Identification of *Armillaria* species on Declined Oak in Britain: Implications for Oak Health

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Abstract

The identity of 51 isolates of *Armillaria* from 15 *Quercus robur* trees in poor health, and a single healthy tree, at nine sites in England, was determined using Multi Locus Sequence Analysis (MLSA) of three gene regions. Sequences of the ITS 1, IGS-1 and EF-1α gene regions were obtained by PCR (Polymerase Chain Reaction) amplification and sequencing, and phylogenetic trees were generated based on Maximum Likelihood and Bayesian inference of

phylogenies. Four *Armillaria* species were isolated: *A. gallica*, *A. mellea, A. ostoyae* and *A. tabescens*. *Armillaria gallica* was most frequently isolated (40/51 isolates), but only from woodland trees. *Armillaria mellea* was isolated infrequently (3/51), from garden trees; *A. tabescens*, was isolated infrequently (4/51), from trees either in a garden or a parkland location. *Armillaria ostoyae* (4/51 isolates) was co-isolated with *A. gallica*, raising interesting questions about the synecology of these species, suggesting that more thorough investigations are required to detect all species present on a single host. The distribution of these *Armillaria* species in Britain and historical information about them on oak are described. It is concluded that further studies are necessary to determine the role of *Armillaria* in Oak Declines; *A. gallica* should be a key focus, but investigations should include polymicrobial interactions with other microorganisms, including other *Armillaria* species.

Keywords

Armillaria-root-rot, forest-pathology, MLSA, polymicrobial-disease, *Quercus robur*

Introduction

The native oak species *Quercus robur* L. (pedunculate oak) and *Q. petraea* (Matt.) Liebl. (sessile oak) are the most important, iconic broadleaf tree species in the UK (Harmer *et al.*, 2010; Rackham, 1993), and continental Europe (Oszako, 1999; Thomas, 2008). They are indispensable, both environmentally and ecologically, critical for woodland structure and biodiversity, valuable and culturally significant, and resilient to numerous adverse conditions. However, native oak trees are often taken for granted (Miles, 2013), with the expectation that they will survive adverse treatment and conditions, ultimately leading to a lack of care and attention needed for them to survive and prosper in ever more challenging

environments brought about by climate change, disturbance, and pest and pathogen threats.

Pest and disease attacks are amongst the greatest threats to the health of oak trees, and especially stressed trees (Desprez-Loustau *et al*., 2006; Haavik *et al*., 2015). Both native oak species are affected by periodic episodes of Oak Declines, which cause high levels of mortality, and *Armillaria* (Fr.) Staude spp. are frequently associated with the traditional view of Oak Declines (Falck, 1918; Day, 1927; Gibbs and Greig, 1997). The traditional concept of Oak Decline regarded it as a complex syndrome, expressed in multiple symptoms, and having both biotic and abiotic agents implicated in causal roles (Delatour, 1983; Landmann, 1993; Gibbs and Greig, 1997; Thomas, 2008). Disease symptoms include progressive decline of the tree, twigs shedding, and small branches die from the top downwards, giving the overall appearance of crown thinning. Chlorotic foliage is sometimes reported, as is the appearance of numerous epicormic shoots in the lower crown, along tree trunks or main branches. Sudden withering and death of foliage has been reported in some countries (see Delatour, 1983). Lammas-shoot formation may take place. Longitudinal cracks may appear on the stems with necrotic underlying inner bark; sometimes dark 'slime flux' seeps from cracks in the bark (Jacquiot, 1949, 1950, 1976; Gibbs and Greig, 1997; Vansteenkiste et al., 2004). In some cases, the bark separates from the stems in large sheets. Beetle damage to the inner bark tissues, specifically that caused by the larvae of *Agrilus biguttatus* (Coleoptera, Buprestidae), also known as the twin spotted oak borer (TSOB) is frequently mentioned, and sometimes gradual recovery of the trees occurs (Delatour, 1983; Cech and Tomiczek, 1986; Donaubauer, 1998). However, some authorities emphasise the regional

differences in factors involved (Delatour, 1983; Lynch *et al*., 2013), and progress on sorting out individual factors involved was not advanced.

Brown *et al*. (2016) highlighted that a number of studies have attempted to identify and separate different types of Oak Decline, principally to get a better understanding of the significance of the different factors at play, including the roles of particular biotic agents such as *Armillaria* spp. The first to apply this approach was Petrescu (1966) who described three types of Decline ('sudden', 'rapid' and 'slow'), based on the rapidity of diminishing tree health and death in Romania. More recently Denman and Webber (2009) distinguished two different types of Oak Decline in the UK, based not only on rate of deterioration, but also on the parts of the tree attacked. Chronic Oak Decline (COD) was akin to the 'slow' decline conceptualised by Petrescu (1966), occurring over decades, with poor root health (including the roles pathogens play in this), suspected of having an underlying cause. Refinement of these definitions and a metric that can be used to identify COD trees is still required. On the other hand, Acute Oak Decline (AOD) was defined as having a faster effect with the above ground parts of the tree being the main organs attacked (Denman and Webber, 2009) and key causal roles for various insect and microbial components. However, as COD and AOD occur on different parts of the tree, it is possible that both could occur on the same individual, either sequentially or even simultaneously, thus the relationship between these two forms of Oak Decline still requires resolution.

In many reports on traditional Oak Decline, *Armillaria* spp. are mentioned as key components of the syndrome (Falck, 1918; Georgévitch, 1926; Yossifovitch, 1926; Day, 1927; Osmaston, 1927; Robinson, 1927; Wargo, 1996; Gibbs and Greig, 1997; Donaubauer,

1998; Thomas, 2008). Other root pathogens including *Gymnopus* (Pers.) Roussel (syn. *Collybia* (Fr.) Staude), (Marçais, *et al*., 1999; Camy *et al*., 2003) and *Phytophthora* de Bary have also been implicated (Blaschke, 1994; Brasier, 1993; Jung *et al*., 2000; Jönsson *et al*., 2005). It is well established that *Armillaria* spp. occur on native oak species in Europe, but information about which species occur on native oak in Britain, their distribution and ecological roles under different conditions, is lacking or outdated. This information is crucial in order to have a better understanding of Oak Declines, as *Armillaria* spp. could contribute to the 'predisposition' stage of the Decline or hasten mortality. In contrast they may behave as primary pathogens, aggressively attacking host tissue, or as saprophytes taking advantage of decaying dead stumps and roots (Baumgartner *et al*., 2011; Guillaumin and Legrand, 2013). Whatever roles they play, a starting point for investigating the disease on native oak species in Britain, would be to determine baseline data on the species of *Armillaria* that occur on these trees, using the most up-to-date and reliable identification methods.

Determining the species identity of *Armillaria* isolates is not straight forward. Since inception of the genus, the identity of many species has changed several times (Watling *et al*., 1991). In the 1970s identification was based on basidiocarp (fruiting body) morphology (Herink, 1973 in Watling *et al*., 1991; Baumgartner *et al*., 2011). Prior to that, all root rots attributable to *Armillaria* spp. were considered to be caused by a single species, *Armillaria mellea* (Vahl ex Fr.)P. Kumm. *sensu lato* (Guillaumin and Legrand, 2013). However, variation amongst the morphotypes implied that multiple species existed and this method of identification was deemed unreliable.

The discovery that phenotypes arising from single spore cultures were morphologically different to those derived from culturing vegetative mycelia (Hintikka, 1973), led to the realisation that the genetic makeup and life history of the fungus were a means of separating species (Korhonen, 1978). This resulted in the development of the biological species concept, which defines a species as a group of individuals that have the ability to interbreed and produce viable offspring. Hyphal mating tests between haploid strains were carried out, and novel *Armillaria* spp. were subsequently described (Korhonen, 1978; Anderson and Ulllrich, 1979).

