Ophiostoma quercus: An unusually diverse and globally widespread tree-infecting fungus

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ABSTRACT

Ophiostoma quercus (*Ascomycota*, *Ophiostomatales*) is a globally widespread, insect-vectored fungus that colonizes a wide diversity of hardwood and conifer hosts. Although the fungus is considered to be non-pathogenic, it is closely related to the fungi that cause Dutch elm disease. We examined the global diversity of *O. quercus* based on a ribosomal RNA marker and three unlinked gene regions. The fungus exhibited substantial morphological diversity. In addition, *O. quercus* had high genetic diversity in every continent from which it was collected, although the fungus was most diverse in Eurasia. There was no evidence of geographical clustering of haplotypes based on phylogenetic and network analyses. In addition, the phylogenetic trees generated based on the different markers were non-congruent. These results suggest that *O. quercus* has been repeatedly moved around the globe, because of trade in wood products, and that the fungal species most likely outcrosses regularly. The high genetic diversity of the fungus, as well as its ability to utilize a wide variety of arthropod vectors and colonize a tremendous diversity of tree host species makes *O. quercus* truly unique among ophiostomatoid fungi.

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1. Introduction

Human activities over the past Century have led to a dramatic increase in the world-wide movement of tree-colonizing fungi (Gladieux et al., 2014; Santini et al., 2018; Wingfield et al., 2016). Although most exotic fungi are benign to tree hosts, a small number can lead to tree mortality, and in extreme cases can cause the extirpation of entire tree species (Fisher et al., 2012). Understandably, the most damaging fungal species have been the focus of the majority of research and control efforts. However, less damaging fungi still present a threat to forest health because of their potential to become pathogenic if they encounter naive or susceptible trees (Hulcr and Dunn, 2011; Ploetz et al., 2013). This is particularly concerning with fungal species that have high genetic diversities, and those that readily outcross or hybridize, because these species are more likely to evolve into aggressive tree pathogens (Brasier, 2000).

Ophiostoma quercus (*Ascomycota*, *Ophiostomatales*) is an ophiostomatoid fungus (Wingfield et al., 1993) that is vectored by bark beetles, nitidulid beetles, and mites (Chang et al., 2017; De Beer et al., 2003; Grobbelaar et al., 2009; Harrington et al., 2001; Kamgan Nkuekam et al., 2012). The fungus has been collected from every continent except Antarctica, although the native range of the fungus is still a source of controversy. *O. quercus* was originally hypothesized to have originated in Europe before being introduced to North America and the Southern Hemisphere (Brasier and Kirk, 1993; Harrington et al., 2001). This hypothesis was partially supported by research on mating type frequencies, which indicated that only one mating type is present in North America, while both are present in Europe (Brasier and Kirk, 1993). However, later studies suggested that the fungus may be native to the Southern Hemisphere, as the fungus was isolated from a large number of native tree species in Africa, Oceania and South America (De Beer et al., 2003).

In addition to having a worldwide distribution, *O. quercus* is remarkable because it has exceptionally high morphological and genetic diversity (De Beer et al., 2003; Grobbelaar et al., 2009). It

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has also been isolated from a wide variety of native and non-native species of hardwoods (Grobbelaar et al., 2009) and conifers (Jankowiak et al., 2017; Jankowiak and Bilański, 2013; Paciura et al., 2010). Although there has been some suggestion that the fungus contributed to oak decline in Europe (Harrington et al., 2001), this fungus is largely considered to be saprotrophic and non-pathogenic. This is because it primarily grows in freshly cut woody material visited by bark beetles and other insects, and does not form large lesions when inoculated into live trees (Geldenhuis et al., 2004; Jankowiak, 2013; Luque et al., 2000).

Although *O. quercus* is not considered to be a pathogen, there is notable interest in this fungus because it belongs to the *Ophiostoma ulmi* species complex, and is therefore closely related to the pathogens that cause Dutch elm disease: *O. ulmi*, *Ophiostoma himal-ulmi*, and the two subspecies of *Ophiostoma novo-ulmi* (De Beer and Wingfield, 2013). It has been suggested that *O. ulmi* displaced *O. quercus* as an associate of elm trees and elm bark beetles while *O. ulmi* spread across Europe (Brasier, 1990). During this time, *O. ulmi* may have hybridized with *O. quercus*, after which *O. ulmi* acquired both vegetative compatibility genes and mycoviruses from *O. quercus* (Brasier, 2001).

The potentially negative impacts of hybridization between *O. quercus* and the fungi that cause Dutch elm disease have been demonstrated. In this case, a single *O. quercus* isolate to which the cerato-ulmin gene from *O. novo-ulmi* was experimentally transferred was pathogenic on elm trees (Del Sorbo et al., 2000). The close genetic relatedness between *O. quercus* and the Dutch elm disease fungi, as well as the ability of some fungi in the *O. ulmi* species complex to hybridize with other species in the species complex (Brasier, 2001), underscores the potential threat that *O. quercus* poses to trees worldwide.

