2) a similar pattern emerged, although the accurate counts of growth rings made in the laboratory indicated that the ring counts made in the field had underestimated the age of the trees and therefore the year in which they died. As before, each of the 27 dead trees had at least one diamond-shaped canker on the stem indicative of *H. fraxineus* infection. Examples of the cankers are shown in Figure 2. With this second set of trees, ring counts revealed that 18 out of the 27 had died by 2008/2009, which corresponded to between 10 and 13 years after being planted out. Dating the callus growth on the trees revealed cankers had been initiated up to 3 years earlier, so the symptoms of ash dieback symptoms could be dated at least as far back as 2004/2005 on one tree and to 2005/2006 for a further two (Table 2).

Additional sampling and analysis

Dating the ~1.0 m length of stem taken from a tree at site 3 in 2015 (close to the area of greatest symptom intensity and tree mortality), indicated the first growth occurred in 2000. Thus assuming a planting stock age of 2 years, this suggests the likely planting date for the compartment at site 3 of 2001/2.

The same section of stem had six sunken cankers, two of which had coalesced and each canker ranged in length from *ca.* 12 to 25 cm. *Hymenoscyphus fraxineus* was isolated from four of the six cankers demonstrating they were active lesions, and it transpired that all *H. fraxineus* isolates were of the same mating type (*MAT1-1*). Despite the sunken and aged appearance of the cankers which were surrounded by high ridges of callus tissue, this proved to be deceptive when estimating canker age. The depth of the cankers was largely due to vigorous growth and formation of wide growth rings between 2012 and 2014 from the uninjured cambium and phloem rather than multiple smaller growth rings over a longer period of time. Overall, four of the active lesions dated from 2012; one lesion for which *H. fraxineus* could not be isolated (inactive lesion) dated from 2010; and another inactive lesion dated from 2011 at the latest (Table 3). This suggests that the fungus is capable of remaining alive and colonizing stem material for upwards of 3–4 years after growing into bark tissue from infected shoots or foliage.

Real-time PCR and end-point PCR

Of the 27 canker samples tested with RT-PCR, *H. fraxineus* was confirmed in six (samples 3, 5, 7, 10, 14 and 20) and the two visually healthy shoots were negative for *H. fraxineus* (Table 2). Of the remaining samples, twelve were negative for *H. fraxineus* and the remaining nine produced an inconclusive result (Table 2). Additional definitive evidence for *H. fraxineus* was obtained in two more samples (7 and 14) with less sensitive conventional PCR using the primers of Johansson *et al.* (2010); partial ITS sequences obtained from these samples both showed 100 per cent identity to the type of *H. fraxineus* (Oth_01; GenBank Acc: GU586904).

Table 3 Timing of lesion development by *Hymenoscyphus fraxineus* on a single live *Fraxinus excelsior* log collected in 2015.

Lesion number	Date of lesion initiation	Earliest year of foliar infection	*H. fraxineus* isolated
Lesion 1	2012	2011	Yes
Lesion 2A	2012	2011	Yes
Lesion 2B	2012	2011	Yes
Lesion 3	2012	2011	Yes
Lesion 4	2010	2009	No
Lesion 5	2011 (at latest[1])	2010	No

[1]Log not cross-sectioned at lesion centre, so infection could have been earlier.

Discussion

To evaluate whether ash planted over the previous 20 years might have included any plants infected by the ash dieback pathogen, *H. fraxineus*, certain criteria were required when selecting sites for study. Ideally, sites needed to be relatively isolated and therefore unlikely to be affected by airborne *H. fraxineus* inoculum originating from any nearby local disease foci initiated either from very recently planted infected ash or from disease outbreaks on mainland Europe. If possible, study sites were also excluded with multiple planting episodes over the life of the plantation, to reduce the opportunities for the oldest trees on the site to be cross-infected by diseased stock introduced several years later.

Although the six sites selected for study (established between 1991 and 2002) did not meet all these criteria completely, we were able to find evidence of infection by *H. fraxineus* that dated back at least to the mid-2000s. Moreover, based on the site characteristics, the most parsimonious explanation for the established infection is that it originated from use of infected planting stock rather than from airborne inoculum. In particular, the location and geographical isolation of sites 1, 2 and 3 in Northumberland, Leicestershire and Devon, made the likelihood of exposure to airborne ascospores from ash dieback outbreaks in mainland Europe very small.

