Pathogen profile

Hymenoscyphus pseudoalbidus, the causal agent of European ash dieback

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SUMMARY

The ascomycete *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*) causes a lethal disease known as ash dieback on *Fraxinus excelsior* and *Fraxinus angustifolia* in Europe. The pathogen was probably introduced from East Asia and the disease emerged in Poland in the early 1990s; the subsequent epidemic is spreading to the entire native distribution range of the host trees. This pathogen profile represents a comprehensive review of the state of research from the discovery of the pathogen and points out knowledge gaps and research needs.

Taxonomy: Members of the genus *Hymenoscyphus* (Helotiales, Leotiomycetidae, Leotiomycetes, Ascomycota) are small discomycetes which form their ascomata on dead plant material. A phylogeny based on the internal transcribed spacers (ITSs) of the rDNA indicated the avirulent *Hymenoscyphus albidus*, a species native to Europe, as the closest relative of *H. pseudoalbidus*.

Symptoms: *Hymenoscyphus pseudoalbidus* causes necrotic lesions on leaves, twigs and stems, eventually leading to wilting and dieback of girdled shoots. Bark lesions are characterized by a typical dark- to cinnamon-brown discoloration.

Life cycle: *Hymenoscyphus pseudoalbidus* is heterothallic and reproduces sexually on ash petioles in the litter once a year. Ascospores are wind dispersed and infect ash leaves during the summer. The asexual spores only serve as spermatia.

Tools and techniques: The most important techniques for fungal handling, such as detection, isolation, culturing, storage, crossing and ascocarp production, are briefly described.

Management: Once the disease is established, management is hardly possible. The occurrence of a small fraction of partially tolerant trees constitutes hope for resistance breeding in the future. Healthy-looking trees should be preserved.

INTRODUCTION

At the beginning of the 1990s, severe dieback symptoms on European ash (*Fraxinus excelsior* L.) were observed for the first time in north-eastern Poland. Currently, a large part of the native distribution area of *F. excelsior* is affected by this potentially lethal disease (Pautasso *et al.*, 2013). The causal agent was detected by Kowalski (2001) and later described as a new mitosporic ascomycete, *Chalara fraxinea* (Kowalski, 2006). The sexual stage was initially morphologically assigned to *Hymenoscyphus albidus*, a widespread discomycete indigenous to Europe (Kowalski and Holdenrieder, 2009b). However, based on molecular data, Queloz *et al.* (2011) recognized the pathogen as a new cryptic species and named its teleomorph *Hymenoscyphus pseudoalbidus*. Recently, morphological and genetic studies have revealed that *H. pseudoalbidus* is a pathogen introduced to Europe and most probably originates from East Asia (Zhao *et al.*, 2012).

Before the current epidemic, dieback symptoms on mature ash trees had occasionally been noted in Europe for a long time, but they were interpreted as abiotic damage (caused by frost or drought), as a consequence of excessive fructification, maturation feeding of bark beetles (*Leperisinus varius* and *Hylesinus* spp.) or combinations of abiotic and biotic factors (Boudier, 1994; Hulden, 1941; Hull and Gibbs, 1991). Similarly, drought and late frost were suspected as inciting factors during the initial stage of the epidemic in Poland, and the presence of numerous more or less pathogenic fungi on affected trees fuelled discussions concerning their role in disease aetiology (Cech, 2006; Przybył, 2002; Pukacki and Przybył, 2005). Although it is sometimes rather difficult to isolate *H. pseudoalbidus* from symptomatic plant tissues, it is now beyond dispute that *H. pseudoalbidus* is the causal agent that threatens European ash (*F. excelsior*) and narrow-leaved ash (*F. angustifolia*) in Europe.

The main aim of this review is to summarize the current state of knowledge concerning the emerging ash pathogen *H. pseudoalbidus* (anamorph: *Chalara fraxinea*). The review provides information on the following topics: (i) pathogen morphology and taxonomy; (ii) population genetics; (iii) life cycle and reproductive mode; (iv) disease symptoms and host range; (v) host–pathogen interaction; (vi) epidemiology; (vii) ecology; (viii) techniques for pathogen detection and handling; and finally (ix) knowledge gaps and research needs based on implications from other pathosystems. The literature review incorporates, to the best of our knowledge, most relevant knowledge concerning *H. pseudoalbidus* published until May 2013.

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MORPHOLOGY AND TAXONOMY

Hymenoscyphus pseudoalbidus is a discomycete that forms numerous white-stalked apothecia (mostly up to 3 mm in diameter, rarely 8 mm) on the leaf litter of the previous year (Fig. 1a,b). The hymenium is composed of cylindrical paraphyses (1.8–2.4 μm thick, enlarged to 3 μm at the apex) and cylindrical-clavate asci (80–107 × 8–10 μm), showing a positive iodine reaction and forming hyaline single-celled ascospores (13–21 × 3.5–5.0 μm) (Fig. 1h). During germination, the ascospores become melanized and single septate in most cases, and appressoria and/or germ tubes are formed (Fig. 1i–l). The asexual stage (*C. fraxinea*) is characterized by brown phialides (16–24 × 4–5 μm) (Fig. 1s–v), which form short cylindrical hyaline single-celled conidia (3.2–4.0 × 2.0–2.5 μm) in slimy spore droplets and, occasionally, also in chains. Only the first-formed conidium is clavate and 6–7 μm long (Kowalski, 2001, 2006) (Fig. 1v). The substrate colonized by *H. pseudoalbidus* (leaf debris in the litter, occasionally also small stems) becomes blackened by a conspicuous pseudosclerotial layer (Fig. 1a–g) on which the apothecia develop during the summer (Gross and Holdenrieder, 2013; Kowalski and Holdenrieder, 2009b).

When Kowalski and Holdenrieder (2009b) recognized the teleomorph of *C. fraxinea*, they tentatively identified it as *H. albidus* morphologically. However, *H. albidus* was described in 1850 as *Peziza albida* Roberge ex Desm. (Rehm, 1896), is considered to be widespread in Europe and has never been reported to be pathogenic. Queloz *et al*. (2011) molecularly identified the pathogen as a separate cryptic species and named it *H. pseudoalbidus* (see also the corrigendum, Queloz *et al*., 2012). The authors collected apothecia from diseased and healthy ash stands and compared them in terms of three genetic sequence markers [internal transcribed spacer (ITS), elongation factor-1-α (EF1-α), calmodulin (CAL)] and by genetic fingerprinting (inter-simple sequence repeat-polymerase chain reaction, ISSR-PCR). Phylogenetic analysis allowed the distinction of two groups, with specimens collected in diseased stands always grouping together and specimens from healthy stands almost exclusively belonging to the other group. Using similar methods, other authors independently confirmed this finding (Feau *et al*., 2011; Husson *et al*.,

2011). Later, Bengtsson *et al*. (2012) and Gross *et al*. (2012b) were able to separate the two cryptic species based on microsatellite (MS) markers. Furthermore, Gross *et al*. (2012c) showed that the two closely related species exhibit different mating systems (see below). Recently, the presence of croziers at the ascus base of *H. pseudoalbidus* has been described as an unequivocal morphological distinguishing feature between the two sister species *H. pseudoalbidus* and *H. albidus* (Zhao *et al*., 2012).

The genus *Hymenoscyphus* belongs to the family Helotiaceae (Helotiales, Leotiomycetidae, Leotiomycetes, Pezizomycotina, Ascomycota). This genus includes more than 150 species worldwide (Kirk *et al*., 2008) and new species continue to be discovered (Han and Shin, 2008; Zheng and Zhuang, 2013). A phylogeny based on the ITS regions of the rDNA indicated the avirulent *H. albidus*, a species native to Europe, as the closest known relative of *H. pseudoalbidus* (V. Queloz, unpublished data). Most *Hymenoscyphus* species are regarded as saprotrophic decomposers of plant material and, before the emergence of ash dieback, none of the described *Hymenoscyphus* species was known to cause plant diseases (Wang *et al*., 2006). However, some forest pathogens within the Helotiaceae are known, e.g. *Gremmeniella abietina*, causes Brunchorstia disease in conifers, *Cenangium ferruginosum* causes Cenangium dieback of pines and *Crumenulopsis sororia* causes bark cankers on pines. Important tree pathogens are also found in other helotialean families, e.g. *Lachnellula willkommii* (Hyaloscyphaceae), which causes cankers on European larch (*Larix decidua*), and *Phacidium infestans* (Phacidiaceae), a snow mould on Swiss stone pine (*Pinus cembra*) (Butin, 2011). In addition, many species within the Helotiales are endophytic and tend to shift to a pathogenic lifestyle if the host is under stress (Sieber, 2007).

