

NEW DISEASE REPORT

First report of *Hymenoscyphus fraxineus* causing ash dieback in Spain

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Email: miguel.nemesio@hotmail.com**KEYWORDS**European ash, *Fraxinus excelsior*

In August 2021, mature *Fraxinus excelsior* trees and associated regeneration with typical symptoms of ash dieback (shoot dieback, wilting of leaves, necrotic rachises and lenticels, and fungal fruiting bodies) were observed in the localities of Oviedo (43.3804, -5.8679) and Bulnes (43.2352, -4.8241) in the principality of Asturias in north-western Spain (Figs. 1–2). The lethal disease is caused by the ascomycete fungus *Hymenoscyphus fraxineus* (anamorph *Chalara fraxinea*), which was introduced into Europe from Asia. Since its introduction in the mid-1990s in north-east Poland, *H. fraxineus* has spread across Europe causing severe decline and increased mortality of European ash (*Fraxinus excelsior*) (Kowalski, 2006; Queloz et al., 2011) and narrow-leaved ash (*Fraxinus angustifolia*).

In both Oviedo and Bulnes, 10–15 necrotic rachises and fruiting bodies were collected in August 2021 (Fig. 3). The rachises were dried and fresh fruiting bodies stored in 99% (v/v) ethanol until further processing. For fungal isolation, five rachises per site were wiped clean with 70% (v/v) ethanol, then small wedges were cut out with a scalpel and laid on ash malt extract agar (five wedges per rachis, i.e. 25 isolates per site; Gross et al., 2014). Fungal cultures were left to grow at 20°C (daylight) for two weeks. Forty-two cultures showed the typical mycelial morphology of *H. fraxineus* on ash malt agar, that is dark discolouration of the agar at the edge of the culture and creamy-white mycelium (Fig. 4), five cultures were contaminated with *Penicillium* and three wedges showed no fungal growth. Phialophores and conidia of the *Chalara* anamorph were observed under the microscope from cultures considered to be *H. fraxineus* (Fig. 5). Four *H. fraxineus*-like isolates and one fruiting body per site were chosen for molecular analysis.

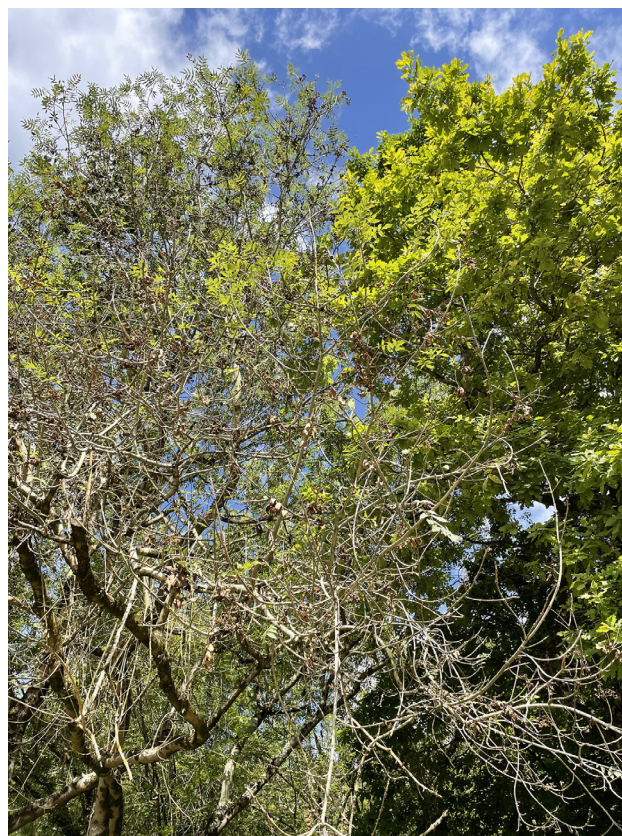


FIGURE 1 Mature *Fraxinus excelsior* tree with ash dieback symptoms in Oviedo, Spain

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FIGURE 2 Regeneration of *Fraxinus excelsior* in Bulnes, Spain showing wilting and necroses