We examined the global genetic diversity of *O. quercus*. Attempts to develop usable microsatellite markers for *O. quercus* have been unsuccessful. This is due to the fact that the developed markers cannot be reliably scored because they contain large numbers of indels in the flanking regions (S. J. Taerum, unpublished data). Therefore, we genotyped the isolates using three nuclear markers as well as a fragment of ribosomal RNA (rRNA). We then conducted phylogenetic analyses to confirm that the isolates examined were those of *O. quercus*, and to test for congruence between the different gene trees. Finally, we used the phylogenetic analyses as well as network analyses to test for geographical patterns in *O. quercus*, and to determine if the fungus had an obvious geographic origin.

2. Methods

2.1. Isolates and morphology

Isolates of *O. quercus* were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa. These isolates had been obtained from a wide variety of tree hosts and geographical locations (Table 1). Among these, twenty isolates representing twenty countries on six continents were selected for the study of morphological diversity. The diversity in the fungal species was examined for both culture and microscopic characteristics. For culture characteristics, isolates were grown in the dark at 25 °C for 14 d on malt extract agar (MEA: 2 % Biolab malt extract, 2 % Difco agar) and oat meal agar (OA: filtered liquid of 30 g cooked oatmeal in 800 ml water, 20 g Difco agar, extra water to make it up to 1 L). For microscopic characteristics, isolates were grown on 2 % MEA under near UV light for 20—24 d at room temperature. Fruiting structures were mounted in microscope slides in water, which was later replaced with 85 % lactic acid in which further observation was

made and images captured. Microscopic features were observed using a Nikon Eclipse N*i* compound microscope and a Nikon SMZ18 stereo microscope. Images were captured with a Nikon DS-Ri2 camera mounted on the microscopes.

2.2. Marker amplification and sequencing

DNA was extracted from fungal isolates as described by Duong et al. (2012). For each *O. quercus* isolate, we amplified a rRNA marker (part of the internal transcribed spacer [ITS1-ITS2]) and three unlinked gene regions (beta-tubulin [βt], elongation factor 1-α [EF], and calmodulin [CAL]). The primers ITS1-F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) were used to amplify ITS1-ITS2, B2a and B2b (Glass and Donaldson, 1995) were used to amplify βt, EF2F (Marincowitz et al., 2015) and EF2R (Jacobs et al., 2004) were used to amplify EF, and CL2F and CL2R (Duong et al., 2012) were used to amplify CAL. PCR and sequencing protocols followed the methods of Duong et al. (2012). For the outgroups in the phylogenetic analyses, we also amplified a representative isolate of *Ophiostoma australiae*, *O. himal-ulmi*, *Ophiostoma karelicum*, *O. novo-ulmi*, *Ophiostoma tasmaniense*, *Ophiostoma tsotsi*, and *O. ulmi*. The βt, EF and ITS1-ITS2 sequences for *Ophiostoma borealis*, *Ophiostoma catonianum*, *Ophiostoma denticiliatum*, and *Ophiostoma undulatum* were downloaded from GenBank (accession numbers shown in Fig. 1 and Fig. S1).

2.3. Phylogenetic analyses

The dataset for each marker was aligned using MAFFT v. 7 (Katoh and Standley, 2013). We used jModelTest v. 2.1.7 (Darriba et al., 2012) to determine the best evolutionary models for each gene region for Maximum likelihood (ML) and Bayesian analyses. ML analyses were conducted using raxML v. 8.0.0 (Stamatakis, 2014), with 1000 bootstrap replicates for statistical support. In addition, Bayesian analyses were conducted using MrBayes v. 3.2.6 (Ronquist and Huelsenbeck, 2003), following the protocol described by Taerum et al. (2013).

2.4. Network analyses and genetic diversity

Network analyses were conducted using only the sequences of *O. quercus*. Median joining networks using PopART v. 1.7 (Leigh and Bryant, 2015) were generated on the individual nuclear gene regions (βt, EF, CAL), as well as a concatenated dataset of the three gene regions. To test for geographical patterns within continents, network analyses were also conducted using concatenated datasets that contained only sequences from continents for which data were available for more than ten isolates (Africa, Asia, Europe, and South America). The networks were then visually examined in an attempt to determine the most likely origin of the fungus and to consider its movement history.

Finally, we determined the genetic diversity of *O. quercus* both globally and within each continent, based on the concatenated dataset. Arlequin v. 3.5 (Excoffier et al., 2005) was used to calculate diversity indices (number of haplotypes, expected heterozygosity, mean allelic diversity). The ratios of isolate number examined to observed haplotypes, or multi-locus genotypes (MLG), to determine the level of clonality for each continent.

3. Results

3.1. Isolates

In total, we obtained 92 *O. quercus* isolates for this study. Of these, 28 were from Africa (three from Malawi, 12 from South

Table 1
Collection and host details, as well as GenBank accession numbers, of all isolates included in this study.