DIVERSITY, POPULATION DIFFERENTIATION AND DISTRIBUTION OF *H. PSEUDOALBIDUS* POPULATIONS

Hymenoscyphus albidus, the indigenous cryptic sister species of the pathogen, is mainly known from Europe (http://data.gbif.org/species/2582400/). However, Hosoya *et al*. (1993) reported

Fig. 1 Morphology of *Hymenoscyphus pseudoalbidus*: (a) mature apothecia on an ash petiole, top view; (b) ascomata, side view; (c) long-stalked apothecia formed in a moist chamber; see also the small ascomata initials on the surface of the pseudosclerotium; (d) immature apothecia on a small ash stem; (e) apothecia formed on pseudosclerotial leaflet veins; (f) surface view of a pseudosclerotial plate; (g) several pseudosclerotia, separated from each other by demarcation zones, on an ash petiole; (h) freshly ejected ascospores (in water); (i–l) germinating ascospores on malt extract agar, forming germ tubes (gt) and appressoria (ap); (m) ascospores on a cover glass forming appressorial rings (ar); (n) ascospore germinating on cellophane on malt extract agar (MEA) with ash leaves; note two types of exudate: a hyaline mucilage (h) surrounding the spore, and a melanin-like exudate (l) surrounding the appressorium; (o, p) germinating ascospores on the upper side of a leaflet; note the formation of appressoria (ap), appressorial rings (ar) and surface hyphae (sh); (q, r) an ascospore that germinated on cellophane on water agar, forming two appressoria (ap), entering the substrate and spreading within the cellophane by flattened hyphae (fh) (surface view and deeper level of the same object, in lactophenol blue); (s–v) anamorphic state (*Chalara fraxinea*): (s,t) groups of phialides formed on the lower side of a necrotic leaflet; (u) phialides formed in culture on MEA; (v) spore droplet on the top of a phialide. Unless otherwise specified, the specimens for microscopy were mounted in lactic acid.
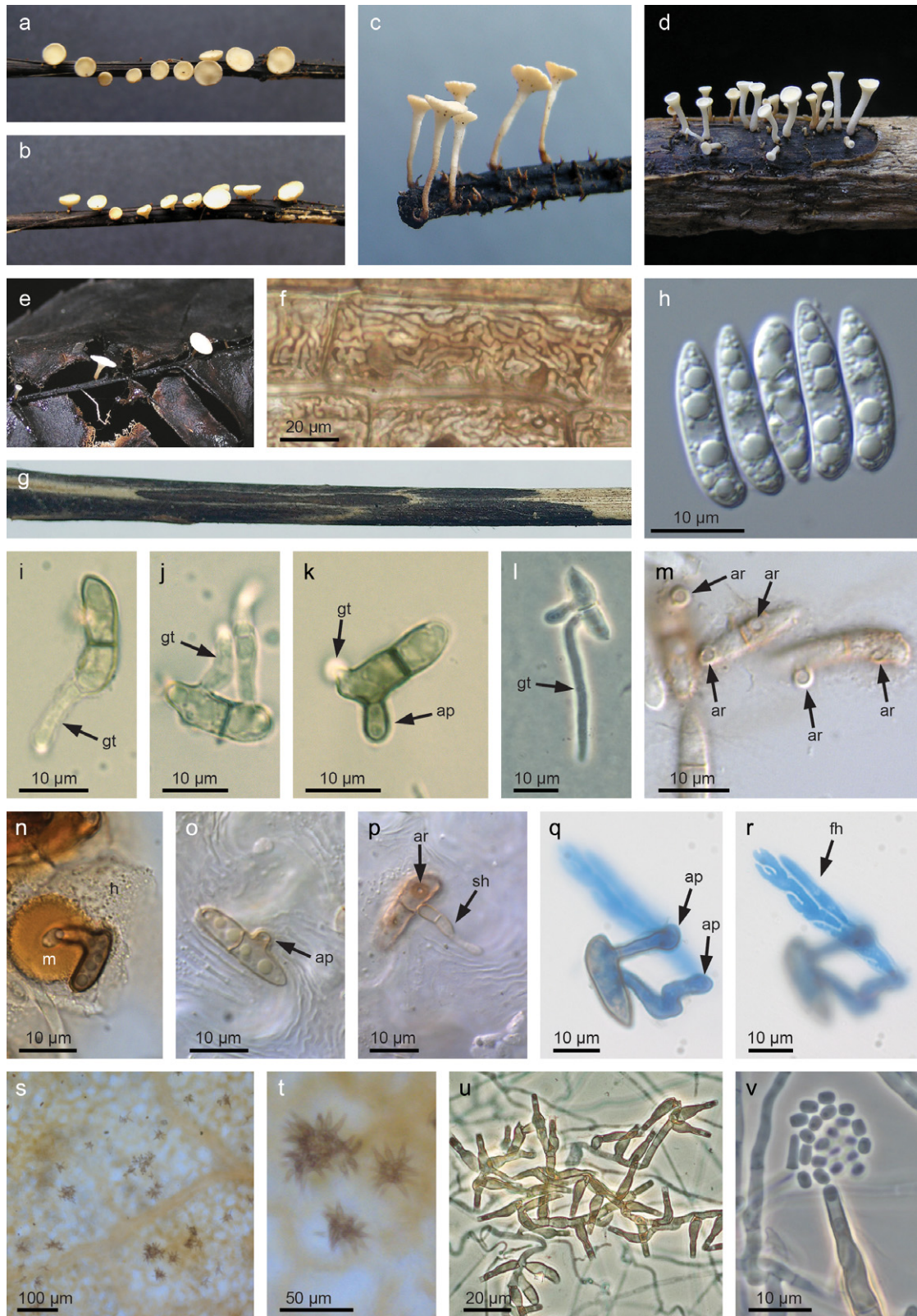
Lambertella albida (a synonym of *H. albidus*) from petioles of decaying leaves of *Fraxinus mandshurica* var. *japonica*, as new to Japan. Recently, Zhao *et al*. (2012) sequenced four genomic DNA markers [ITS, the D1/D2 region of the large subunit (LSU) rDNA gene (28S rDNA), the CAL gene and the translation factor EF1-α] of six specimens of Japanese *L. albida*, including the three specimens originally identified by Hosoya *et al*. (1993). Combining the dataset of Queloz *et al*. (2011), who also used ITS, CAL and EF1-α for species delimitation, with the DNA sequences of Japanese specimens, Zhao *et al*. (2012) showed that *L. albida* from Japan is actually conspecific with *H. pseudoalbidus*, and that the sequence diversity among Japanese specimens is considerably higher than that among European ones. A subsequent study incorporating large Japanese and European *H. pseudoalbidus* populations confirmed the finding of a greater genetic diversity in Japan (Gross *et al*., 2012a). This led to the conclusion that the fungus has been introduced to Europe from eastern Asia.

Although more detailed knowledge about Asian *H. pseudoalbidus* population genetics is yet to be obtained, some data are already available for Europe. Using ISSR-PCR fingerprinting, Rytkönen *et al*. (2011) distinguished 14 haplotypes among 32 isolates that originated from Finland (20), Estonia (11) and Latvia (1). No measures of population differentiation were provided, but the observed high genotypic diversity indicated sexual reproduction. Kraj *et al*. (2012) also found a high genotypic diversity within 159 Polish isolates originating from five different regions, which were screened with five different ISSR-PCR primers. The authors further detected a significant distinction between highland and lowland populations. This remains the only study that has found evidence for a genetic structure among *H. pseudoalbidus* populations in Europe. Other analyses assessed the population biology of the pathogen using MS markers. These markers are easily reproducible, co-dominant and evolve rapidly. They facilitate the study of allelic frequencies and are thus more powerful than ISSR-PCR fingerprinting techniques (Schlotterer, 2004; Sunnucks, 2000). Bengtsson *et al*. (2012) used a combination of arbitrarily primed PCR (AP-PCR) and eight MS markers to characterize a broad range of isolates derived from 10 different European countries, and found high genotypic diversity, very low allelic richness and near-zero pairwise population differentiation ($F_{ST} \leq 0.028$). Similar results were obtained by Gross *et al*. (2012b), who used a set of 18 polymorphic MS markers to screen two larger populations of *H. pseudoalbidus* originating from Germany. Notably, as in the study of Bengtsson *et al*. (2012), populations revealed near-zero differentiation in pairwise comparisons ($F_{ST} = 0.022$) and the majority of MS loci revealed a bi-allelic nature. Taken together, the population genetic data point to an outbreeding mating system and a dispersal through ascospores (high genotypic diversity), virtually no population structure (very low F_{ST} values; but see Kraj *et al*., 2012) and signs of a founder effect (low allelic richness).

LIFE CYCLE

To date, most research on ash dieback has focused on the pathosystem *F. excelsior–H. pseudoalbidus*. If not otherwise specified, we hereafter only refer to this pathosystem.

The entire life cycle of *H. pseudoalbidus* is completed on *Fraxinus* leaves. Apothecia are produced during the summer on leaf debris of the previous year (Kirisits and Cech, 2009; Kowalski and Holdenrieder, 2009b; Timmermann *et al*., 2011). Most apothecia are formed on petioles and rachises (hereafter called petioles) but, occasionally, apothecia are also found on small stems lying on the ground (Kirisits and Cech, 2009; Kirisits *et al*., 2012; Kowalski and Holdenrieder, 2009b) (Fig. 1d). The main sporulation period is from June to early September, but, under favourable conditions, sporulation can start earlier and last until October (Hietala *et al*., 2013; Kirisits and Cech, 2009; Kowalski and Holdenrieder, 2009b; Timmermann *et al*., 2011).