Using mating tests and phenotypic attributes (for example substrate utilisation and growth rates), in combination with morphology, Rishbeth (1982) identified five *Armillaria* species present in England: *Armillaria mellea* (Vahl ex Fr.) P. Kumm *s.l.*; *A. ostoyae* (Romagn.); *A. tabescens* (Scop. Ex Fr.); *A. bulbosa* (Barla) Romagn., (= *A. gallica* Marxm. & Romagn.) and an unknown species. Most of these were isolated from conifers, but some also occurred on unspecified broadleaved species only, with specific mention of *A. tabescens* from oak. Rishbeth (1982) used haploid isolates of known identity in mating tests, crossing diploid cultures of *Armillaria* that were derived from fruitbodies, rhizomorphs or infected material. However, Guillaumin *et al.* (1991) and others observed that results of haploid-diploid, and even haploid-haploid crosses can be difficult to interpret leading to uncertain or unresolved identification, raising some doubt about the veracity of the identification by Rishbeth (1982).

With developments in molecular technology, DNA sequencing became an obvious tool to use for species identification in order to overcome the difficulties associated with mating

tests and morphological based identifications (Baumgartner *et al*., 2011). Pérrez-Sierra *et al.* (1999) used PCR (polymerase chain reaction) RFLPs (restriction fragment length polymorphisms) of the IGS-1 (intergenic spacer one) region of the ribosomal RNA operon of 66 *Armillaria* isolates originating from a range of host species in the UK; only two of these isolates were from oak. Using this method, Pérrez-Sierra *et al.* (1999) confirmed five *Armillaria* species present in the UK, with *A. gallica* and *A. mellea* identified on pedunculate oak, but intraspecific variation was noted within *A. mellea* isolates, casting some uncertainty as to whether it represented a single species or not. It was concluded that the development of specific primers would be needed to give a quicker, accurate diagnosis (Pérrez-Sierra *et al.*, 1999).

Mulholland *et al.* (2011) applied a PCR-based diagnostic assay on North European *Armillaria* species, using species-specific primers that bind to the Elongation Factor 1-alpha (EF-1α) gene. Twenty five *Armillaria* isolates from various hosts, including three isolates from *Q. robur* in the UK, were tested together with a number of European *Armillaria* isolates of known identity. All three of the oak isolates were identified as *A. gallica* (Mulholland *et al.*, 2011). The method was shown to be quick and effective but intra-strain sequence heterogeneity was encountered, and these isolates could not be analysed. It was recommended that multiple gene regions be sequenced to resolve the true identity of isolates of *Armillaria* (Mulholland *et al.*, 2011).

We collected a number of *Armillaria* isolates from pedunculate oak trees in England with symptoms of poor, thin crowns and/or crown die-back, and/or stem bleeding. The key objective of this study was to identify the isolates to species level. In view of the lack of a

single gene to ensure completely reliable identification to species level as outlined above but also mentioned by Mulholland *et al.*, (2011) and Tsykun *et al.* (2013), a multi locus sequence analysis (MLSA) approach was adopted to determine the identity of these *Armillaria* isolates from English oak. The aims of the study were thus to determine the identity of the isolates; document their distribution and symptoms shown by colonised host trees, and consider their potential role in Oak Declines based on information available in the literature.

Methods

Sites, symptoms and sampling

Symptomatic trees were brought to our attention either through cases reported to the Forestry Commission's 'Tree Health Disease Diagnosis and Advisory Service' (THDAS), Forest Research (FR), Alice Holt, Farnham, Surrey, England; or during investigation activities in the Oak Decline research project at FR. Nine sites were visited and information about the site and management of the symptomatic tree(s) were recorded (Fig. 1 and Table 1). In total, 16 trees were examined. In the Forest of Dean, four trees at Chestnuts Wood, and three trees near the Speculation car park were sampled. Two trees each, from Moorend Common and Runswood, but single trees only from Grafton Wood, Hatchlands, Kent, Oakhill and Reading, were tested. Crown condition of affected trees was assessed on a scale of 1-5 with 1 being very poor and 5 being very healthy. The Hessian method of crown morphology assessment of old oaks (Hessian Forest Research Institute Hahn, Münden, Germany) was used as a guide for winter assessment when the crown was bare, and the Forestry Commission's Field Book 12 (Innes, 1990) was used as a guide in summer when trees were in full leaf. Diameter at breast height (DBH i.e. 1.3 m) was measured using a Forestry Suppliers metric diameter

Table 1. Origin, reference and identity of *Armillaria* isolates

Site Name	Tree Number	Our Tree Reference	Isolate Reference	Identity	Management and tree crown condition ranked 1-5[a]
Forest of Dean Chestnuts Wood	1	T1	CW18; CW19	*A. gallica*	Plantation. T1 - Napoleonic oak (*Q. robur*) approximately 190 years old. T2, T3 and T4 were 60 year old *Q. robur*. Trees were managed for timber production. Crown Condition: T1, T2,T3 and T4=crown ranked 3
	2	T2	CW21; CW25	*A. gallica*	
	3	T3	CW27; CW30; CW33; CW36; CW37; CW41	*A. gallica*	
	4	T4	CW49; CW50; CW51; CW52	*A. gallica*	
			CW48; CW53; CW54; CW55	*A. ostoyae*	
Forest of Dean Speculation Cannop	5	T5	FOD5-30; FOD5-31; FOD5-32; FOD5-33	*A. gallica*	Plantation. All Napoleonic oak (*Q. robur*) approximately 190 years old and managed for timber production. Crown Condition: T5, T8,T16= crown ranked 1
	6	T8	FOD8-13; FOD8-14; FOD8-18; FOD8-21	*A. gallica*	
	7	T16	FOD16-6	*A. gallica*	
Grafton Wood	8	T6	GW19; GW20	*A. gallica*	Woodland. Amenity wood managed for conservation, biodiversity and limited amenity access. Crown Condition: T6= crown ranked 3
Hatchlands	9	T1	H21	*A. tabescens*	Parkland. Wooded part of parkland on heritage estate, tree situated close to public walk way through

					wood. Managed for amenity. Crown Condition: T1= crown ranked 4
Kent	10	T2	MCollins12; MCollins15	*A. mellea*	Garden. Urban home in a flower bed. The bed was recently built up with soils and compost, covering the base of the stem with shrubs recently planted (and some died) in the flower bed. Crown Condition: T2= crown ranked 3
Moorend Common	11	T1	MC3; MC5; MC22; MC23;MC24; MC25; MC26; MC27	*A. gallica*	Woodland. Unmanaged woodland, amenity and conservation. Mature trees estimated between 100-150 years old. Crown Condition: T1 and T2= crown ranked 2
	12	T2	MC28	*A. gallica*	
Oakhill, Kent	13	T5	OH86; OH87; OH88	*A. tabescens*	Garden. Single tree approximately 100 years old in a flowerbed surrounded by shrubs. Highly managed gardens. Crown Condition: T5 = crown ranked 4
Reading	14	T1	MK7	*A. mellea*	Garden. Single mature tree approximately 200 years old growing in an unmanaged domestic garden on

					the boundary of a highly managed garden. Shrubs in flower bed present in the managed garden. Many of these shrubs had died. Crown Condition: T1= crown ranked 3
Runswood	15	T3	RW1; RW2;RW3; RW4; RW5	*A. gallica*	Woodland. Mature oak woodland come into recent management for timber. Tree H1 was a healthy tree. Crown Condition: T3= crown ranked 1; H1= crown ranked 5
	16	H1	RW60	*A. gallica*	

[a]**Crown ranking: 1** = Dead (or just about dead having lost >95% of its crown); **2** = 80-95% canopy missing; **3** = Moderate decline (leaves yellowing, canopy thinning, 35-75% missing, gaps in canopy) including minor dead wood (100mm diameter in outer crown); **4** = Minor reduction in canopy health (10%-30% missing); **5** = Healthy canopy.