Continent	Country	species	host	CMW#	Accession numbers			
					βt	CAL	EF	ITS
Africa	Malawi	*Ophiostoma quercus*	*Eucalyptus grandis*	17139[a]	MH248389	MH248558	MH248623	MH248724
Africa	Malawi	*Ophiostoma quercus*	*Prunus africana*	18228	MH248405	MH248559	MH248629	MH248725
Africa	Malawi	*Ophiostoma quercus*	*Prunus africana*	26206	MH248431	MH248518	MH248651	MH248761
Africa	South Africa	*Ophiostoma quercus*	*Quercus robur*	862	MH248379	MH248478	MH248576	MH248675
Africa	South Africa	*Ophiostoma quercus*	*Quercus robur*	867	MH248380	MH248479	MH248577	MH248676
Africa	South Africa	*Ophiostoma quercus*	*Eucalyptus* sp.	2520	MH248382	MH248480	MH248647	MH248718
Africa	South Africa	*Ophiostoma quercus*	*Eucalyptus* sp.	2521	MH248383	MH248554	MH248578	MH248719
Africa	South Africa	*Ophiostoma quercus*	*Eucalyptus* sp.	3069	MH248384	MH248481	MH248579	MH248720
Africa	South Africa	*Ophiostoma quercus*	*Pinus* sp.	3119	MH248385	MH248482	MH248609	MH248721
Africa	South Africa	*Ophiostoma quercus*	*Rapanea melanophloeos*	14279[a]	MH248438	MH248483	MH248621	MH248723
Africa	South Africa	*Ophiostoma quercus*	*Pinus patula*	19361	MH248457	MH248492	MH248642	MH248702
Africa	South Africa	*Ophiostoma quercus*	*Pinus elliottii*	19365	MH248455	MH248542	MH248603	MH248759
Africa	South Africa	*Ophiostoma quercus*	*Trichocladus crinitus*	40337	MH248461	MH248489	MH248636	MH248730
Africa	South Africa	*Ophiostoma quercus*	*Rapanea melanophloeos*	40338	MH248462	MH248490	MH248637	MH248731
Africa	South Africa	*Ophiostoma quercus*	*Curtisia dentata*	40343	MH248463	MH248491	MH248638	MH248732
Africa	Tanzania	*Ophiostoma quercus*	*Eucalyptus* sp.	17691	MH248464	MH248568	MH248632	MH248755
Africa	Tanzania	*Ophiostoma quercus*	*Eucalyptus* sp.	17694[a]	MH248465	MH248540	MH248633	MH248756
Africa	Tanzania	*Ophiostoma quercus*	*Eucalyptus* sp.	17795	MH248466	MH248569	MH248634	MH248757
Africa	Uganda	*Ophiostoma quercus*	*Acacia mearnsii*	5826	MH248412	MH248499	MH248616	MH248740
Africa	Uganda	*Ophiostoma quercus*	*Acacia mearnsii*	5910	MH248413	MH248501	MH248617	MH248741
Africa	Uganda	*Ophiostoma quercus*	*Acacia mearnsii*	5917	MH248414	MH248500	MH248593	MH248688
Africa	Uganda	*Ophiostoma quercus*	*Acacia mearnsii*	5932	MH248415	MH248502	MH248594	MH248742
Africa	Uganda	*Ophiostoma quercus*	*Acacia mearnsii*	5943	MH248416	MH248503	MH248595	MH248689
Africa	Uganda	*Ophiostoma quercus*	*Acacia mearnsii*	5952[a]	MH248417	MH248504	MH248618	MH248743
Africa	Uganda	*Ophiostoma quercus*	*Acacia mearnsii*	14307	MH248421	MH248556	MH248622	MH248694
Africa	Zimbabwe	*Ophiostoma quercus*	*Eucalyptus grandis*	40060	MH248408	MH248487	MH248605	MH248727
Africa	Zimbabwe	*Ophiostoma quercus*	*Eucalyptus grandis*	40061	MH248391	MH248493	MH248582	MH248728
Africa	Zimbabwe	*Ophiostoma quercus*	*Eucalyptus grandis*	40063	MH248409	MH248488	MH248604	MH248729
Asia	Azerbaijan	*Ophiostoma quercus*	*Castanea sativa*	8283	MH248418	MH248505	MH248624	MH248744
Asia	Azerbaijan	*Ophiostoma quercus*	*Castanea sativa*	8284[a]	MH248419	MH248506	MH248625	MH248745
Asia	Azerbaijan	*Ophiostoma quercus*	*Quercus longipes*	9255	MH248397	MH248511	MH248626	MH248707
Asia	Azerbaijan	*Ophiostoma quercus*	*Castanea sativa*	9259	MH248451	MH248535	MH248596	MH248746
Asia	Azerbaijan	*Ophiostoma quercus*	*Quercus* sp.	9474	MH248448	MH248536	MH248627	MH248747
Asia	Azerbaijan	*Ophiostoma quercus*	*Quercus* sp.	13870	MH248453	MH248513	MH248602	MH248750
Asia	Azerbaijan	*Ophiostoma quercus*	*Quercus longipes*	13872	MH248437	MH248537	MH248628	MH248751