The wind-dispersed ascospores (Kowalski and Holdenrieder, 2009b; Timmermann *et al*., 2011) adhere to the leaf surface via a secreted mucilage, which is more or less hyaline around the spore (Fig. 1m,n) and conspicuously melanized around appressoria formed on cellophane on ash leaf agar (Fig. 1n). However, on cellophane laid over water agar, no mucilage is visible (Fig. 1q). Ascospores penetrate the ash leaf cuticle via appressoria (Fig. 1k,o) (Cleary *et al*., 2013). However, in most cases, the appressorium is reduced to a melanized appressorial ring at the ventral side of the spore (Fig. 1m,p; O. Holdenrieder, unpublished data). In moist chambers, initial lesions on the leaves of ash seedlings occurred 2 weeks after inoculation (Cleary *et al*., 2013). The further colonization of the leaf has yet to be studied in detail. After massive ascospore inoculation on the upper leaf surface under field conditions (using small Petri dishes containing sporulating apothecia, which were positioned on individual leaflets lying on a support pad), tiny necrotic lesions (approximately 1–3 mm in diameter) developed within 12 days and the inoculated leaflets were shed a few days later without inducing rachis infection (O. Holdenrieder, unpublished data). Leaf shedding was also observed after wound inoculation of the rachis (Kräutler and Kirisits, 2012). On detached leaflets incubated in a moist chamber, necrotic lesions developed within 10 days after inoculation of the adaxial side (O. Holdenrieder, unpublished data). The necrotic lesions on the leaflet lamina frequently spread along the leaf veins and rachis in a proximal direction (Fig. 2b,d) and, occasionally, the pathogen crossed the junction between the petiole and stem, thereby initiating a necrotic lesion on the stem (Kirisits *et al*., 2009). Stem infection is normally a dead-end in the life cycle of the pathogen, because fructification occurs only very rarely on dead stems (see above).

After leaf fall in the autumn, the fungus starts to produce a characteristic black pseudosclerotial plate on the petioles, occasionally also on leaflet veins and small stems (Fig. 1d–g; Gross and

Holdenrieder, 2013; Kirisits *et al*., 2009; Kowalski and Holdenrieder, 2009b). The pseudosclerotial plate is formed a few cell layers below the substrate surface and the peripheral host tissues are degraded by other organisms until the spring, so that the melanized wall of the pseudosclerotium becomes exposed (Fig. 1a–g). In the following vegetation period, during the second or even the third year after leaf fall, new apothecia develop on the pseudosclerotia and the cycle is closed (Gross and Holdenrieder, 2013; O. Holdenrieder, unpublished data).

The asexual stage occurs during the autumn and winter on ash petioles in the litter, preferentially near developing pseudosclerotial plates (Kowalski and Bartnik, 2010; O. Holdenrieder, unpublished data). Occasionally, phialides can also be found on necrotic leaves (Fig. 1s,t), on wound-inoculated stems (Kowalski and Holdenrieder, 2009a) and on incubated wood discs (Husson *et al*., 2012). *In vitro*, asexual sporulation can be induced on malt extract agar (MEA) by prolonged incubation (1–2 months) at low (5–15 °C) temperatures (Hosoya *et al*., 1993; Kowalski, 2006; Kowalski and Bartnik, 2010) or on MEA amended with ash leaves (Kirisits *et al*., 2013). The minute size of the phialoconidia and their inability to germinate on various substrates led to the hypothesis that they act as spermatia (Hosoya *et al*., 1993; Kirisits *et al*., 2009). Indirect proof of spermatial function was provided by a parenthood analysis of different apothecia on a single petiole, which showed that not all paternal (fertilizing) strains are found on the same petiole. Consequently, fertilization must occur by the transfer of spermatia from strains on other petioles (Gross *et al*., 2012c). We do not know during which time of the year fertilization occurs. However, pseudosclerotial petioles collected in January 2013 in Zurich and incubated at 20 °C under 12 h near-UV light/ 12 h darkness developed numerous fertile apothecia within 4 weeks (O. Holdenrieder, unpublished data).

REPRODUCTIVE MODE

Taking into account the high genotypic diversity of *H. pseudoalbidus*, Bengtsson *et al*. (2012) suggested a heterothallic mating system. However, outcrossing also occurs in homothallic fungi (Billiard *et al*., 2012; Pérez *et al*., 2010; Pontecorvo *et al*., 1953) and heterothallic fungi are sometimes able to fertilize themselves (Marra *et al*., 2004). Therefore, the high genotypic diversity of a fungus *per se* is only an indication of an outbreeding reproductive mode, but no proof of a heterothallic mating system. The molecular characterization of the mating type locus of *H. pseudoalbidus* and its confirmation *in vivo* by crossing experiments provided the final proof of the heterothallic mating system of *H. pseudoalbidus* (Gross *et al*., 2012c). Moreover, the even distribution of both mating types in two populations originating from the current epidemic periphery (Switzerland) and two populations from the centre (Poland) convincingly demonstrated random mating of all populations and ruled out the hypothesis of

an initial clonal distribution (Gross *et al*., 2012c). In contrast, the avirulent species, *H. albidus*, has a homothallic mating system (Gross *et al*., 2012c), does not form an anamorph (Kirisits *et al*., 2013) and shows a lower genetic diversity (Queloz *et al*., 2011).

DISEASE SYMPTOMS AND HOST RANGE

Ash dieback is essentially a lethal disease, affecting all age classes from saplings to mature trees (Fig. 2). Symptom development on susceptible hosts starts in late summer with the formation of necrotic lesions on the leaflets (Fig. 2a). The lesions expand preferentially along veins in the proximal direction (Fig. 2b) and spread into the rachis. Thereby, the tissues surrounding the base of the petiolule sometimes remain green at the beginning and the lesion appears first at a short distance from the leaflet or the leaflet scar (Fig. 2d). As soon as the rachis is girdled, the distal part of the leaf wilts and desiccates (Bakys *et al*., 2009a) (Fig. 2a). Occasionally, direct infection of the rachis might also occur, which is visible as small necrotic lesions without contact to leaflets (Cleary *et al*., 2013; T. Kowalski, University of Agriculture in Krakow, Krakow, Poland, unpublished data).

The incubation period following ascospore inoculation lasts 10–14 days, but excessive infection pressure leads to premature leaf abscission (see above). Similarly, wound-infected leaves of ash trees potted outdoors showed initial symptoms after 2 weeks. However, the few necroses developing on the stem originated from natural infections and none developed from the strains used for leaf inoculation (Kräutler and Kirisits, 2012).

In nature, most infected leaves are shed before the pathogen reaches the stem. However, in some cases, the mycelium of the pathogen grows from the petiole into the stem and spreads in the axial direction in the inner shoot tissues (Schumacher *et al*., 2010). Hyphae preferentially colonize the medullary sheath and the ray parenchyma, but also occur in other tissues (Fig. 3). The broad hyphae, up to 7 μm wide, grow intracellularly and occasionally form intrahyphal hyphae. At least during the initial stage of infection, no disintegration of the plasmalemma and cell wall of the host cells was observed (Dal Maso *et al*., 2012; Schumacher *et al*., 2010).

The xylem infection spreads in the axial direction preferentially within the central tissues and is frequently associated with a brownish discoloration (Fig. 2j,k).