Fig. 1. Map showing the distribution of the sites sampled for isolation of *Armillaria* from oak trees in England.
1 = Speculation car park, Forest of Dean
2 = Chestnuts Wood, Forest of Dean
3 = Grafton Wood
4 = Hatchlands
5 = Kent (MCollins)
6 = Moorend Common
7 = Kent (Oakhill)
8 = Reading (MK)
9 = Runswood, Wattlington

tape. Photographs documenting both external and internal symptoms were taken. To sample the trees a sharp, surface disinfected chisel was used to expose infected tissue and remove panels from necrotic buttress roots and stem bark and where landowner permission was granted, larger areas of stem and buttress root bark were removed to obtain insights into the extent of colonisation. The panels were approximately 10 cm x 15 cm (LxB), taken to a depth of 4–5 cm, and two to four panels per tree were removed. Panels were placed in a clean polythene bag, kept cool and taken to the laboratory where they were kept at 4°C for processing which took place within 1-3 days of sampling. During sampling, between trees all sampling equipment was thoroughly surface disinfected with 95% industrial methylated spirits (IMS).

Fungal isolation, culture and preparation for identification

Immediately prior to isolation the bark panels were triple surface disinfected in the laboratory by submerging in 70% ethanol (EtOH) for 1min, followed by 1 min in 0.25% sodium hypochlorite (NaOCl) solution prepared from commercial bleach (with 5% NaOCl content) and finally placing them in 70% EtOH again for 1 min., before rinsing twice in sterile water for 1 min each. Bark panels were then allowed to air dry before isolations were carried out. Small chips (2–3 mm in length and in breadth) of infected tissue cut from the dead-live junction of necrotic lesions were placed on a non-selective culture medium, Malt agar (MA) made according to the manufacturers instructions and/or on a selective MAT (malt-antibiotic-thiabendazole) culture medium (Malt extract, 10 gL^{-1}; agar, 15 gL^{-1}; Penicillin-G, 0.05 gL^{-1}; Streptomycin sulphate, 0.05 gL^{-1}; Polymyxin, 0.025 gL^{-1}; Thiabendazole lactate (23 %) 1 mlL^{-1}), which was autoclaved for 15 mins at 121 p.s.i., cooled to 50°C then dispensed in 20 ml aliquots into disposable 9 cm diameter Petri plates. Plates containing

tissue pieces were incubated under aerobic conditions at room temperature for two to three weeks, and when fungal growth emerged from the wood chips, they were sub-cultured by transferring to MA. Each fungal colony developing from a chip of bark constituted an isolate. Although both MA and MAT isolation media were used, MAT isolation medium was the most successful for obtaining *Armillaria* isolates (more than 90% success rate – data not shown). The *Armillaria* isolates were maintained on 2% potato dextrose agar (PDA) (Ford *et al*., 2015), or 3.3% MA slopes amended with streptomycin (10mg ml^{-1}), (MA+S), and kept at 25°C in the dark for short term storage. For long term storage, isolates were transferred to PDA or MA plates when colonies were sizable, 0.5 cm diameter discs with mycelium were placed in 2 ml cryovials under sterile 30% glycerol (Fisher cat: 10795711 diluted in sterile water) and stored at -80°C (recommended by Kathryn Ford - James Hutton Institute, Dundee, Scotland, UK, pers. comm.). As additional long term storage, discs were stored as described above but the 30% glycerol solution was replaced with sterile water only and cultures were kept at 4°C in the dark.

DNA extraction

For DNA extraction, isolates were cultured on sterile squares of clear cellophane (5 cm x 5 cm) placed on MA. The isolates were then incubated at 25°C in the dark until sufficient mycelial growth was obtained and cultures could easily be scraped off the cellophane. Genomic DNA was extracted using the FASTDNA® Spin Kit system (MP Biomedicals, USA) according to the manufacturer's instructions. DNA was stored at -20°C until required.

PCR and sequencing

The IGS-1 region was amplified using primers 0-1 (Duchesne and Anderson, 1990) and P-1 (Hsiau, 1996). Amplicons from the ITS region (including the ITS-1, 5.8S gene and ITS-2 regions) were obtained using primer pair ITS-1/ITS-4 (White *et al*., 1990). A portion of the EF-1α gene was amplified using primers EF595F and EF1160R (Kauserud and Schumacher, 2001).

PCR reactions were conducted using a HotStarTaq® DNA Polymerase kit (Qiagen, UK). PCR mixtures comprised 10x PCR buffer, dNTPs (2mM each), primers (50 µM each), 0.5 µl HotStarTaq DNA Polymerase (2.5 U reaction-1), 1 µl template DNA (20 – 100 ng) and 19 µl sterile MilliQ water. PCR was performed using a Mastercycler® pro Vapo protect (Eppendorf, USA). PCR amplification cycles were: 5 min initial denaturation at 95°C, 30 cycles of 1 min denaturation at 95°C, primer annealing for 30 s at 55°C (ITS and IGS-1), or 48°C (EF-1α) and extension for 1 min at 72°C, a final extension for 5 min at 72°C was included to complete the reaction. PCR products were visualised after electrophoresis on a 1.5% agarose (Sigma, USA) gel, stained with GelRed (Biotium cat: 41003). PCR products with the expected size were purified using DNA Clean & Concentrator kits (Zymo Research, USA) and the amplicons sequenced in both directions at Source BioScience, Cambridge, UK. Sequences were inspected and assembled using Sequencher 5.2.4 software (Genecodes, USA). The sequences generated in this study were submitted to GenBank (ITS: file ITS_Armillaria_Genbank.sqn:
KX618532 - KX618582, IGS-1: file Armillaria_IGS1.sqn: KX618583 - KX618633 and file Armillaria_EF-1α: KX674465-KX674515).

Table 2. Origin, Identification method and GenBank Accession numbers of strains used

