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Rapid invasion by an aggressive pathogenic fungus (*Hymenoscyphus pseudoalbidus*) replaces a native decomposer (*Hymenoscyphus albidus*): a case of local cryptic extinction?

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ABSTRACT

Ash dieback caused by the infectious fungus *Hymenoscyphus pseudoalbidus* currently threatens the common ash, *Fraxinus excelsior*, in Europe. An intriguing aspect is the morphological and ecological similarity between *H. pseudoalbidus* and the native saprotroph *Hymenoscyphus albidus*. We revisited four localities where *H. albidus* apothecia were collected from 1989 to 2005 and established the current relationship of the species in these Danish ash stands based on microsatellites and differences in *ITS* sequences (used as CAPs marker). Scottish collections from 2010 supported the hypothesis that Danish herbarium samples prior to 2005 are identical to *H. albidus* still found in Scotland. The markers further revealed that herbarium samples from 1989 to 1994 were all *H. albidus*, while the latest collection (2005) was *H. pseudoalbidus*, which coincides with the first Danish symptom observations. Collections from 2010 were purely *H. pseudoalbidus*. We suggest that expanding *H. pseudoalbidus* excludes *H. albidus* from its niche resulting in *H. albidus* becoming a rare species in Denmark, which is perhaps already locally extinct.

Introduction

The ecological and evolutionary consequences of invasive species on native species are numerous and include hybridisation, introgression, niche displacement, competitive exclusion and extinction (Mooney & Cleland 2001). Invasive species may also disturb well-established mutualistic relationships (Bond & Slingsby 1984) and invading parasites that have undergone a host shift can cause severe consequences for the new host (Oldroyd 1999). In plant pathogens, such host shifts are known as one of the triggers for emerging infectious diseases (EID's) after introductions caused by human behaviour (Stukenbrock & McDonald 2008). Forest tree species are also affected by new fungal diseases with immediate effects on tree mortality followed by long-term evolutionary and ecological effects (Loo 2009). In Europe, Dutch elm disease caused by *Ophiostoma novo-ulmi* has caused massive eradication of mature elm trees (Brasier 1991). Other European forest tree species are currently affected by pathogenic fungi (*Phytophthora* species on alder, oak and beech; Brasier *et al.* 2004a,b; Jung *et al.* 2005; Xu *et al.* 2009). While the negative impacts of new non-indigenous fungi on host species are widely recognised, their effects on the native fungal species have not been given much attention (Desprez-Loustau *et al.* 2007).

Until recently, *Hymenoscyphus albidus*, an ascomycete known from Europe, has been considered a harmless

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decomposer of ash litter (Kowalski & Holdenrieder 2009). The species did not attract much interest until 2010, when it was suggested to be involved in the devastating dieback of *Fraxinus excelsior* (Kowalski & Holdenrieder 2009), affecting populations in most of continental Europe (Bakys *et al.* 2009). Epidemic symptoms of *F. excelsior* dieback were first recorded in Poland in the 1990's (Przybyl 2002) and since 2001 have been observed spreading to Central, North and Western Europe. To our knowledge, so far no symptoms have been recorded in the British Isles. The stepwise spread of symptoms through the continent indicates the effects of an invasive pathogen, and the fungus *Chalara fraxinea* was first suggested as the causal agent in 2006 (Kowalski 2006). The teleomorph was identified 3 yr later as *H. albidus* by the same scientists (Kowalski & Holdenrieder 2009). However, recently Queloz *et al.* (2011) showed that the anamorph, *C. fraxinea*, was genetically different from the teleomorph *H. albidus*. In addition, they found the true teleomorph of *C. fraxinea* and described it as *Hymenoscyphus pseudoalbidus* sp. nov. Although *H. albidus* and *H. pseudoalbidus* are morphologically very similar, the two species can be separated based on molecular analysis and sequencing of the *calmodulin* gene, *translation elongation factor 1-α*, the internal transcribed spacers of the rDNA genes (*ITS*) and *ISSR* fingerprinting (Queloz *et al.* 2011). Inter-specific polymorphisms have also been found with the marker *FG740* (a single copy protein-coding homologue) (Feau *et al.* 2011), and recent studies of *H. albidus* and *H. pseudoalbidus* based on microsatellite markers also suggest clear genetic differentiation between the species (Gross *et al.* 2011; Bengtsson *et al.* 2012).