The infection is not systemic, but fungal DNA was occasionally detected up to 10 cm ahead of visible necrotic lesions in apparently healthy inner bark tissue (McKinney *et al*., 2012b). The radial expansion of the infection leads to the death of bark tissue (Fig. 2j), which is associated with initially dark necrotic lesions that subsequently become characteristically cinnamon brown (Fig. 2e). Bark death is preceded by cambial necrosis (Fig. 2j) (Kowalski, 2001; Kowalski and Holdenrieder, 2008). The lesions on the bark mostly become visible during late autumn and winter, but
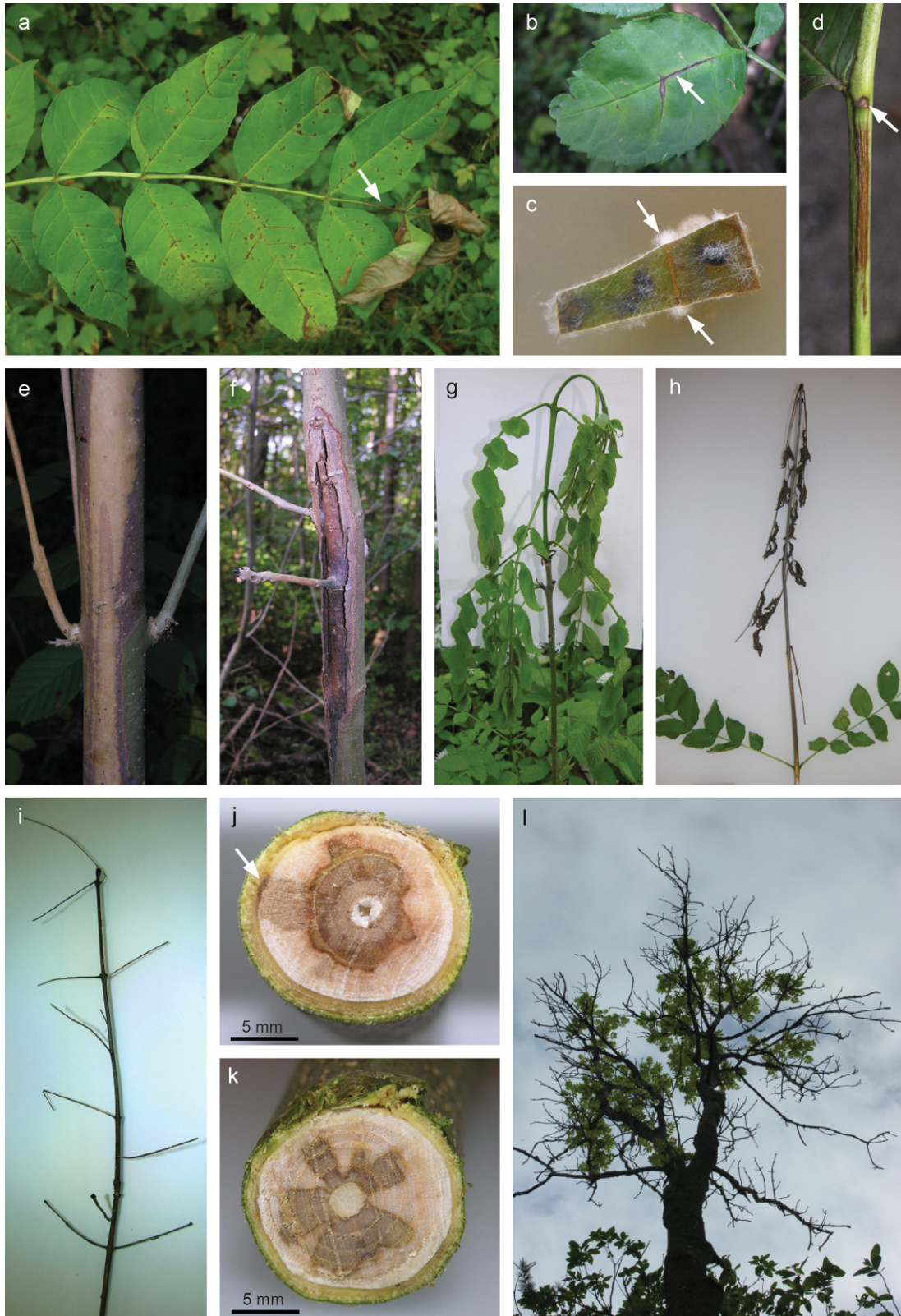
Fig. 2 Symptoms of ash dieback: (a) initial stage of leaf infection with many small necrotic spots; from one leaflet, the infection has already spread to the rachis and triggered wilting of distal leaf parts (arrow); (b) necrotic lesion extending along the main leaflet vein in proximal direction (arrow); (c) isolation of fungal mycelia from a surface-disinfected leaflet fragment containing a necrotic vein as shown in (b); the mycelium of *Hymenoscyphus pseudoalbidus* is outgrowing only from the vein cuts (arrows); the other mycelia belong to endophytic fungi; (d) initial necrotic lesion on a rachis after infection via the petiolule; the scar left by the shed leaflet is marked by an arrow; (e) necrotic bark lesion on a young stem; (f) necrotic bark lesion on an older stem; (g) wilting shoot in spring; (h) withered shoot distal to a necrotic lesion during summer; (i) withered shoot with attached petioles (and rachises) during winter; (j) internal xylem necrosis initiating a cambial necrosis (arrow); (k) central necrosis still surrounded by healthy peripheral xylem tissues; (l) mature tree dying off and forming epicormic branches.
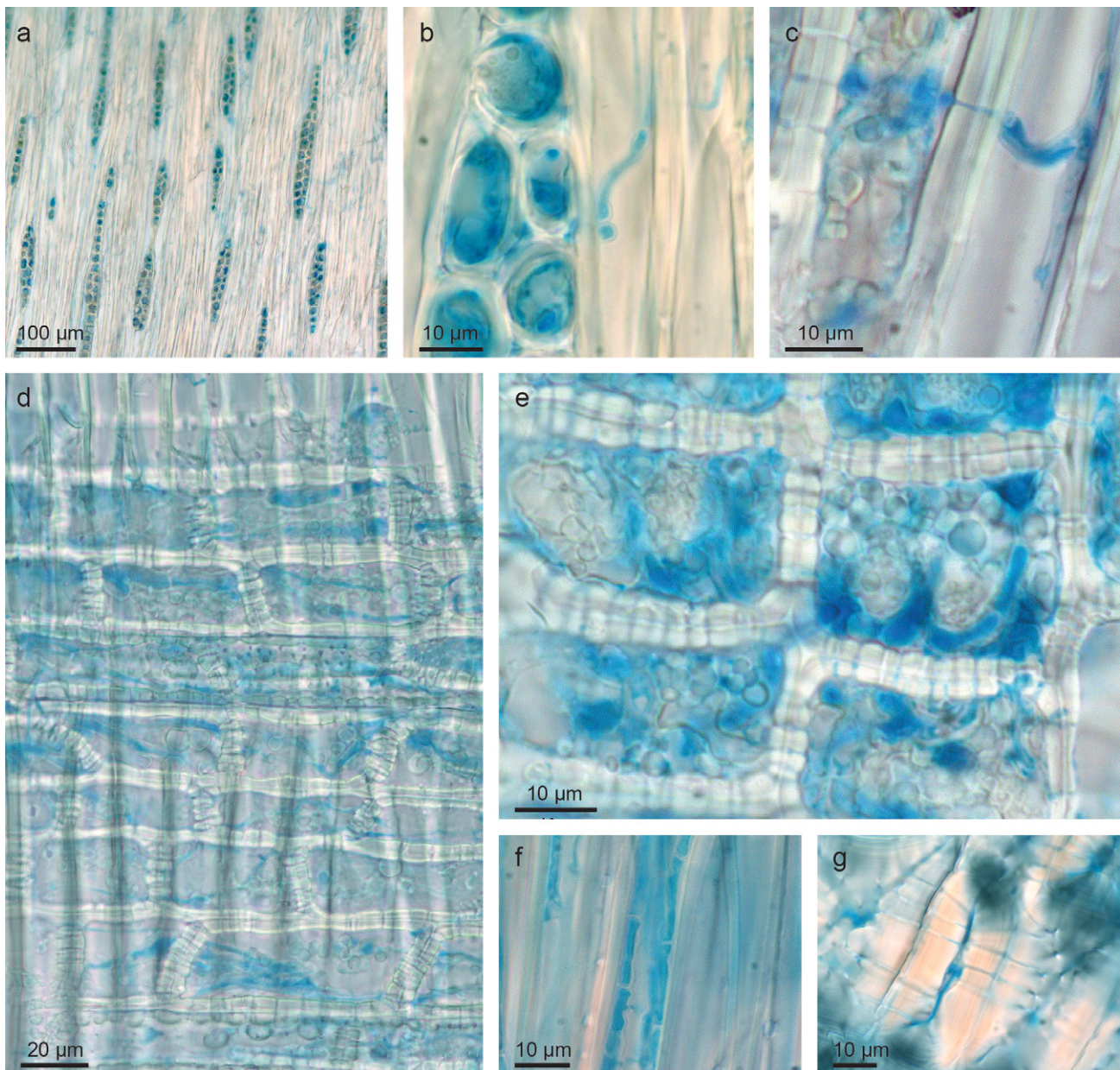
Fig. 3 Mycelium of *Hymenoscyphus pseudoalbidus* in *Fraxinus excelsior* tissues (stained with lactophenol blue): (a, b) hyphae concentrated in xylem rays (tangential section); (c) hypha growing through a cell wall separating a ray cell from a fibre cell; (d, e) hyphae in xylem ray cells (radial section); (f) hyphae growing in a xylem fibre in an axial direction; (g) hyphae growing within the lumen and pit channels of a bark sclereid.

sometimes not until the spring. This constitutes a risk of pathogen dispersal by facilitating the trade of latently infected plants (Husson *et al.*, 2012; Kirisits *et al.*, 2009). On the necrotic bark, opportunistic fungi (*sensu* Kirk *et al.*, 2008) develop quickly and complicate diagnosis (see below). As soon as the shoot becomes girdled, the distal part dies. If girdling occurs during late summer, leaflets desiccate, disintegrate and the petioles remain attached during the following winter (Fig. 2i) and are easy to recognize against the background of snow. If girdling occurs during the winter, the buds distal to the infection do not sprout during the following spring. Girdling during the spring or early summer triggers wilting (Fig. 2g). The pathogen continues to spread in the proximal direction and also affects shoot segments older than 1 year. The bark necrosis can cease at the shoot base or a node, but if the stem is split axially, it can be frequently observed that the xylem necrosis expands beyond that point. Even the main stem can become infected via a side branch (Fig. 2e,f). Severely affected trees respond to branch dieback by the formation of epicormic shoots (Fig. 2l) (Engesser *et al.*, 2009; Kowalski and Holdenrieder, 2008; Skovsgaard *et al.*, 2010). If such shoots develop at the stem base, they are perfect entry points for new infections and the tree can die rapidly (Engesser and Meier, 2012; Husson *et al.*, 2012; Metzler and Witzel, 2012). It has been suspected that, under moist conditions at the stem base, lenticels or small wounds can also act as entry points (Husson *et al.*, 2012), but definite proof for this is lacking. Inoculation experiments with colonized wood blocks attached with Parafilm on unwounded annual shoots in summer yielded no infections (O. Holdenrieder, unpublished data).