Species and Culture number	Origin, country	Identification method(s)	GenBank accession numbers		
			IGS-1	ITS	EF-1a
Armillaria borealis					
A1	Single-spore culture, Finland	Haploid tester strains (Guillaumin et al. 1991)	JN657440	JN657467	JN657494
A5	Single-spore culture, Germany	Haploid tester strains (Guillaumin et al. 1991)	JN657441	JN657468	JN657495
A2	Single-spore culture, Finland	Haploid tester strains (Guillaumin et al. 1991)	HQ232279	HQ232287	HQ285901
A618	Rhizomorph, Switzerland	Haploid-diploid pairing, PCR-RFLP and sequence analysis of IGS-1, ITS, and EF-1a	JN657442	JN657469	JN657496
A722	Rhizomorph, Switzerland	Haploid-diploid pairing, PCR-RFLP and sequence analysis of IGS-1, ITS, and EF-1a	JN657443	JN657470	JN657497
Armillaria cepistipes					
B2	Single-spore culture, Finland	Haploid tester strains (Guillaumin et al. 1991)	HQ232280	HQ232288	HQ285902
B3	Single-spore culture, Finland	Haploid tester strains (Guillaumin et al. 1991)	JN657418	JN657445	JN657472
B5	Single-spore culture, Italy	Haploid tester strains (Guillaumin et al. 1991)	JN657419	JN657446	JN657473
C13AE	Rhizomorph, Ukraine	PCR-RFLP, sequence analysis of IGS-1, ITS, and EF-1*α*	JN657420	JN657447	JN657474
C19AS2	Diploid culture, Ukraine	PCR-RFLP, sequence analysis of IGS-1, ITS, and EF-1*α*, haploid-diploid pairing	JN657421	JN657448	JN657475
Y16AE	Rhizomorph, Ukraine	PCR-RFLP, sequence analysis of IGS-1, ITS, and EF-1*α*	JN657422	JN657449	JN657476
C5C-S1	Diploid culture, Ukraine	PCR-RFLP, sequence analysis of IGS-1, ITS, and EF-1*α*, haploid-diploid pairing	JN657423	JN657450	JN657477
S11A-E	Rhizomorph, Ukraine	PCR-RFLP, sequence analysis of IGS-1, ITS, and EF-1*α*	JN657424	JN657451	JN657478
Armillaria gallica					
E5	Single-spore culture, France	Haploid tester strains (Guillaumin et al. 1991)	HQ232283	HQ232291	HQ285905
E4	Single-spore culture, France	Haploid tester strains (Guillaumin et al. 1991)	JN657425	JN657452	JN657479
E6	Single-spore culture, France	Haploid tester strains (Guillaumin et al. 1991)	JN657426	JN657453	JN657480
HY1	Single-spore culture, Ukraine	PCR-RFLP and sequence analysis of IGS-1, ITS, and EF-1*α*, haploid-haploid pairing	JN657427	JN657454	JN657481
HY2a	Single-spore culture, Ukraine	PCR-RFLP and sequence analysis of IGS-1, ITS, and EF-1*α*, haploid-haploid pairing	JN657428	JN657455	JN657482

C1A_S	Rhizomorph, Ukraine	PCR-RFLP and sequence analysis of IGS-1, ITS, and EF-1*α*	JN657429	JN657456	JN657483
Y11D-S1	Diploid culture, Ukraine	PCR-RFLP and sequence analysis of IGS-1, ITS and EF-1*α* haploid-diploid pairing	JN657430	JN657457	JN657484
Y7C-S1	Diploid culture, Ukraine	PCR-RFLP and sequence analysis of IGS-1, ITS and EF-1*α* haploid-diploid pairing	JN657431	JN657458	JN657485
Armillaria mellea					
D1	Single-spore culture, France	Haploid tester strains (Guillaumin et al. 1991)	JN657437	JN657464	JN657491
D5	Single-spore culture, France	Haploid tester strains (Guillaumin et al. 1991)	JN657438	JN657465	JN657492
D4	Single-spore culture, France	Haploid tester strains (Guillaumin et al. 1991)	HQ232282	HQ232290	HQ285904
HY-3	Single-spore culture, Ukraine	Haploid-haploid pairing, PCR-RFLP and sequence analysis of IGS-1, ITS, and *EF-1α*	JN657439	JN657466	JN657493
Armillaria ostoyae					
C5	Single-spore culture, France	Haploid tester strains (Guillaumin et al. 1991)	HQ232281	HQ232289	HQ285903
C2	Single-spore culture, France	Haploid tester strains (Guillaumin et al. 1991)	JN657432	JN657459	JN657486
C4	Single-spore culture, France	Haploid tester strains (Guillaumin et al. 1991)	JN657433	JN657460	JN657487
Y17DS	Rhizomorph, Ukraine	PCR-RFLP and sequence analysis of IGS-1, ITS, and *EF-1α*	JN657434	JN657461	JN657488
HpAg1	Single-spore culture, Ukraine	Haploid-haploid pairing, PCR-RFLP and sequence analysis of IGS-1, ITS, and *EF-1α*	JN657435	JN657462	JN657489
D20	Mycelial fans, Switzerland	Haploid-diploid pairing, PCR-RFLP and sequence analysis of IGS-1, ITS, and EF-1*α*	JN657436	JN657463	JN657490
Armillaria tabescens					
HAt1S5	Single-spore culture, Ukraine	Basidiocarp morphology, PCR-RFLP and sequence analysis of IGS-1, ITS, and EF-1a	HQ232284	HQ232292	HQ285906
HAt2S5	Single-spore culture, Ukraine	Basidiocarp morphology, PCR-RFLP and sequence analysis of IGS-1, ITS, and EF-1a	HQ232285	HQ232293	HQ285907
HAt5S3	Single-spore culture, Ukraine	Haploid-haploid pairing, sequence analysis of IGS-1, ITS, and EF-1a	HQ232286	HQ232294	HQ285908

Multilocus sequence analysis

A selection of DNA sequences for European species of *Armillaria* published by Tsykun *et al*., (2013) was obtained from GenBank (Table 2). The sequences that were selected included those from isolates that serve as reference strains for sexual compatibility tests (i.e. in delineating biological species) (Tsykun *et al.*, 2013). *Moniliopthora roreri* was used as outgroup species to root the phylogenetic trees. Multiple sequence alignments were done with the online version of MAFFT (Katoh and Standley, 2013) using the default settings. Nucleotide substitution models that best fitted the data matrices were determined using jModelTest version 2.1.6 (Darriba *et al*., 2012) and incorporated in subsequent phylogenetic analyses.

Phylogenetic trees were generated for each locus based on Maximum Likelihood (ML) and Bayesian inference of phylogenies (BI). Maximum likelihood analyses were done using PHYML version 3.1 (Guindon *et al*., 2010). Node support was determined using bootstrap analyses (1000 replicates).

MrBayes version 3.2.3 (Ronquist *et al*., 2012) was used to determine phylogenies based on Bayesian inference. Markov chains were run twice for 20 million generations with sampling of every 100th tree. Effective sampling size (ESS) as a measure of convergence was determined using Tracer version 1.5 (http://tree.bio.ed.ac.uk/software/tracer/). In total, 25% trees with low likelihood were discarded (burn-in) prior to calculating posterior probability values in MrBayes. Trees were viewed in FigTree version 1.4 (http://tree.bio.ed.ac.uk/software/figtree/). Trees generated in this study were submitted to TreeBase (Accession number: S19411).

Results

Sites and trees sampled

Site distribution: Buttress root or stem panels were obtained from sixteen trees spread over nine sites in England (Fig. 1). Five of the sites were located in woodlands or plantations (Table 1) where eleven trees with poor crown condition and/or stem bleeding, and one healthy looking tree, were analysed. A further three sites each had a single symptomatic tree in a garden setting, and the ninth site had a single tree in a parkland setting (Table 1). Three of the five woodland/plantation sites *viz*. Speculation Cannop and Chestnuts Woods in the Forest of Dean (FOD), Gloucestershire and Runswood (Norfolk) were managed for timber production, while the remaining woodland sites, *viz*. Grafton Wood (Worcestershire) and Moorend Common (Oxfordshire) were managed for conservation and amenity purposes. The garden sites were in Reading (single site) and Kent (two sites), and the affected trees were situated in flower beds in the gardens, while the tree in the parkland was in a wooded part of the park where a foot path allowed visitor access through the open grown woodland with grass understory on a heritage estate near Guildford, Surrey.