Asia	Bhutan	*Ophiostoma quercus*	*Quercus griffithii*	8221	MH248446	MH248530	MH248661	MH248690
Asia	Bhutan	*Ophiostoma quercus*	*Quercus griffithii*	8527[a]	MH248445	MH248564	MH248665	MH248691
Asia	China	*Ophiostoma quercus*	*Pinus yunnanensis*	11756	MH248447	MH248509	MH248662	MH248733
Asia	China	*Ophiostoma quercus*	*Tsuga* sp.	12122	MH248440	MH248553	MH248663	MH248736
Asia	China	*Ophiostoma quercus*	*Tsuga dumosa*	12287[a]	MH248430	MH248551	MH248666	MH248722
Asia	China	*Ophiostoma quercus*	*Tsuga* sp.	12382	MH248444	MH248528	MH248667	MH248711
Asia	China	*Ophiostoma quercus*	*Pinus kesiya*	41715	MH248468	MH248566	MH248654	MH248766
Asia	China	*Ophiostoma quercus*	*Pinus kesiya*	41732	MH248469	MH248567	MH248660	MH248767
Asia	China	*Ophiostoma quercus*	*Pinus kesiya*	41748[a]	MH248467	MH248525	MH248664	MH248717
Asia	Japan	*Ophiostoma quercus*	*Betula platyphylla*	1564[a]	MH248441	MH248494	MH248589	MH248677
Europe	Austria	*Ophiostoma quercus*	*Fagus sylvatica*	12605	MH248452	MH248552	MH248630	MH248748
Europe	Austria	*Ophiostoma quercus*	*Fagus sylvatica*	12609	MH248401	MH248549	MH248631	MH248749
Europe	Austria	*Ophiostoma quercus*	*Fagus sylvatica*	16561	MH248439	MH248550	MH248583	MH248753
Europe	Austria	*Ophiostoma quercus*	*Fagus sylvatica*	16564	MH248403	MH248538	MH248598	MH248709
Europe	Austria	*Ophiostoma quercus*	*Fagus sylvatica*	16570[a]	MH248404	MH248539	MH248639	MH248697
Europe	Austria	*Ophiostoma quercus*	*Quercus robur*	16573	MH248398	MH248557	MH248640	MH248698
Europe	Austria	*Ophiostoma quercus*	*Carpinus betulus*	16609	MH248449	MH248516	MH248641	MH248754
Europe	Austria	*Ophiostoma quercus*	*Ulmus glabra*	22909	MH248407	MH248517	MH248644	MH248760
Europe	Finland	*Ophiostoma quercus*	*Betula pendula*	23111	MH248429	MH248562	MH248659	MH248716
Europe	France	*Ophiostoma quercus*	*Fagus sylvatica*	2463	MH248393	MH248510	MH248601	MH248736
Europe	France	*Ophiostoma quercus*	*Quercus robur*	2465	MH248424	MH248560	MH248653	MH248734
Europe	France	*Ophiostoma quercus*	*Quercus* sp.	2467	MH248450	MH248523	MH248591	MH248737
Europe	France	*Ophiostoma quercus*	*Quercus petraea*	13245[a]	MH248402	MH248521	MH248620	MH248708
Europe	Norway	*Ophiostoma quercus*	*Populus* sp.	19178	MH248425	MH248565	MH248655	MH248712
Europe	Norway	*Ophiostoma quercus*	*Quercus* sp.	19214	MH248471	MH248526	MH248584	MH248699
Europe	Norway	*Ophiostoma quercus*	*Quercus* sp.	19241	MH248443	MH248563	MH248646	MH248758
Europe	Norway	*Ophiostoma quercus*	*Quercus* sp.	19242	MH248406	MH248547	MH248643	MH248700
Europe	Norway	*Ophiostoma quercus*	*Quercus* sp.	19260	MH248454	MH248541	MH248635	MH248701
Europe	Norway	*Ophiostoma quercus*	*Salix* sp.	19264	MH248426	MH248561	MH248656	MH248713
Europe	Norway	*Ophiostoma quercus*	*Betula* sp.	19272[a]	MH248427	MH248519	MH248657	MH248714
Europe	Norway	*Ophiostoma quercus*	*Betula* sp.	22055	MH248428	MH248520	MH248658	MH248715
Europe	Poland	*Ophiostoma quercus*	*Pinus sylvestris*	40202	MH248410	MH248527	MH248587	MH248710
Europe	Poland	*Ophiostoma quercus*	*Fraxinus exselsior*	40208	MH248411	MH248543	MH248599	MH248762
Europe	Poland	*Ophiostoma quercus*	*Fagus sylvatica*	43227	MH248432	MH248546	MH248588	MH248763
Europe	Poland	*Ophiostoma quercus*	*Abies alba*	43658	MH248456	MH248544	MH248600	MH248764
Europe	UK	*Ophiostoma quercus*	*Quercus* sp.	27847[a]	MH248423	MH248522	MH248586	MH248765
North America	USA	*Ophiostoma quercus*	*Juglans cinerea*	3108	MH248433	MH248524	MH248645	MH248679