The pathogen can also colonize parts of the root system (Kowalski, 2001; Kowalski and Lukomska, 2005; Schumacher, 2011; Schumacher *et al.*, 2010), but the roots of severely infected trees are prone to attack by opportunistic fungi (e.g. *Armillaria* spp.), which accelerates tree death (Bakys *et al.*, 2009b, 2011; Enderle *et al.*, 2013; Husson *et al.*, 2012; Lygis *et al.*, 2006; Skovsgaard *et al.*, 2010). Predisposed trees are also attacked above ground by bark beetles and numerous opportunistic fungi (see below).

HOST RANGE

The natural (original) hosts of *H. pseudoalbidus* appear to be *F. mandshurica* and *F. chinensis*, as these ash species are the only hosts reported from Asia (Hosoya *et al.*, 1993; Zhao *et al.*, 2012). The fungus is not known as a pathogen in Japan (Zhao *et al.*, 2012). In Europe, the known hosts showing disease symptoms include the European ash species *F. excelsior* (Kowalski, 2006) and *F. angustifolia* (Kirisits *et al.*, 2010; Schumacher *et al.*, 2007), the North American *F. nigra*, *F. pennsylvanica* and *F. americana* and the Asian *F. mandshurica* (Drenkhan and Hanso, 2010). Although *F. nigra*, *F. excelsior* and *F. angustifolia* are heavily affected by the disease, *F. pennsylvanica* is only moderately susceptible, followed

by *F. mandshurica* and *F. americana*, which appear to be largely tolerant in Estonia. The flowering ash, *F. ornus*, apparently does not develop the disease under field conditions (Kirisits *et al.*, 2009; see also various contributions in FRAXBACK, 2012), but fructification on naturally infected petioles has been observed (O. Holdenrieder, unpublished data) and *F. ornus* has been shown to be moderately susceptible in artificial leaf and stem inoculation experiments (Kirisits *et al.*, 2009; Kräutler and Kirisits, 2012). The Asian *F. chinensis* and *F. bungeana*, as well as the American *F. latifolia*, *F. pennsylvanica* and *F. velutina*, developed no lesions after wound inoculation on twigs of mature trees in Germany (G. Aas and O. Holdenrieder, unpublished data). However, nursery stock of *F. pennsylvanica* from Germany was infected naturally and stem inoculation experiments on the remaining healthy plants showed that they were also susceptible (A. Gross, ETH Zurich, Zurich, Switzerland, unpublished data). Unfortunately, data concerning the susceptibility of exotic ash species are very scarce. Moreover, the determination of ash species is difficult and misidentifications frequently occur (Koch *et al.*, 2012). Stem inoculation experiments on other Oleaceae (*Forsythia* sp., *Ligustrum vulgare*, *Olea europaea*, *Syringa vulgaris*) revealed no necrotic lesions (O. Holdenrieder, unpublished data). The same applies to *Acer pseudoplatanus* and *Sambucus nigra* (Kowalski and Holdenrieder, 2009a).

HOST–PATHOGEN INTERACTIONS

Inoculation experiments revealed that *H. albidus*, which is native to Europe, does not cause necrotic lesions on European ash (*F. excelsior*), whereas the introduced *H. pseudoalbidus* is pathogenic (Husson *et al.*, 2011; A. Gross and O. Holdenrieder, unpublished data). However, little attention has been paid to virulence differences of *H. pseudoalbidus* strains so far. Artificial stem inoculations demonstrated the pathogenicity of *H. pseudoalbidus* in different studies, where all tested isolates were capable of inducing necrotic lesions (Bakys *et al.*, 2009a; Husson *et al.*, 2011; Kowalski and Holdenrieder, 2009a; McKinney *et al.*, 2011; Ogris *et al.*, 2009; Szabo, 2009). Variations in lesion length were observed among strains, but were not significantly different (Bakys *et al.*, 2009a; Husson *et al.*, 2011; Kowalski and Holdenrieder, 2009a). There is a need for virulence assays that incorporate carefully genotyped *H. pseudoalbidus* isolates and ash clones to balance the factor of host genotypic variability.

Recent studies have shown the presence of partially resistant European ash genotypes in Germany (Enderle *et al.*, 2013; Metzler *et al.*, 2012a), Denmark (Kjær *et al.*, 2012; McKinney *et al.*, 2011), Sweden (Stener, 2012), Lithuania (Pliūra *et al.*, 2011) and Poland (Kowalski *et al.*, 2012). Notably, early leaf senescence correlated significantly with low susceptibility of ash trees in two independent studies (McKinney *et al.*, 2011; Stener, 2012; but see also Kirisits and Freinschlag, 2012). Early leaf shedding might

prevent the fungus from growing into the stem. Moreover, a significantly slower necrosis development was observed on early-senescing ash trees relative to late-senescing ones (McKinney *et al*., 2012b), pointing to an additional genetically determined resistance factor. The potential involvement of early leaf shedding in resistance suggests that the speed of growth of *H. pseudoalbidus* might be a potentially crucial virulence trait. Different isolates of *H. pseudoalbidus* show a great variability in growth rates on MEA (Kowalski, 2006; Kowalski and Bartnik, 2010). However, these experiments were carried out with virulent isolates, as all were isolated from necrotic lesions.

Many plant pathogens use toxins to kill host cells (Horbach *et al*., 2011). In *H. pseudoalbidus*, the phytotoxin viridiol and several viridiol-related secondary metabolites have been discovered (Andersson *et al*., 2010, 2012, 2013; Grad *et al*., 2009). Viridiol was previously only known from *Trichoderma* spp. (Jones and Hancock, 1987; Wipf and Kerekes, 2003). The compound has been proven to have phytotoxic effects on ash seedlings (Andersson *et al*., 2010, 2013; Grad *et al*., 2009). However, the nonpathogenic *H. albidus* had a similar phytotoxic effect as the pathogenic *H. pseudoalbidus* on sterile *Lemna minor* plants cultured in close proximity to the fungus on MEA in Petri dishes (O. Holdenrieder, unpublished data), and viridiol production was very high in a Swiss *H. albidus* strain (Junker *et al*., 2013). Therefore, the production of viridiol is probably not the critical virulence factor. Phytotoxic volatile lactones might also be involved in pathogenicity (Metzler *et al*., 2012b). Recently, using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, the so-called chalarafraxinines were detected, which represent a group of as yet uncharacterized secondary metabolites produced by the pathogen (Pham *et al*., 2013). In culture, the mycelium of *H. pseudoalbidus* shows a positive Bavendamm reaction, indicating the presence of extracellular polyphenoloxidases (Metzler *et al*., 2012b; Schumacher, 2011; Schumacher *et al*., 2010), which are involved in the infection process, in defence against competing microorganisms and in lignin degradation (Baldrian, 2006; Mayer and Staples, 2002). However, the same is true for the nonpathogenic *H. albidus* (O. Holdenrieder, unpublished data). Shotgun pyrosequencing of the *H. pseudoalbidus* genome revealed numerous genes encoding cell wall-degrading enzymes (V. Queloz & M. Schlegel, unpublished data), which are typical of necrotrophic fungi (Kämper *et al*., 2006). However, in its native range, *H. pseudoalbidus* does not induce symptoms on *F. mandshurica*. Apparently, on susceptible exotic hosts, the necrotrophic behaviour is accentuated, whereas, on the native host, the hemibiotrophic lifestyle prevails. The mechanism driving this shift is still unknown.

EPIDEMIOLOGY

An epidemic requires: (i) an accumulation of susceptible host individuals; (ii) an aggressive pathogen with a high reproduction rate; and (iii) environmental conditions suitable for the pathogen. It definitely appears that these conditions are fulfilled in Europe over entire regions. European ash is the most widespread *Fraxinus* species in Europe (FRAXIGEN, 2005), but the standing tree volume is estimated to be low in most European countries (except for the UK with 15%; DEFRA, 2013). Nevertheless, *F. excelsior* is the second or third most frequent deciduous tree species after European beech (*Fagus sylvatica*) in central European countries (Kirisits and Cech, 2010; Speich and Brändli, 2011). Moreover, young trees are often abundant, but do not appear in forest inventories, i.e. the number of individual ash trees is much higher than suggested by inventories. European ash is highly susceptible to the pathogen, as can be deduced from the high rate of spread and destructiveness of the ongoing epidemic and the results of infection experiments. The pathogen is highly virulent because it did not co-evolve with European ash (Zhao *et al*., 2012). However, a small fraction of ash genotypes are less susceptible to the disease (Enderle *et al*., 2013; Kjær *et al*., 2012; Kowalski *et al*., 2012; McKinney *et al*., 2011; Metzler *et al*., 2012a; Pliūra *et al*., 2011; Stener, 2012). The tolerance is heritable, giving reason for hope for the recovery of *F. excelsior* (Pliūra *et al*., 2013). In Poland, two resistance-associated molecular markers have been detected (Kowalski *et al*., 2012).