Identification of isolates collected

Fifty-one isolates from sixteen trees were analysed in this study. Based on the phylogenetic analyses four species were identified: *A. gallica, A. mellea, A. ostoyae* and *A. tabescens*. Phylogenetic trees generated from ITS, IGS-1 and EF-1α grouped isolates MCollins12, MCollins15 and MK7 in a cluster together with sequences of *A. mellea* (Figs. 2-4); and isolates OH86, OH87, OH88 and H21 with sequences of *A. tabescens* (Figs. 2-4). Elongation Factor -1α phylogeny grouped isolates CM48, CM54, CM55 and CM59 together with sequences representing *A. ostoyae* (Fig. 2) while phylogenetic trees obtained from the ITS

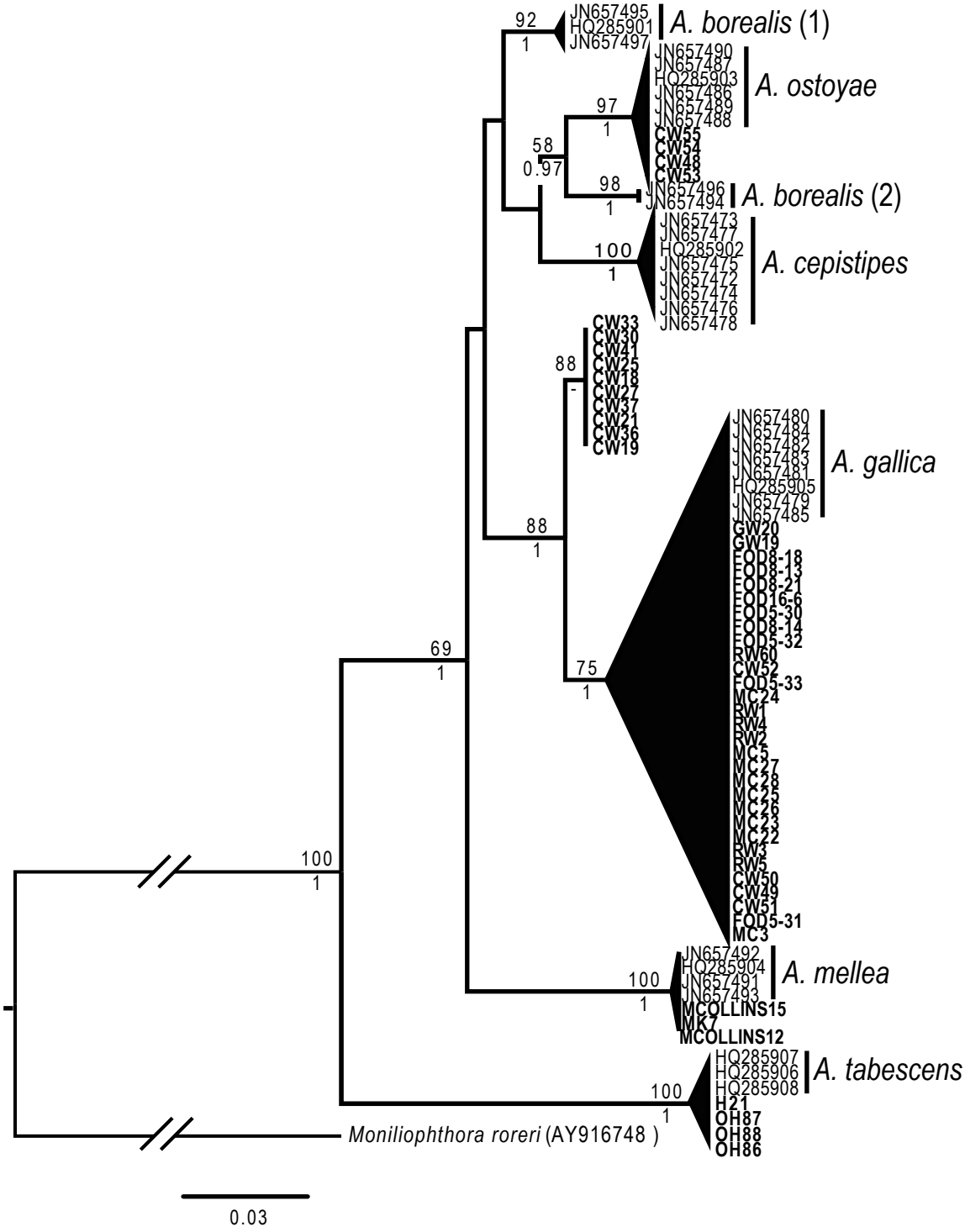

Fig. 2. Phylogram showing the phylogenetic relationships of isolates from this study and sequences of *Armillaria* species from GenBank. The phylogenetic tree was generated from TEF-1α DNA sequences using maximum likelihood. Bootstrap values greater than 60% are indicated above the branches, values below the branches are posterior probability values from a Bayesian analysis (PP > 0.95). The tree is rooted to *Moniliophthora roreri* (GenBank accession number: AY916748). Scale bar: nucleotide substitutions per site.