(continued on next page)

Table 1 (continued)

Continent	Country	species	host	CMW#	Accession numbers			
					βt	CAL	EF	ITS
North America	USA	Ophiostoma quercus	Juglans cinerea	3109[a]	MH248434	MH248496	MH248606	MH248680
North America	USA	Ophiostoma quercus	Juglans cinerea	3111	MH248435	MH248508	MH248607	MH248681
North America	USA	Ophiostoma quercus	Juglans cinerea	3112	MH248442	MH248497	MH248608	MH248682
Oceana	Australia	Ophiostoma quercus	Eucalyptus globulus	24771	MH248390	MH248486	MH248585	MH248705
Oceana	New Zealand	Ophiostoma quercus	Pinus radiata	24632[a]	MH248458	MH248484	MH248580	MH248726
Oceana	New Zealand	Ophiostoma quercus	Pinus radiata	24638	MH248459	MH248555	MH248652	MH248703
Oceana	New Zealand	Ophiostoma quercus	Pinus radiata	24643	MH248460	MH248485	MH248581	MH248704
South America	Brazil	Ophiostoma quercus	unknown hardwood	2542[a]	MH248381	MH248495	MH248590	MH248678
South America	Brazil	Ophiostoma quercus	unknown hardwood	3120	MH248386	MH248498	MH248592	MH248683
South America	Chile	Ophiostoma quercus	Pinus radiata	9481[a]	MH248387	MH248512	MH248648	MH248692
South America	Chile	Ophiostoma quercus	Eucalyptus nitens	14268[a]	MH248420	MH248507	MH248597	MH248752
South America	Chile	Ophiostoma quercus	Eucalyptus globulus	15858	MH248388	MH248514	MH248649	MH248695
South America	Chile	Ophiostoma quercus	Eucalpytus regnans	15861	MH248422	MH248515	MH248650	MH248696
South America	Colombia	Ophiostoma quercus	Eucalyptus sp.	3122	MH248436	MH248529	MH248610	MH248684
South America	Colombia	Ophiostoma quercus	Quercus humboltii	11278[a]	MH248392	MH248548	MH248619	MH248693
South America	Uruguay	Ophiostoma quercus	Eucalyptus grandis	5625	MH248399	MH248531	MH248611	MH248738
South America	Uruguay	Ophiostoma quercus	Eucalyptus grandis	5627	MH248394	MH248532	MH248612	MH248685
South America	Uruguay	Ophiostoma quercus	Eucalyptus grandis	5629	MH248400	MH248545	MH248613	MH248686
South America	Uruguay	Ophiostoma quercus	Eucalyptus grandis	5631[a]	MH248395	MH248533	MH248614	MH248739
South America	Uruguay	Ophiostoma quercus	Eucalyptus grandis	5633	MH248396	MH248534	MH248615	MH248687
		Ophiostoma australiae		2579	MH248472	MH248572	MH248670	MH248769
		Ophiostoma himal-ulmi		22729	MH248475	−	MH248674	MH248770
		Ophiostoma karelicum		23099	MH248476	MH248575	MH248671	MH248773
		Ophiostoma novo-ulmi		10573	MH248473	MH248573	MH248672	MH248771
		Ophiostoma tasmaniense		1033	MH248470	MH248570	MH248668	MH248735
		Ophiostoma tsotsi		18134	MH248477	MH248571	MH248669	MH248768
		Ophiostoma ulmi		43874	MH248474	MH248574	MH248673	MH248772

[a] Cultures used for photoplates in Fig. 2.