Genomic DNA was extracted from fungal cultures and from fruiting bodies using LGC reagents (LGC Biosearch Technologies, UK) and Kingfisher 96 Flex (Thermo Fisher Scientific, USA) according to the manufacturers' instructions. The ITS (ITS1/ITS4) (White *et al.*, 1990) region was amplified using the JumpStart REDTaq ReadyMix (Sigma Aldrich, Germany) with the following reaction concentrations: 12.5 μ l JumpStart REDTaq ReadyMix, 8.5 μ l molecular-grade water, 1 μ l of each primer and 2 μ l DNA template. PCR conditions were as follows: 2 min at 94°C for initial denaturation, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C, and a final elongation for 10 min at 72°C. PCR products were sent to Microsynth (Switzerland) for forward Sanger sequencing using the primers ITS1 and ITS4. A total of five clean sequences were obtained from mycelium and two clean sequences from direct DNA-Extraction from fruiting bodies. The sequences were analysed using the BLASTn search which revealed 99–100% identity with *H. fraxineus* species in GenBank (e.g. KJ820631, KJ820658, KJ820670). ITS sequences were deposited in GenBank (OL455802–OL455808) confirming the presence of *H. fraxineus* in Spain.

In France, *H. fraxineus* was first reported in 2008 in the north-east of the country (Anonymous, 2008). The pathogen has since spread south and west, reaching the French Pyrenees in 2020 (<https://draaf.paca.agriculture.gouv.fr/Carte-nationale-des-premiers>). Based on its progression it is likely to be detected in the Spanish Pyrenees in the next few years. Both *F. excelsior* and *F. angustifolia* are native to Spain and susceptible to ash dieback. Based on previous studies, the epidemic is



FIGURE 3 Necrotic rachis with fruiting bodies

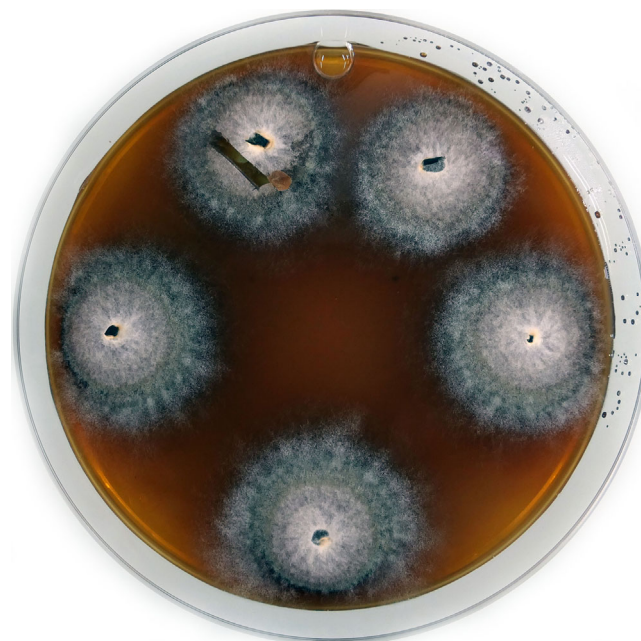


FIGURE 4 Mycelial growth of *Hymenoscyphus fraxineus* isolates on ash malt agar

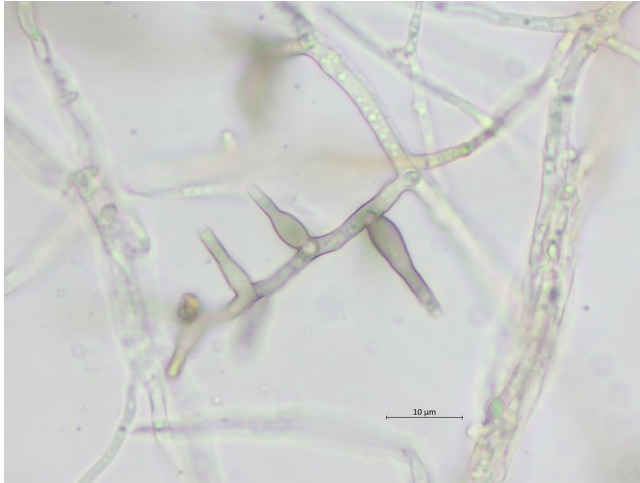


FIGURE 5 Phialophores and conidia of the *Chalara* anamorph under the light microscope

certain to cause severe damage to ash populations (Coker *et al.*, 2019). High summer temperatures and differences in climate in the different regions of Spain make it difficult to predict the spread of the pathogen. A monitoring system should be implemented to track the progression of the disease and to evaluate its impact. Breeding efforts are required to develop gene banks that can act as a source of tolerant plant material in the future.

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