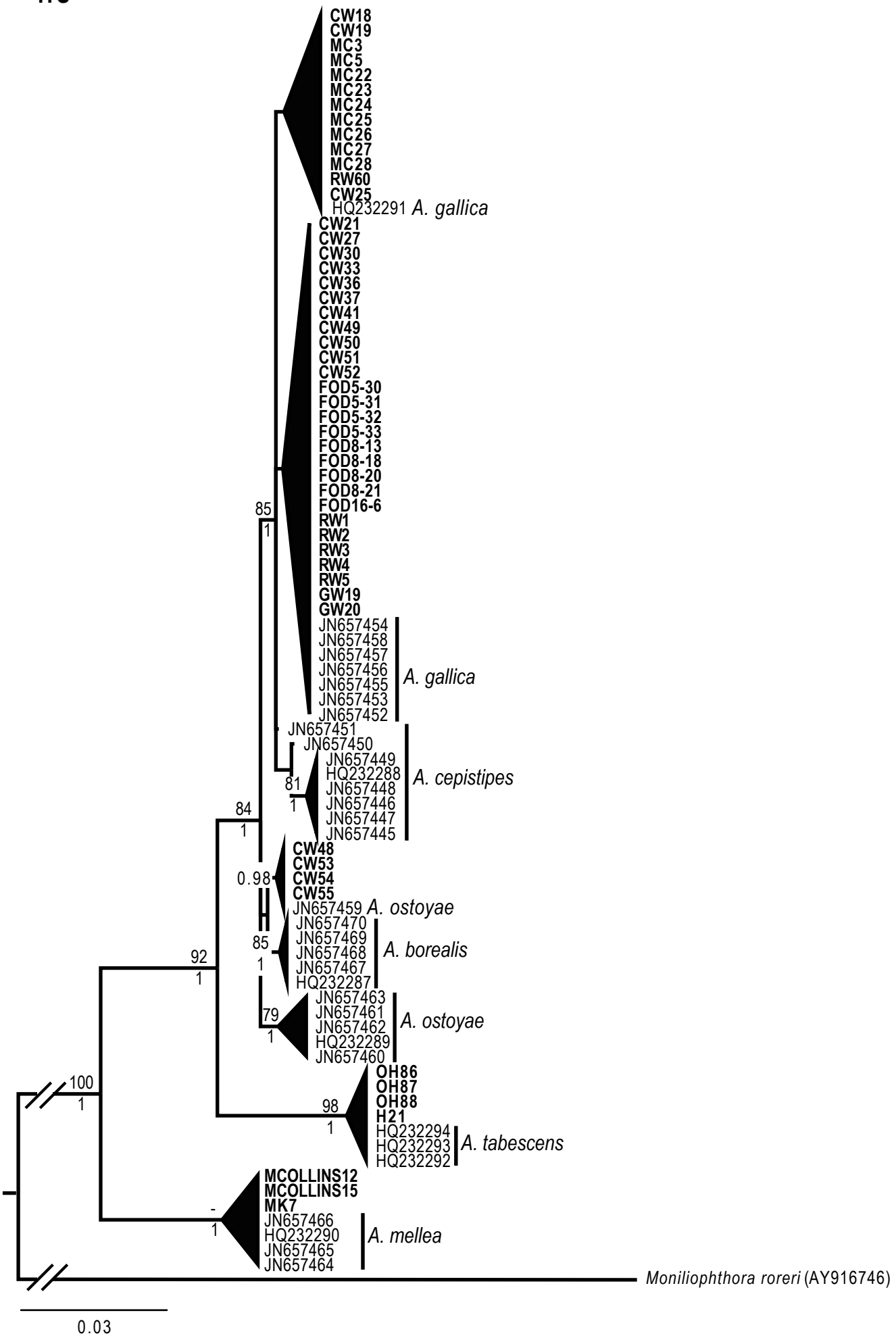

Fig. 3. Phylogenetic tree generated from maximum likelihood analysis based on the ITS sequence matrix. Bootstrap values greater 60% are indicated above the branches, values below the branches are posterior probability values (PP > 0.95). The tree is rooted to *Moniliophthora roreri* (GenBank accession number: AY916746). Scale bar: nucleotide substitutions per site.

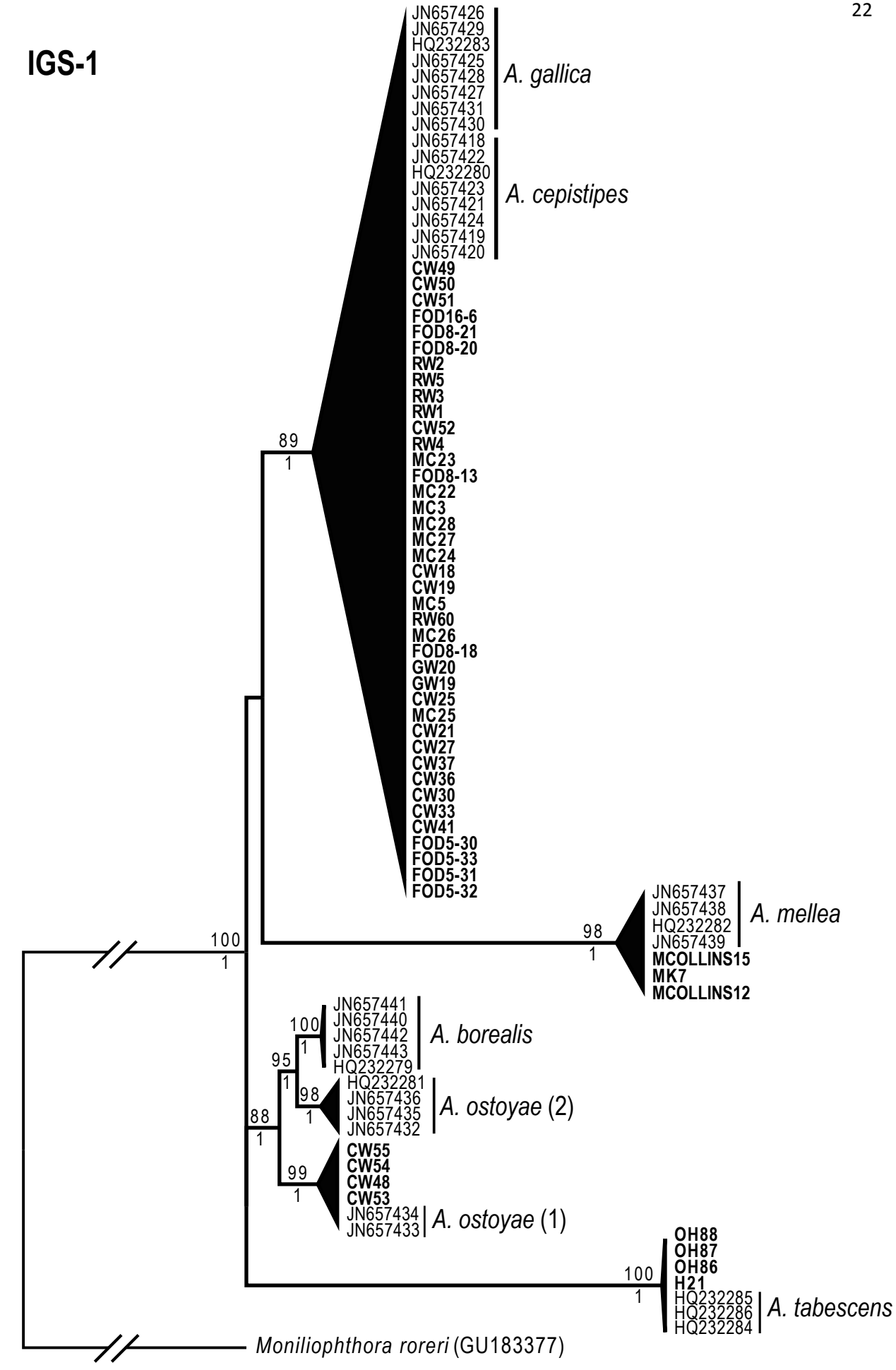

Fig. 4. Phylogenetic tree generated the IGS-1 data matrix using maximum likelihood analysis. Bootstrap values greater 60% are indicated above the branches, values below the branches are posterior probability values (PP > 0.95). The tree is rooted to *Moniliophthora roreri* (GenBank accession number: GU183377). Scale bar: nucleotide substitutions per site.

region grouped these isolates with only one sequence of *A. ostoyae* (JN657459), the remainder of the *A. ostoyae* sequences grouped separate from this cluster (Fig. 3). Trees generated from the IGS-1 data matrix grouped the isolates with two sequences of *A. ostoyae* (JN657434 and JN657433), while the rest of the isolates of *A. ostoyae* from GenBank formed a separate cluster (Fig. 4). The remainder of the isolates grouped with *A. gallica* based on EF-1α sequences (Fig. 2) but with some isolates from Forest of Dean Chestnuts Wood forming a sister group with sequences of *A. gallica* (Fig. 2). However, phylogenetic trees obtained from the ITS and IGS-1 data matrices grouped these isolates with sequences representing *A. gallica* and *A. cepistipes* (Figs. 2-4).

Prevalence and distribution of isolates and species isolated

Armillaria gallica was isolated most frequently (40/51) and was obtained from all the woodland and plantation sites but not from the garden sites (Table 1). It was isolated from superficial rhizomorphs of the single healthy tree (from Runswood), as well as from trees with poor crown condition (Table 1). *Armillaria mellea* (3/51 isolates) was isolated from garden trees only at two of the garden sites, both trees showed moderate crown thinning (Table 1). *A. tabescens* was isolated from an affected tree in a parkland setting (1 isolate) where slight thinning was evident in the crown, but it was also isolated from a garden tree growing in a flower bed at Oakhill, Kent, with a similar crown condition and surrounded by shrubs and herbaceous perennials (3 isolates), totalling 4/51 isolates tested (Table 1). Four isolates of *Armillaria ostoyae* were obtained from only one of four trees sampled at a plantation in the Forest of Dean, and they were isolated together with *A. gallica*; the crown condition of the tree was thinning moderately (but becoming advanced) (Table 1).