The disease front of ash dieback moved from eastern Poland to Switzerland (i.e. over more than 1200 km) within 16 years (1992–2008), showing a mean dispersal rate of 75 km per year. Similar estimates have been obtained on the basis of detailed disease progression maps of France (B. Marçais, INRA, Nancy Université, Champenoux, France, personal communication). This is more than twice as fast as the spread of chestnut blight, another introduced disease, in North America (37 km per year) or Italy (29 km per year) (Roane *et al*., 1986; Shain, 1982), but similar to the dispersal rate of cereal mildew (Limpert *et al*., 1999). Ash dieback dispersed somewhat less rapidly at the northern and southern limits of the distribution area of *F. excelsior*. Dispersal rates were 30 km per year in Norway and 50–60 km per year in north-eastern Italy (Luchi *et al*., 2012; Solheim *et al*., 2011). Ascospores are produced in abundance during several months in late spring and summer, i.e. the infectious period is rather long (Kirisits *et al*., 2009; Timmermann *et al*., 2011). Ascospores of *H. pseudoalbidus* are drought sensitive and freshly ejected spores usually have a germination capacity of 90%–100%. The spores died within 24 h in a desiccator, but 62% survived for 24 h at room conditions (approximately 20 °C and 30% relative humidity). For spores deposited on a cellophane sheet and exposed to outdoor conditions in July during a rainy period (but protected from rain and direct sunlight), the germination rate declined to 6%–10% within 5–6 days (O. Holdenrieder, unpublished data). The conspicuous melanization of the ascospores after ejection and the attachment to the host surface (see above) indicate that the spores can probably withstand adverse conditions at this stage rather well (Henson *et al*., 1999).

Pathogen dispersal can also occur through long-distance transport of latently infected plants (see above). This probably occurred in Ireland [Department of Agriculture Food and the Marine (Ireland), 2012]. Moreover, pseudosclerotial leaf fragments can be easily dispersed as contaminants of trade goods. The initiation of an epidemic through infected substrate requires the presence of both mating types. This can be achieved by multiple introductions, but also by joint dispersal of several genotypes within a single substrate or by transportation of a single, already fertilized genotype (Gross *et al*., 2012c). Even ash seeds have been suspected as a potential inoculum source, because the fungus can be detected on them by molecular means (Cleary *et al*., 2012). However, despite extensive ash seed transfer from Europe to the USA, the pathogen has not yet been reported from North America.

Both disease incidence and severity increased rapidly in most countries after the first observation of the disease (Engesser and Meier, 2012; Metzler, 2011; Rytkönen *et al*., 2011; Solheim *et al*., 2012a; Thomsen and Skovgaard, 2012). Disease severity in most ash clones monitored by McKinney *et al*. (2011) increased steeply from 36% loss of foliage in 2008 to 56% in 2009, but a few clones maintained a low level of damage. In Austria, disease severity (percentage of crown volume affected) increased only moderately from 11% to 16% within 2 years and very few trees died (Kessler *et al*., 2012). On mature trees, disease progress is slower than on seedlings or young trees (Kirisits and Freinschlag, 2012; Kowalski and Holdenrieder, 2008). This suggests that infection pressure is lower and leaf quality and/or microclimatic conditions are less suitable for infection in the crown.

So far, the epidemic in Europe has progressed according to a sigmoid function. The lag phase lasted almost 10 years, whereas the exponential (log) phase, during which the disease spread over most of northern and central Europe, was of a comparatively short duration (Fig. 4). The northern limits of the distribution area of European ash have already been reached in Scandinavia, whereas there are still some disease-free areas in eastern, southern and western Europe. Westwards dispersal was probably delayed because of the prevalence of westerly winds. Dispersal to the south, i.e. central Italy, southern France and the Pyrenees, might be hampered by unsuitable climatic conditions (Hauptman *et al*., 2013). The situation at the epidemic front in eastern Europe is less well known. At least the Baltic States are severely affected (EPPO, 2013), and first infections have been observed in the Ukraine (Davydenko *et al*., 2012) and Russia near St. Petersburg (Selikhovkin *et al*., 2013).

ECOLOGICAL CONSIDERATIONS

The large-scale occurrence of the pathogen shows that it tolerates a wide range of environmental conditions. However, the effects of only a few environmental factors have been studied. The mycelial growth rate of *H. pseudoalbidus* in pure cultures is highest at
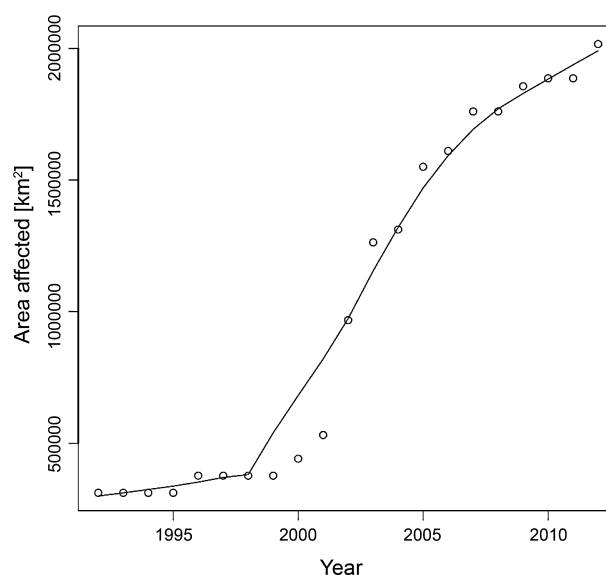
Fig. 4 Progress of ash dieback in Europe. The whole area of a country was added to the area affected by the disease in the year of the first report of the disease in that country. The year of the first report was according to fig. 1 in Timmermann *et al*. (2011). Curve was fitted using LOWESS (*f* = 0.4) (Cleveland, 1981).

about 20 °C and ceases at about 30 °C (Kowalski and Bartnik, 2010; Pham *et al*., 2013). In ash tissues, however, the fungus is more heat sensitive and survival depends not only on temperature, but also on exposure time. In addition, host tissues tolerate a slightly higher temperature than the fungus. Therefore, the development of curative hot water treatments for small plants appears to be possible (Hauptman *et al*., 2013). The mycelium of *H. pseudoalbidus* (from cultures on MEA) is rather resistant to frost and drought: it tolerates freezing at −20 °C for at least 2 months and survives even at −70 °C at least for 1 month, showing normal growth after recovery. Mycelia dried at room temperature survived for 7 weeks, but showed a reduced growth rate after transplantation to fresh medium (O. Holdenrieder, unpublished data).

The climate requirements of *F. mandshurica* in north-eastern Asia are similar to those of *F. excelsior* in Europe, except that *F. mandshurica* tolerates lower minimum temperatures during the winter and is less late frost resistant than *F. excelsior* (Schenck, 1939; Wang and Dai, 1997). Thus, the climate in most of the range of *F. excelsior* is suitable for the pathogen.

Disease resistance or tolerance in *F. excelsior* is a rare phenomenon. Ash dieback will thus dramatically reduce the size of its host populations in Europe, thereby threatening not just ash, but also the organisms depending on *F. excelsior* (Pautasso *et al*., 2013). This also applies to microorganisms, as shown by the example of *H. albidus* (McKinney *et al*., 2012a), and is proposed for various lichens (Ellis *et al*., 2012; Jönsson and Thor, 2012). In host tissues weakened or killed by the pathogen, endophytic and opportunistic

fungi develop very quickly. On aerial plant parts, the most frequently detected genera are *Alternaria*, *Cladosporium*, *Diplodia*, *Fusarium*, *Phoma* and *Phomopsis*. Some of these can apparently replace *H. pseudoalbidus*, thereby preventing its expansion. However, such opportunistic fungi can also be pathogenic on their own and might contribute to lesion enlargement (Bakys *et al*., 2009a, b; Jankovsky and Holdenrieder, 2009; Kowalski and Holdenrieder, 2009a, b; Kowalski and Lukomska, 2005; Lygis *et al*., 2006; Przybył, 2002; Pukacki and Przybył, 2005; Schumacher *et al*., 2007). Among these, *Botryosphaeria stevensii* (anamorph *Diplodia mutila*) is of special interest with respect to climate warming, because this fungus is known as an opportunistic pathogen from warm regions (Ragazzi *et al*., 1999; Sánchez *et al*., 2003; Schumacher *et al*., 2007, 2010). The root system of affected trees is also attacked by opportunistic fungi, e.g. *Armillaria* spp., which considerably accelerate ash tree death (Bakys *et al*., 2011; Husson *et al*., 2012; Lygis *et al*., 2006; Skovsgaard *et al*., 2010).