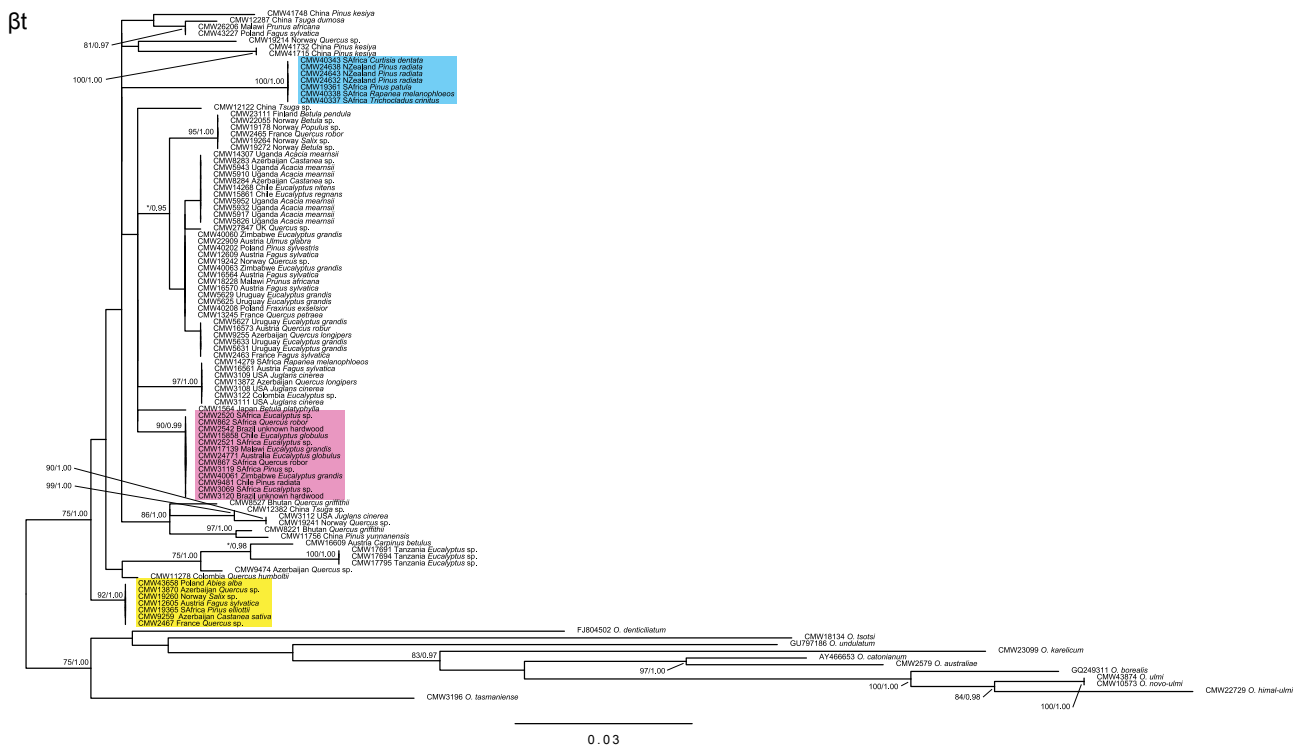**Fig. 1.** Maximum likelihood (ML) phylograms of *O. quercus* isolates based on beta-tubulin (βt), calmodulin (CAL), and elongation factor (EF), with other fungi in the *O. ulmi* species complex as an outgroup. Species names are preceded by their CMW culture collection codes or their GenBank accession number. The numbers next to the nodes represent statistical support, with the first values representing ML bootstrap proportions (if greater than 75), and the second values representing Bayesian posterior probabilities (if greater than 0.9). Three monophyletic clades are highlighted in the βt tree (in blue, yellow, and pink). The isolates within those clades are highlighted with the same colours in the EF and CAL trees. (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