Undertaking accurate counts of annual growth rings in the laboratory to determine the year of tree death as well as the year in which cankers were initiated on the main stem of affected trees, allowed disease symptoms to be dated back to at least 2004/2005 on a site planted in 1991/1992 and to 2005/2006 on a site planted in 1996. Ring dating undertaken in the field also indicated that the disease was longstanding but gave less accurate results for the date of first canker symptoms, possibly because of the difficulty in distinguishing individual annual rings from early and late stage annual growth. In addition, with older samples the dead wood tended to be of poor quality again affecting count accuracy. Thomsen and Jorgensen (2011) reported that the size of growth of rings can reduce markedly following high infection levels of *H. fraxineus* again making such assessments difficult.

Molecular analyses also confirmed the presence of *H. fraxineus* in about 22 per cent of the symptomatic trees, at least one of which had died in 2004/2005 and three more which had died in 2008/2009 and had canker symptoms that had been initiated one or two years earlier. The failure to detect *H. fraxineus* in more of the samples with the typical ash dieback cankers that died prior to 2008 could have several explanations. It may be that *H. fraxineus* was not present in those samples and the symptoms were caused by other canker causing agents that affect ash (Kowalski and Lukomska, 2005). There is also the prospect that the DNA extracted from older symptomatic material had become too degraded to amplify and detect following invasion by wood decay fungi and bacteria, or over time the wood had accumulated inhibitors which affected the PCR process. Either is a possibility given the failure in RT-PCR testing to detect both plant DNA as well as *H. fraxineus* DNA from some of the sampled plant material. Despite this, the combined evidence of typical symptoms of ash dieback that could be reliably dated back to the mid-2000s and PCR-based confirmation of the presence of *H. fraxineus* in some of this material, indicate that the visible signs of ash dieback had started to occur almost a decade before the first formal reports of ash dieback in the UK in 2012.

If ash dieback was indeed present on some ash stock planted as early as 1991, it raises the question of why disease development appears to have been relatively slow. Only low levels of mortality were observed some 12–18 years after planting and disease spread to neighbouring woodland and hedgerow ash trees was limited based on the findings of the 1.5 km radius surveys. Assuming that *H. fraxineus* was introduced to each site on infected planting stock and laboratory analysis dates some ash dieback cankers to 2004/2005, it follows that there must have been pseudosclerotial material for the formation of apothecia and some spore production on site at least in the previous year (2003/2004) and probably earlier. However, earlier cankers are likely to have been present on smaller dead plants but easily overlooked. In addition, by time of investigation such material would be too degraded for analysis (e.g. site 1, Table 1), or restricted to fine shoots which would have broken off and disintegrated long ago. Studies in nurseries, however, have shown that pseudosclerotia giving rise to apothecia can form at the root collars and lower stems of infected ash plants after death (Kowalski and Holdenrieder, 2009a,b; Kirisits *et al.*, 2012), suggesting that some infected saplings with *H. fraxineus* lesions could sustain spore production leading to disease spread. A similar observation was made in Carmarthenshire, where apothecia were found on a basal lesion caused by *H. fraxineus* (Figure 3). Field observations in England have also shown that apothecia can be found on woody shoot material in the litter layer (M Biddle, B Wylder, J. Webber, unpublished), and Kirisits *et al.* (2014) showed that a small proportion of ash shoots with fresh *H. fraxineus* lesions which were then exposed naturally by placing on the soil surface for a year could go on to form apothecia.

The time between planting of infected stock and a spreading ash dieback outbreak will also be dependent on the amount of time taken for apothecial formation. This requires both mating types of the fungus to be present and come into physical contact with each other so that sexual reproduction can occur (Gross *et al.*, 2012) and only then can apothecial formation occur sometime later. However, even when compatible mating types are present, initial levels of ascospore production are likely be very low. The formation of new *H. fraxineus* cankers on the main stems of affected trees may also be delayed as much of

Figure 3 *Fraxinus excelsior* tree planted in 2007 at a site in Carmarthenshire found with apothecia growing from a *Hymenoscyphus fraxineus* basal lesion in 2014. Image supplied courtesy of Menna Langford, Natural Resources Wales.

the infected foliage is now known to be shed before the pathogen can extend into the bark of shoots and stems to cause girdling cankers (Kirisits and Freinschlag, 2012; Krautler and Kirisits, 2012).