The control of established ash dieback in the forest is practically impossible and silvicultural recommendations are limited to avoiding the loss of the value of mature ash stands (for a review, see Metzler *et al*., 2013). Only if the pathogen has been introduced recently through infected nursery stock and there are no infections in the surrounding ecosystems is removal of infected plants recommended (DEFRA, 2013). In principle, ash leaf litter might be removed from nurseries and parks as a prophylactic measure, but this would make sense only in the absence of extraneous inoculum. Chemical control might be possible for small plants, but is counterproductive, as it makes no sense to preserve susceptible plants, which would fuel the epidemic after outplanting. In addition, sublethal fungicide treatments might prevent symptom development and thereby facilitate long-distance dispersal with latently infected plants. However, there is some hope for the evolution of resistance (see above), which can be facilitated by breeding and appropriate forest management.

TECHNIQUES FOR PATHOGEN DETECTION AND HANDLING

Isolation of *H. pseudoalbidus* from necrotic lesions on the bark is reported to be straightforward (Kowalski, 2006; Queloz *et al*., 2011), although very low isolation rates have sometimes been found (Bakys *et al*., 2009a; Jankovsky and Holdenrieder, 2009). A small tissue sample is aseptically taken from the xylem close to the pith, preferentially from the necrotic zone close to healthy tissues. The samples are incubated on an agar-based culture medium. However, isolation success greatly depends on the season and the age of the necrotic lesion. Fresh necrotic bark lesions usually appear in the autumn and winter, and isolation from the xylem usually yields good results (approximately 75% from late October to April). During the winter months, the isolation success from fresh lesions remains high, but slowly decreases during the spring

until it drops to near zero between July and September (A. Gross, unpublished data). Isolation from pseudosclerotia produces good results (90%–100%), regardless of the season. Pseudosclerotial petioles are air dried for a few hours and surface sterilized using absolute ethanol, which evaporates quickly and does not soak into the pseudosclerotium. Once the petioles are dry, the pseudosclerotial plate (Fig. 1f,g) is removed tangentially using a sterile scalpel and small tissue samples are taken and incubated on culture medium (Gross and Holdenrieder, 2013).

The isolation of the pathogen from necrotic leaflets is difficult, because of the presence of numerous fast-growing fungi (Fig. 2c). Nevertheless, timely transfer of mycelia emerging from the vein cut can yield pure cultures. *Hymenoscyphus pseudoalbidus* was only very rarely isolated from green leaflets in the autumn (U. Oggenfuss, ETH Zurich, Zurich, Switzerland, unpublished data), but frequently from asymptomatic leaf stalks collected in September (Bakys *et al*., 2009a).

Methods to isolate single fungal spores have been discussed elsewhere (see, for example, Booth, 1971). Apothecia can be readily generated in the laboratory by incubating pseudosclerotia collected in nature. Gross *et al*. (2012c) induced ascocarp formation using an incubator with near-UV light at around 20 °C, as described by Leach (1971). Pseudosclerotia were placed into polystyrol Petri dishes on moist paper tissues. No difference in the viability of spores produced under UV was detected in comparison with field-grown spores.

Controlled crosses are possible with strains of opposite mating types on sterilized petioles (Gross *et al*., 2012c). For mating type determination, a multiplex PCR is available (Gross *et al*., 2012c).

Hymenoscyphus pseudoalbidus grows slowly on standard culture media, such as MEA (mean radial growth rate of 0.52 mm/day; Kirisits *et al*., 2013) or potato dextrose agar (PDA), and shows great variability on MEA (Fig. 5d). The growth rate of the mycelium is considerably enhanced by adding fresh or frozen ash leaves to MEA (Fig. 5a,b) (Gross *et al*., 2012c; Kirisits *et al*., 2013). *Hymenoscyphus pseudoalbidus* and *H. albidus* cultures MEA supplemented with ash leaves reveal a characteristic dark-reddish brown discoloration of the medium and show less variation in colony morphology compared with growth on MEA (Fig. 5b,e). During the winter, frozen leaves can be used instead of fresh leaves, but the growth-stimulating effect on *H. pseudoalbidus* is lost slowly with increasing freezing time of the ash leaves (A. Gross, unpublished data). Moreover, germination of *H. pseudoalbidus* spores was inhibited on a medium in which long-frozen (>5 months) ash leaves were used, and we recommend the use of MEA for spore viability tests. On cellophane sheets overlaid on agar medium, in addition to superficial mycelium, lobed, flattened irregular hyphae grow within the cellophane (Fig. 5f). However, in plant tissues, such hyphae could not be found (O. Holdenrieder, unpublished data). As for other fungi (Borman *et al*., 2006), long-term storage of *H. pseudoalbidus* in water is
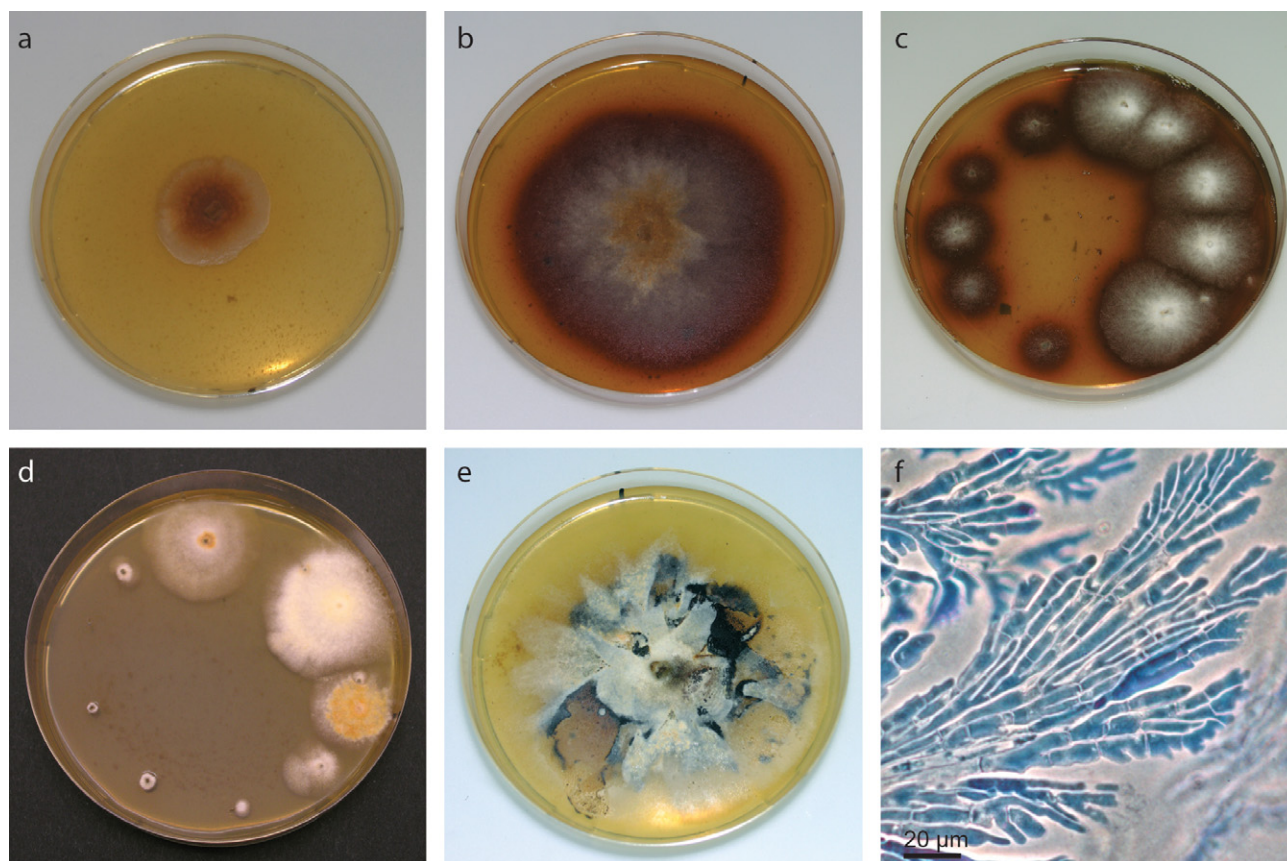
Fig. 5 Morphology of *Hymenoscyphus pseudoalbidus* in culture: (a) colony of the type strain (CBS 122504) growing on malt extract agar (MEA) for 3 weeks at 20 °C (diameter of Petri dish, 9 cm); (b) colony of the type strain growing on ash leaf agar for 3 weeks at 20 °C; (c) isolation from a necrotic shoot (incubated for 3 weeks at 20 °C); the smaller colonies on the left originated from pith tissue, the faster growing colonies on the right originated from central xylem tissue; (d) single spore cultures on MEA (36 days old), showing the variability in growth rate and colony morphology; (e) old culture (stored for several months at 5 °C) on MEA; (f) flattened hyphae growing within a cellophane sheet on ash leaf agar (stained with lactophenol blue).

possible for at least 4 years (O. Holdenrieder, unpublished data). Ascocarps of *H. pseudoalbidus* can be differentiated from *H. albidus* by the presence of a crozier at the ascus base (Zhao *et al*., 2012), which, however, is difficult to observe. A secure differentiation is achieved by sequencing the ITS region (Queloz *et al*., 2011). Real-time PCR assays for the *in planta* detection of *H. pseudoalbidus* have been developed by Chandelier *et al*. (2010) and Ioos *et al*. (2009). Alternatively, a species-specific ITS PCR for *H. pseudoalbidus* detection in tissues of *F. excelsior* is available (Johansson *et al*., 2010). These methods were developed prior to the separation of *H. albidus* and *H. pseudoalbidus*, and the specificity was not tested for these cryptic species at that time. However, the specificity of the real-time PCR assays was shown subsequently (A. Chandelier, Walloon Agricultural Research Centre, Gembloux, Belgium, personal communication; Husson *et al*., 2011) and a specific cleaved amplified polymorphism (CAP) marker was developed, based on the ITS PCR of Johansson *et al*. (2010). For the detection of infections on the basis of secondary

metabolites, MALDI-TOF mass spectrometry has been suggested (Pham *et al*., 2013).