In France, Husson *et al.* (2011) have analysed sequences of *ITS* and three single-copy genes from samples collected in Northeastern France where ash dieback is present, and in Western and Central France, where no disease symptoms have been observed. Two types were found, which correspond to *H. pseudoalbidus* and *H. albidus*. Furthermore, Husson *et al.* (2011) analysed the pathogenicity of both species and found that only *H. pseudoalbidus* showed strong pathogenicity towards ash seedlings. They concluded that, although both types are found in the study, their distributions are very specific, and they suggest that *H. pseudoalbidus* is a recent invader of the area spreading from the north-eastern part of the country. *H. pseudoalbidus* samples were only found in this area, suggesting that the species was not present in France before the onset of disease. These results disagree with those of Queloz *et al.* (2011), who found *H. pseudoalbidus* in herbarium samples collected from 1978 to 1987 in Switzerland where no symptoms had been reported until recent years. It remains unclear whether the two species co-exist in infected areas and whether the rapid spread of *H. pseudoalbidus* has influenced the occurrence of the native decomposer *H. albidus*. In the present paper, we compare herbarium samples collected in 1989, 1993, 1994 and 2005 with systematic collections made in 2010 at the same localities to determine the effect on *H. albidus* of the invasion of the aggressive *H. pseudoalbidus* in Denmark. We further establish whether the first occurrence of *H. pseudoalbidus* in herbaria collections coincides with the first observations of ash dieback in the country (in 2002–2003; Skovsgaard *et al.* 2010; Thomsen & Jørgensen 2011). Finally, we include samples collected in Scotland, where the ash dieback disease caused by *H. pseudoalbidus* is apparently absent, to determine if early Danish herbarium collections of *H. albidus* are identical to the harmless Scottish decomposer, and to detect whether *H. pseudoalbidus* is present in areas without symptomatic trees. For these purposes we used a CAPs marker (cleaved amplification polymorphisms) based on observed differences in the *ITS*-region, and seven microsatellites from Bengtsson *et al.* (2012), to distinguish the species in Denmark and Scotland, to determine the level of intraspecific variation in Danish *H. pseudoalbidus* populations, and to look for signs of hybridisation and introgression.

Methods

Herbarium specimens, collection sites and sampling

Specimens were obtained from the Herbarium of Fungi at the Natural History Museum of Denmark, in Copenhagen. These specimens had been sampled at four Danish localities in four different years (Table 1). Three localities were forest stands in Jutland whereas the last location (Kalvebod Fælled) was a semi-natural wooded area just south of Copenhagen.

The Danish localities were revisited on Aug. 4th 2010 (except Kalvebod Fælled which was revisited Aug. 27th 2010). Disease symptoms were observable at all localities. At each forest locality eight sample sites were selected (Table 2), and four sample sites were selected at Kalvebod Fælled. GPS coordinates were registered at each site and based on these we estimated the approximate size of the sampled area. At each of the eight (for Kalvebod Fælled, four) sample sites 10 sclerotified petioles with apothecia were collected from the forest floor within approximately 2–3 m^2.

From each sample site, three apothecia from three separate petioles were used to generate single spore cultures. The remaining apothecia were stored at −20 °C until DNA extraction. Apothecia for single spore cultures were fixed on lids of

Table 1 – Specimens obtained from the Herbarium of Fungi in Copenhagen. DNA was extracted from all obtained apothecia and analysed by PCR and *Sma* I digest. The last column represents the results of all tested apothecia of the respective collection no., where *H. a.* is *Hymenoscyphus albidus* and *H. pa.* is *H. pseudoalbidus* sp. nov.

Site	Collector	Date	Specimen no.	No. of apothecia	PCR Sma I type
Åbenrå Nørreskov	Ronald Toft	1989, Aug. 17th	C-F-10680	4	*H. a*
Vejle Nørreskov	Jan Vesterholt	1993, Jul. 29th	C-F-20481 (A)	4	*H. a*
Vejle Nørreskov	Jan Vesterholt	1993, Jul. 29th	C-F-20481 (B)	4	*H. a*
Kalvebod Fælled	Benny T. Olsen	1994, Aug. 28th	C-F-25888	6	*H. a*
Viuf Skov	Jan Vesterholt	2005, Aug. 6th	C-F-44389	4	*H. pa*