CAL

100/1.00 — CMW12122 China *Tsuga* sp.
CMW24638 NZealand *Pinus radiata*
CMW2521 SAfrica *Eucalyptus* sp.
100/1.00 — CMW16573 Austria *Quercus robur*
CMW18228 Malawi *Prunus africana*
CMW17139 Malawi *Eucalyptus grandis*
CMW14307 Uganda *Acacia mearnsii*
*/0.91 — CMW8221 Bhutan *Quercus griffithii*
CMW3122 Colombia *Eucalyptus* sp.
93/1.00 — CMW9259 Azerbaijan *Castanea sativa*
CMW19365 SAfrica *Pinus elliottii*
CMW19260 Norway *Salix* sp.
CMW43658 Poland *Abies alba*
77/0.92
CMW5625 Uruguay *Eucalyptus grandis*
CMW16570 Austria *Fagus sylvatica*
CMW5627 Uruguay *Eucalyptus grandis*
CMW9474 Azerbaijan *Quercus* sp.
CMW17694 Tanzania *Eucalyptus* sp.
CMW13872 Azerbaijan *Quercus longipers*
89/1.00 — CMW5633 Uruguay *Eucalyptus grandis*
CMW16564 Austria *Fagus sylvatica*
CMW40208 Poland *Fraxinus exselsior*
CMW5631 Uruguay *Eucalyptus grandis*
CMW5629 Uruguay *Eucalyptus grandis*
CMW43227 Poland *Fagus sylvatica*
CMW19178 Norway *Populus* sp.
CMW8527 Bhutan *Quercus griffithii*
CMW17691 Tanzania *Eucalyptus* sp.
CMW17795 Tanzania *Eucalyptus* sp.
100/1.00 — CMW12287 China *Tsuga dumosa*
CMW12605 Austria *Fagus sylvatica*
100/1.00 — CMW12609 Austria *Fagus sylvatica*
CMW11278 Colombia *Quercus humboltii*
CMW16561 Austria *Fagus sylvatica*
CMW41715 China *Pinus kesiya*
CMW41732 China *Pinus kesiya*
*/0.98
CMW19242 Norway *Quercus* sp.
CMW12382 China *Tsuga* sp.
CMW26206 Malawi *Prunus africana*
CMW22909 Austria *Ulmus glabra*
*/0.93 — CMW13870 Azerbaijan *Quercus* sp.
CMW16609 Austria *Carpinus betulus*
CMW15858 Chile *Eucalyptus globulus*
CMW9481 Chile *Pinus radiata*
99/0.99 — CMW15861 Chile *Eucalyptus regnans*
CMW9255 Azerbaijan *Quercus longipers*
CMW2463 France *Fagus sylvatica*
CMW19272 Norway *Betula* sp.
CMW22055 Norway *Betula* sp.
*/1.00 — CMW41748 China *Pinus kesiya*
CMW13245 France *Quercus petraea*
CMW27847 UK *Quercus* sp.
90/1.00 — CMW11756 China *Pinus yunnanensis*
CMW2542 Brazil unknown hardwood
CMW3120 Brazil unknown hardwood
CMW14268 Chile *Eucalyptus nitens*
CMW8284 Azerbaijan *Castanea* sp.
CMW5826 Uganda *Acacia mearnsii*
CMW5917 Uganda *Acacia mearnsii*
CMW5943 Uganda *Acacia mearnsii*
CMW3109 USA *Juglans cinerea*
CMW1564 Japan *Betula platyphylla*
CMW5952 Uganda *Acacia mearnsii*
CMW5932 Uganda *Acacia mearnsii*
85/* — CMW3112 USA *Juglans cinerea*
CMW8283 Azerbaijan *Castanea* sp.
CMW5910 Uganda *Acacia mearnsii*
99/1.00 — CMW3111 USA *Juglans cinerea*
CMW2467 France *Quercus* sp.
97/1.00 — CMW3108 USA *Juglans cinerea*
CMW19241 Norway *Quercus* sp.
CMW2465 France *Quercus robor*
100/1.00 — CMW23111 Finland *Betula pendula*
CMW19264 Norway *Salix* sp.
CMW40202 Poland *Pinus sylvestris*
CMW19214 Norway *Quercus* sp.
100/1.00 — CMW40061 Zimbabwe *Eucalyptus grandis*
CMW2520 SAfrica *Eucalyptus* sp.
CMW867 SAfrica *Quercus robor*
CMW3119 SAfrica *Pinus* sp.
CMW24771 Australia *Eucalyptus globulus*
CMW3069 SAfrica *Eucalyptus* sp.
CMW862 SAfrica *Quercus robor*
CMW14279 SAfrica *Rapanea melanophloeos*
CMW40063 Zimbabwe *Eucalyptus grandis*
CMW24643 NZealand *Pinus radiata*
*/1.00 — CMW40338 SAfrica *Rapanea melanophloeos*
CMW40337_390 SAfrica *Trichocladus crinitus*
CMW40343 SAfrica *Curtisia dentata*
CMW24632 NZealand *Pinus radiata*
CMW40060 Zimbabwe *Eucalyptus grandis*
CMW19361 SAfrica *Pinus patula*