MS markers for *H. pseudoalbidus* genotyping and population genetics have been developed by Gross *et al*. (2012b) and Bengtsson *et al*. (2012).

The virulence of *H. pseudoalbidus* can be determined by inoculation of *F. excelsior* shoots and stems (Kowalski and Holdenrieder, 2009a) or by leaf rachis infections (Kräutler and Kirisits, 2012). Ascospore inoculation (Cleary *et al*., 2013) can be used to study the natural infection process of *H. pseudoalbidus*. However, high spore concentrations led to early leaf shedding and no stem necroses were produced following artificial inoculation with ascospores (see above).

Significant insights into pathogenesis and host response can be expected from genomic, transcriptomic, proteomic and metabolomic approaches in the near future, and various genomic projects have been launched (Solheim *et al*., 2012b; Stenlid *et al*., 2012). Illumina sequence data have been made public without

prior analysis on a website at oadb.tsl.ac.uk as well as a public GitHub repository (https://github.com/ash-dieback-crowdsource), comprising assembled transcriptomic and genomic data of a single *H. pseudoalbidus* isolate, the 'interaction transcriptome' of four twig lesions and the transcriptome of an uninfected ash tree (MacLean *et al*., 2013). Initial analyses presented on the website identified: (i) a full-length Nep1-like protein (NLP) homologue (Dong and Kamoun, 2012), which is a putative virulence factor (Gijzen and Nürnberger, 2006); (ii) a secreted protein with four LysM domains belonging to a family of fungal effectors (de Jonge and Thomma, 2009); (iii) 3778 unique pfam domains, including 71 domains linked to copper oxidases and transporters (Crossman, 2012); and (iv) signatures of compositionally non-homogeneous domains (CNHDs) (MacLean, 2013) frequently associated with effector genes in filamentous fungi (Raffaele and Kamoun, 2012). In addition, the full-length mating type 1 sequence containing a gene with similarity to a DNA polymerase zeta catalytic subunit has been identified (Saunders, 2013). Research concerning ash dieback in Europe is currently coordinated by the FRAXBACK action (http://www.fraxback.eu; see also FRAXBACK, 2012), funded by the European Cooperation in Science and Technology (COST). An initial ash genome assembly (454 technology) is available for BLAST searches at http://www.ashgenome.org.

IMPLICATIONS FROM OTHER PATHOSYSTEMS AND OUTLOOK

Although the nature of the *F. excelsior–H. pseudoalbidus* pathosystem appears to be unique, comparisons with related pathosystems might provide useful insights into the understanding and control of ash dieback. Pathosystems can be related to ash dieback because: (i) the pathogen is phylogenetically related to *H. pseudoalbidus*; or (ii) the disease is functionally related to ash dieback.

Phylogenetically related host–parasite systems occur within the Helotiales, a group of fungi including a number of pathogens and endophytic fungi. *Hymenoscyphus pseudoalbidus* is not known to show a pathogenic behaviour in its native range. The behavioural switch to a pathogenic lifestyle in Europe represents the key to understanding its pathogenicity. An example of an endophyte in the Helotiales, which occasionally becomes pathogenic, is *Cenangium ferruginosum*. This fungus can cause pine dieback when conditions become favourable to the fungus or unfavourable to the host (Jurc *et al*., 2000), and is a frequently isolated endophyte of symptomless needles of *Pinus* spp. (Helander *et al*., 1994). Studies of environmental factors associated with needle colonization by *C. ferruginosum* have been performed, showing, for example, that the fungus was more frequent on the lower part of a mountain ridge (Sieber *et al*., 1999). The pine canker pathogen *Crumenulopsis sororia* shows a versatile behaviour depending on site conditions,

such as elevation, exposure or slope (Hayes *et al*., 1981), and the age of host tissues (Ennos and McConnell, 2003). A review of studies on the variation in infectivity and aggressiveness of pathogens in wild systems concludes that such variation is ubiquitous in space and time (Tack *et al*., 2012). Investigations along landscape gradients are needed for *H. pseudoalbidus* to assess environmental variation in ash dieback severity (see Holdenrieder *et al*., 2004). From studies on environmental variation associated with various pine endophytes (Terhonen *et al*., 2011), it can be hypothesized that any gradients in ash dieback severity might be related to variation in the ash endophytic community counteracting *H. pseudoalbidus*. The role of endophytes in preventing tree disease has also been emphasized for *Gremmeniella abietina* on pines (Ranta *et al*., 1995; Santamaría *et al*., 2007).

Many functionally related diseases are caused by introduced pathogens just as selective and lethal as H. pseudoalbidus. For example, Dutch elm disease (DED) has involved two consecutive pandemics across the Northern Hemisphere caused by the ascomycetes Ophiostoma ulmi and Ophiostoma novo-ulmi, both probably introduced from east Asia (Brasier, 2000). The disease has killed the majority of mature elms. As they have spread, both pathogens have been through remarkable evolutionary events involving horizontal gene transfer (Brasier *et al*., 2004). Many different management approaches have been used, but because of the explosive intensity of the disease and the high cost of the management applications, wide-scale eradication and control attempts were rapidly abandoned internationally and have been replaced by longer-term strategies such as breeding and conservation programmes (Dunn, 2000; Potter *et al*., 2011). The DED pathogens reproduce asexually as well as sexually, are transmitted by vector bark beetles (Webber, 2004) and cause a systemic infection. Some elm species can reproduce freely from root suckers and some can flower at a relatively early age. In the aftermath of the heavy losses of mature elms caused by O. novo-ulmi, millions of small recruitment elms have grown up from suckers and seeds across Europe and North America. A new 'balance' has been established between host, pathogen and vector, with abundant small elms now under constant attack by the pathogen, but part of the population usually surviving long enough to reproduce (Brasier, 1986). In contrast, F. excelsior usually does not form root suckers and only a few saplings survive the epidemic. Moreover, the environment seems to influence disease expression distinctly (Enderle *et al*., 2013).

Butternut canker and dogwood anthracnose, caused by the ascomycetes *Ophiognomonia clavigignenti-juglandacearum* and *Discula destructiva*, respectively, show symptoms in North America similar to European ash dieback (Broders *et al*., 2012; Holzmueller *et al*., 2010). However, reproduction of these pathogens is predominantly asexual, and therefore the evolutionary potential of these pathogens is much lower than that of *H. pseudoalbidus* (McDonald and Linde, 2002).

Chestnut canker, which is caused by the introduced ascomycete *Cryphonectria parasitica*, eradicated its new host *Castanea dentata* in North America across much of its range. However, in Europe, co-existence between pathogen and host (*Castanea sativa*) became possible because of virus-mediated hypovirulence (Robin and Heiniger, 2001). Therefore, a search for such viruses in *H. pseudoalbidus* has been initiated (Pliūra *et al.*, 2013).

Other novel and host-specific tree pathosystems are the emerald ash borer (*Agrilus planipennis*), an insect that seriously threatens all North American *Fraxinus* species, and the pine wood nematode (PWN, *Bursaphelenchus xylophilus*), which kills *Pinus* spp. in Asia and Western Europe. The emerald ash borer was introduced into North America from East Asia. It was also recently detected in the Moscow region (Selikhovkin *et al.*, 2013) and stimulated research on comparative transcriptomics and secondary metabolites of American and Asian *Fraxinus* spp., as well as on resistance breeding of ash (e.g. Rebek *et al.*, 2008; Rivera-Vega *et al.*, 2012; Whitehill *et al.*, 2012). For the PWN pathosystem, significant advances in understanding have been achieved through the application of modern molecular techniques (Shinya *et al.*, 2013), and much can be learned from such approaches concerning ash dieback research.

A lesson from these examples is the importance of avoiding further introductions of pathogen strains, so as to reduce the chances of pathogen recombination and the emergence of even more virulent strains. Therefore, even if *H. pseudoalbidus* has already been dispersed across much of Europe, it still makes sense to prove its origin more precisely and to stop importing infected plant material from the natural distribution area of *F. mandshurica*. In addition, it is important to study the biology and ecology of the pathogen in its native range.

The examples mentioned above also demonstrate that not much can be done to prevent the spread of invasive tree diseases, once introduction and establishment have occurred and in the presence of overwhelming inoculum production and a general absence of host resistance. This requires the avoidance of new pathogen introductions, interdisciplinary research on emerging diseases and creative strategies for the mitigation of the impacts of plant diseases also in near-natural ecosystems (Desprez-Loustau *et al.*, 2010; Pautasso *et al.*, 2010; Santini *et al.*, 2013).

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