Symptoms

There was a lack of consistency in the appearance of stem bleeding, but when it did occur it was fairly distinctive. All four of the 60 year old trees at Chestnut Woods, and one tree each at Grafton Wood and Runswood had stem bleeds, and *A. gallica* was isolated from these trees. The crowns of all of these trees were thinning moderately – severely (Table 1). The stem bleeds were distinctive features. Externally, fluid seeping from each proximal fissure between bark plates along the leading edge of the mycelial front of the affected area was notable (Fig. 5a). The underlying bark was not macerated (as in AOD) but remained intact as sheets of mycelial fans colonised the phloem tissue and killed a large portion of bark up the one side of the tree. This caused the outer bark to become detached from the stem inner bark, and the entire sheet dropped off the side of the tree when touched (Fig. 5b). The mycelial fans on the stems were linked with those on the roots indicating, a soil/root origin (Fig. 5c). At Runswood where a large sheet of bark had fallen off the stem, the tree was barely alive but there were two wet oozing patches on one of the moss covered buttress roots next to the area where the bark fell away. In other *A. gallica* cases where no stem bleeding symptoms were evident, for example at Speculation (FOD), the trees were very mature (150-200 years old) and they were co-infected with *G. fusipes* (syn: *Collybia fusipes* (Bull.) Quél. [Spindle Toughshank]). They had undergone major twig shedding, and the crowns had symptoms of severe dieback to the scaffold limbs, leaving very poorly foliated branches (Fig. 5d). The roots were rotted and inner bark stem tissues had dried out. They were pale in colour and had a spongy-fibrous texture, often with thin black ‘veins’ or zone lines present, which were remnant evidence of host response to invasion by the mycelium of *Armillaria*. At Moorend Common one of the trees was dead with fruitbodies clustered around the stem base as well as up the stem of the tree, and no evidence of stem bleeding

Fig 5a: Fluid seeping from proximal fissures between bark plates along the mycelial front of *Q. robur* infected with *Armillaria*. *Photograph credit: G. Battell.*

Fig 5b: Mycelial fans killing the inner bark up the one side of the tree, outer bark detached from the stem, entire bark sheet drops off when disturbed. Inner bark is not mascerated.

Fig 5c: Mycelial fans on stems link with roots indicating a soil/root origin.

Fig 5d: Crown showing major twig shedding, severe dieback and very poor foliation on *Q. robur* co-infected with *A. gallica* and *G. fusipes.*

Fig 5e: Profuse fluid (blue arrow) produced by *Q. robur* infected with *A. mellea*. Note black zone lines (red arrows).

Fig 5f: Insects attracted to, and feeding on fluid produced by *A. mellea* infected trees.

Fig 5g: Mycelium of *A. mellea* penetrating inner bark in layers. Tree response expressed as dark zone lines (red arrows).

Fig 5h: Crown thinning on oak attacked by *A. mellea*, remaining foliage often appearing in tufts on branch tips.

Fig 5i: Light stem bleeding on *Q. robur* infected by *A. tabescens*.

Fig. 5. Symptoms of oak tree affected by *Armillaria* species.

Fig. 5a. Fluid seeping from proximal fissures between bark plates along the mycelial front of *Q. robur* infected with *Armillaria tabescens*. Photograph credit: G. Battell.

Fig. 5b. Mycelial fans killing the inner bark up the one side of the tree, outer bark detached from the stem, entire bark sheet drops off when disturbed. Inner bark is not macerated.

Fig. 5c. Mycelial fans on stems link with roots indicating a soil/root origin.

Fig. 5d. Crown showing major twig shedding, severe dieback and very poor foliation on *Q. robur* co-infected with *A. gallica* and *G. fusipes*.

Fig. 5e. Profuse fluid (blue arrow) produced by *Q. robur* when infected with *A. mellea*. Note black zone lines (red arrows).

Fig. 5f. Insects attracted to, and feeding on fluid produced by *A. mellea* infected trees.

Fig. 5g. Mycelium of *A. mellea* penetrating inner bark in layers. Tree response expressed as dark zone lines (red arrows).

Fig. 5h. Crown thinning on oak attacked by *A. mellea*, remaining foliage often appearing in tufts on branch tips.

Fig. 5i. Light stem bleeding on *Q. robur* infected by *A. tabescens*.

was apparent. The other tree was co-infected with a single AOD lesion and stem bleed occurring on the stem far removed from the collar region where *Armillaria* attack/colonisation was present with no evidence of stem bleeds. The rhizomorphs were superficial, not penetrating into the live layers of tissue and mycelial fans were not present, but the bark immediately below the rhizomorphs and in the collar region was pale and slightly friable. The crowns of these trees were thin.

By contrast, the trees infected with *A. mellea* displayed profuse stem weeping, which was dark on the tree trunk but clear inside the tree (Fig. 5e). On one tree, the fluid attracted many insects which fed on the sap, which in some places had turned a lighter coffee colour and had a definite alcohol scent, which was caused by yeast colonisation and fermentation of the fluid (Fig. 5f). The inner bark underlying weeping patches was still intact as described above for *A. gallica*; but it was notable that the mycelium appeared to penetrate the inner bark in layers, with the tree having a reaction expressed as dark zones (Fig. 5g). The crowns of the trees were thinning with the remaining foliage often appearing in tufts on branch tips (Fig. 5h). Trees infected with *A. tabescens* occurred in south east England, in Kent and Surrey (near Guildford). Symptoms shown by trees attacked by *A. tabescens* were similar, but not quite as severe, as those attacked by *A. mellea* (Fig. 5i).

Discussion

The aim of identifying isolates of *Armillaria* from native oak in England was achieved using a MLSA approach, which revealed four species colonising the trees. These were *A. gallica*, *A. mellea*, *A. ostoyae*, and *A. tabescens*, all of which were previously reported on oak in the UK

(*A. gallica*, *A. mellea* by Pérrez-Sierra *et al*., 1999; *A. tabescens* by Rishbeth, 1982; *A. ostoyae* and *A. gallica* (as *A. bulbosa*) by Thompson and Boddy, 1983). Results from our study indicated that there are no new *Armillaria* species affecting pedunculate oak in England, but in view of the evolution of identification methods and indeed, the species names, it is worthwhile to document the species of *Armillaria* as they currently occur on oak trees at the sites that were sampled.

The gene trees generated in this study sometimes provided incongruent topologies in terms of grouping sequences of *A. ostoyae, A. borealis, A. gallica* and *A. cepistipes*. The gene trees generated from EF-1α sequences, differentiated isolates of *A. gallica* and *A. cepistipes* into two groups, while gene trees obtained from ITS and IGS-1 sequences grouped isolates representing these species into a single group. *Armillaria gallica* and *A. cepistipes* are phylogenetically closely related species (Tsykun *et al.,* 2012) and are morphologically very similar (Antonin *et al.,* 2009). Previous studies showed that the EF-1α gene provides better variation and resolution than the ITS and IGS-1 regions for species delineation (Antonin *et al.,* 2009; Mullholland *et al.,* 2012; Ross-Davis *et al*., 2012; Tsykun *et al.,* 2013). Therefore, based on the grouping of the isolates from Britain with *A. gallica* in the EF-1α gene phylogeny, they were considered being conspecific in this study. Similarly, isolates from Britain that grouped with sequences of *A. ostoyae* in the EF-1α gene phylogeny were identified as belonging to this species.