In addition it has been recognized that incidence and severity of dieback observed at different locations in mainland Europe can vary depending on site-specific ecological conditions (Pliūra *et al.*, 2011; Schumacher, 2011; Skovsgaard *et al.*, 2017). To a great extent this probably relates to how suitable climatic conditions are for apothecia production and ascospore release (Gross and Holdenreider, 2013). The characteristics of individual sites may also influence disease spread. For example, many of the newly established ash sites in England are on farmland where the ground cover is initially bare soil that later develops into a vigorous grass sward which envelops any pseudosclerotial rachises and may hinder apothecia formation and spore release. It can also take more than one year before infected rachises and shoot material go on to produce apothecia of *H. fraxineus*, especially if environmental conditions are unfavourable (Gross and Holdenreider, 2013; Kirisits *et al.*, 2014).

The evidence that *H. fraxineus* was present and infecting ash in England as early as 2004/2005 not only pre-dates by a number of years the first formal record of the pathogen in the UK but also the earliest acknowledgements of a new pathogen in other European countries (Sansford, 2013). It also pre-dates much of our understanding of the pathogen life cycle of the fungus (summarized by Gross *et al.*, 2014), and the naming of the causal agent of ash dieback by Kowalski (2006). The significance of lesions or other symptoms on planting stock in the UK are therefore unlikely to have been recognized or reported. It is also notable, that out of the six sites that we studied, five were apparently stocked with ash plants from British nurseries and not from directly imported plants from mainland Europe where the disease is acknowledged to have been established for much longer. The practice of sourcing ash seed in the UK and then exporting it to mainland Europe for plant production before importing the saplings back to the UK for growing on and planting (Downing, 2012) could explain this discrepancy. However, even if a ban on the movement of ash from mainland Europe into the UK had been put in place immediately after the first description of the new pathogen in mainland Europe (Kowalski, 2006) rather than in October 2012 after the first findings in the wider environment in England, our study indicates that infected ash plants were already in the UK. Once sporulation of *H. fraxineus* began to take place at geographically diverse locations across the UK, the spread of the fungus was likely to be inevitable.

Conclusions

Our evidence indicates that ash dieback has been present in the UK much earlier than previously understood – prior even to the first description of the pathogen in mainland Europe in 2006.

Our observations suggest there may be a lag period of several years from the time of introduction on infected plants to an obvious wider environment outbreak of ash dieback.

Sporulation from these infected plants must have occurred to allow disease spread, probably through the formation of apothecia on the woody stems of affected plants. This process is likely to have occurred many times at other sites where infected ash has been planted in the UK.

Hymenoscyphus fraxineus can remain active in stem lesions 3–4 years after infection despite an active callus response from infected trees.

It proved possible to extract and detect DNA of *H. fraxineus* from old cankers, in one instance up to twelve years after tree death, but more often material of that age (and younger) was too degraded for successful detection of the pathogen.

Acknowledgements

We thank colleagues at Forest Research including Prof. Clive Brasier for advice on site investigation and Andrew Jeeves for careful preparation of ash samples for dating. We also thank our colleagues from Forestry Commission England, Alan Ockenden, Emily Fensom and Helen Carter, for assisting with gathering samples from one of the sites investigated and Iben Thomsen and Thomas Kirisits for their advice and suggestions during discussions about our findings. The kind cooperation of Lilburn Estate, John Greenslade, National Forest, Woodland Trust, Salle Farms Estate, Diana MacMullen and Mr and Mrs Barrett is also acknowledged.

Conflict of interest statement

None declared.

Funding

Support from Forestry Commission England, Forestry Commission (Corporate and Forestry Services) and Defra (Project TH0119) enabled this study to be carried out.

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