In terms of distribution and symptoms present with the different *Armillaria* species, the sample size was too small to be able to carry out statistical testing and make firm conclusions about either, but it is interesting to note the following trends:

(a) In this study *A. mellea* was found at sites in the southerly regions of England, only on trees with profuse stem bleeding and poor crown condition. It has been isolated from areas further north, because Rishbeth (1982) reported it from the Midlands, south east England and east England as far as Norfolk, and the west of England (Gloucestershire); and Beal *et al*., (2015) reported it from infected woody plants (not oak) in the north west (Cumbria and Manchester), Lancashire, Shropshire and Yorkshire. This fungus thus appears to have a wide distribution throughout England and is very likely in other parts of Great Britain as well because it has a wide host range (Rishbeth, 1985). In this study it was isolated only from garden oaks growing in flower beds planted with woody shrubs and herbaceous annuals, and at one site, the owner particularly mentioned that a number of the shrubs in the flower bed had died previously. Similarly, Rishbeth (1982) commented that "*A. mellea* was always present in gardens where more than one kind of broad-leafed tree or shrub had been attacked" (Rishbeth, 1982). He noted the very pathogenic nature of this species (Rishbeth, 1985) but in studies on detecting rhizomorph foci in woodlands, remarked that "it (*A. mellea*) was not found at all" (Rishbeth, 1985). This he attributed to limited growth of rhizomorphs of *A. mellea* (Rishbeth 1982, 1985). Thompson and Boddy (1983) were also unable to isolate *A. mellea* from two UK oak woodland sites (The Forest of Dean and Savernake Forest). They were thoughtful about the reasons for this, listing (1) isolation failure, (2) absence from the trees tested but not necessarily from the site, (3) presence at an earlier stage of root colonisation but displaced by succession organisms, and (4) not present at the sites (Thompson and Boddy, 1983). As *A. mellea* has such a broad host range, is highly pathogenic and has been isolated from woodland species such as *Acer* (maple and sycamore), *Betula* (birch), *Fraxinus* (ash), *Pinus* (pine), *Salix* (willow), *Thuja* and *Tsuga* in the

UK (Rishbeth, 1982; 1985; Perez Sierra *et al*., 1999) it is important to establish whether or not it occurs in oak woodland and plantation environments, and if present, determine its prevalence. The results of the present study together with that of Rishbeth (1982) point to a possible link between *A. mellea* and presence of shrubs and broadleaved trees in gardens. There is a need for a comprehensive review of management practices to avoid the risk of loss to *Armillaria* in general, but in view of the repeated reporting of *A. mellea* from woody shrubs in gardens over most of England and the risk it poses to oak, it seems prudent to be mindful of precautionary best management practice. Aspects of this include avoid disposing of dead or dying shrubs in woodlands.

(b) *Armillaria gallica* was the most commonly isolated species in this study, it occurred on the single healthy tree as well as on those deteriorating visibly, and thus appears to be a prevalent *Armillaria* species on pedunculate oak in England. *A. gallica* was isolated from trees in a variety of environments, especially forests and woodlands. In Britain it has a wide distribution with reports from Scotland, in Dalkeith and Fife (Mulholland *et al*., 2011), as well as throughout England (Perez Sierra *et al*., 1999; Beal *et al*., 2015) where it is particularly prevalent in woodlands (Rishbeth, 1982), but nothing is documented about *Armillaria* species on oak in Wales.

Apparently *A. gallica* can use several different ecological strategies. In this study it was isolated mostly from trees that were declining or nearly dead, and some trees had stem bleeds implying that *A. gallica* could be a primary pathogen'. However, it was also isolated from rhizomorphs attached superficially to the buttress roots of a healthy tree. In other

cases it was co-isolated with known pathogens. For example, the trees at Speculation were declining or almost dead, but were also colonised by *G. fusipes.* *Gymnopus fusipes* is a well-documented aggressive root pathogen of oak (Marçais *et al*., 1999), suggesting that the cause of death of these trees was in large part, due to it. A second example of co-infection was evident at Chestnut Woods where both *A. gallica* and *A. ostoyae*, the latter of which is known as a highly virulent species on conifers (Rishbeth, 1982; 1985; Guillaumin and Legrand, 2013), were isolated from the same necrotic areas on the tree. It is unknown whether other trees on the same site were also infected with *A. ostoyae*. However, co-occupation of host roots, and sharing the same resource raises interesting questions about the pathogenicity and ecological roles and interaction of these species on oak. These two examples thus appear to fit the 'saprogen-opportunistic weak pathogen' model posed for the life style of *A. gallica* (Rishbeth, 1982; 1985; Thompson and Boddy, 1983; Guillaumin and Legrand, 2013), and raises the question of synergistic effects on hosts and polymicrobial necrosis. Rishbeth (1987) stated that "our knowledge of the biology of a forest pathogen is incomplete unless we have some knowledge of its relationships with other microorganisms in the vicinity". A key knowledge gap in the pathogenicity of *Armillaria* species on oak is whether or not they change their relationship with their hosts, and if so, what triggers the apparent change in behaviour, and whether microbial interactions have an effect on behaviours.

(c) *Armillaria tabescens* was isolated from oak with stem bleeds and minor crown thinning, in the south of England. Little is known about the distribution of this species in the UK although it appears to occur in both woodlands (Rishbeth, 1982 and the current study) and

gardens (current study). This study reports *A. tabescens* from southeast England but more recently we have isolated it from oak with severe stem bleeding in a woodland near Thorington, in Suffolk (pers. obs), indicating that it does occur further north. This is interesting as *A. tabescens* has been described as "… a thermophilic species common in southern Europe" preferring warmer Mediterranean climes (Guillaumin and Legrand, 2013). *A. tabescens* is also considered "a saprophyte, mostly colonising dead stumps of Mediterranean oaks, rarely pathogenic in forests, but can cause serious damage in orchards and amenity plantations" (Guillaumin and Legrand, 2013). In this study *A. tabescens* seemed to be causing a serious amount of bark death, but it has also been reported to cause root decay on oak in Britain (Strouts in Rishbeth, 1982; Rishbeth, 1985) making trees susceptible to wind-throw. Therefore in the UK, *A. tabescens* may have the potential to cause significant damage especially as the climate warms, and it may spread from tree to tree in forests and plantations, as Rishbeth (1982) reported that it can spread a considerable distance.

The present study suggests that *A. gallica* is most abundantly associated with native oak in England where it was isolated from trees with symptoms of both AOD and COD as well as from a healthy tree, however a wider sampling is required to confirm this as fact. Since the impacts of Oak Declines on tree survival are of great concern in the UK, where a survey conducted between 1984 and 2005 indicated a marked deterioration of general oak health (Hendry et al., 2005), a better understanding of the main biotic factors that impact the health of oak would help focus research attention, and deliver results that may lead to proactive management to improve oak health and resilience. The role of *Armillaria* in Oak Declines is not yet resolved, and in this study it appeared to have differing modes of colonising oak, but firm evidence of its relationship(s) with other microbes and its oak host

is required to understand its role in Oak Decline diseases. Insights obtained from this study suggest that a far more discerning method of isolating and investigating *Armillaria* infections on oak should be undertaken, as this study has demonstrated that multiple *Armillaria* species, which cannot be visually differentiated in host tissue, can isolated from the same lesion. We have also discovered this phenomenon in other cases (unpublished data). Furthermore, as *A. mellea* and *A. tabescens* are in Britain, and both appear to be highly damaging pathogens of oak, prevention of spread and ingress into forests and woodlands must be a precautionary, preventative management objective until more is known about the risk these species pose to woodlands. More information about their pathogenicity on oak, epidemiology, survival and spread is required, and research must address these knowledge gaps. The pathogenicity of *A. ostoyae* on oak, singly or in combination with other *Armillaria* species, and indeed with other microbes, is yet to be determined. It is clear that future studies investigating the role of *Armillaria* in Oak Decline should focus on *A. gallica*, and the role of this species in the overall health and function of oak in woodlands in Britain, but interaction with other microbes also requires clarification.

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