100/1.00 — CMW23099 *O. karelicum*
100/1.00 — CMW2579 *O. australiae*
CMW10573 *O. novo-ulmi*
CMW43874 *O. ulmi*
*/0.98
97/1.00 — CMW18134 *O. tsotsi*
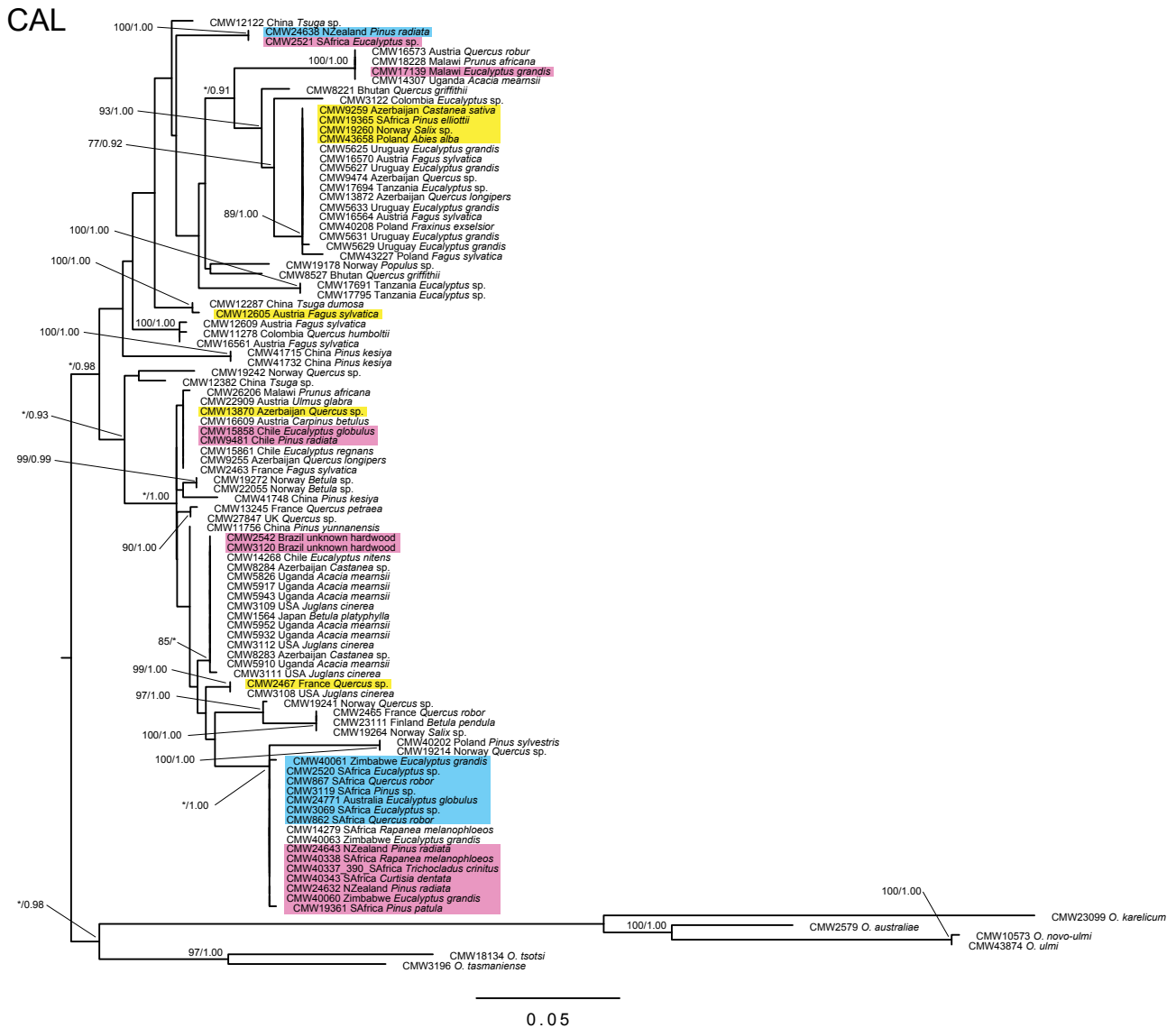CMW3196 *O. tasmaniense*

0.05

Fig. 1. (*continued*).

Africa, three from Tanzania, seven from Uganda, and three from Zimbabwe), 17 from Asia (seven from Azerbaijan, two from Bhutan, seven from China, and one from Japan), 26 from Europe (eight from Austria, one from Finland, four from France, eight from Norway, four from Poland, and one from the UK), four from North America (all from the USA), four from Oceania (one from Australia, and three from New Zealand), and 13 from South America (two from Brazil, four from Chile, two from Colombia, and five from Uruguay). The host species and collection details are summarized in Table 1.

3.2. Morphology

The twenty isolates selected for the examination of fungal morphology showed a broad diversity in both culture and microscopic characteristics. Cultures reached the edge of 60 mm petri dish in 10–14 d, showing circular growth with smooth margins. Mycelia varied from being submerged to abundantly aerial in velvety to cottony textures (Fig. 2 A–H). Some cultures showed sectoring (different form of growth in sectors), concentrically zonating or with scattered pigmented patches. Colour of cultures was diverse from showing no visible colour or white to different shades of brown. Cultures produced both sexual and asexual states. The sexual state was observed in a single isolate from Uruguay (CMW 5631; Fig. 3 A,B). Asexual states had two distinct morphs, which could be distinguished by conidiophores: sporothrix-like (mononematous; Fig. 3 K) and pesotum-like (synnematous; Fig. 3 CeH). Most of the isolates produced both of these asexual morphs, but a few isolates produced sporothrix-like conidiophores only.

In the pesotum-like asexual state, synnematous conidiophores were composed of a few to several individual stipes that were tightly packed. These were produced individually or in tufts, were gregarious, and were lightly or darkly pigmented throughout their length and paler towards the apex. The number of conidiophores produced in cultures varied from none to several (Fig. 2 I–P). Conidiogenous apparatuses were unbranched or mildly branched (Fig. 3 C,D,I), or branched extensively in many tiers (Fig. 3 E–H,J), which gave different appearances of conidiophores from tuft-like to brush-like. Conidia were ellipsoidal to oblong with a pointed base and their shapes were uniform in individual isolates (Fig. 3 L,M). In the sporothrix-like asexual state, conidiogenous cells were blastic
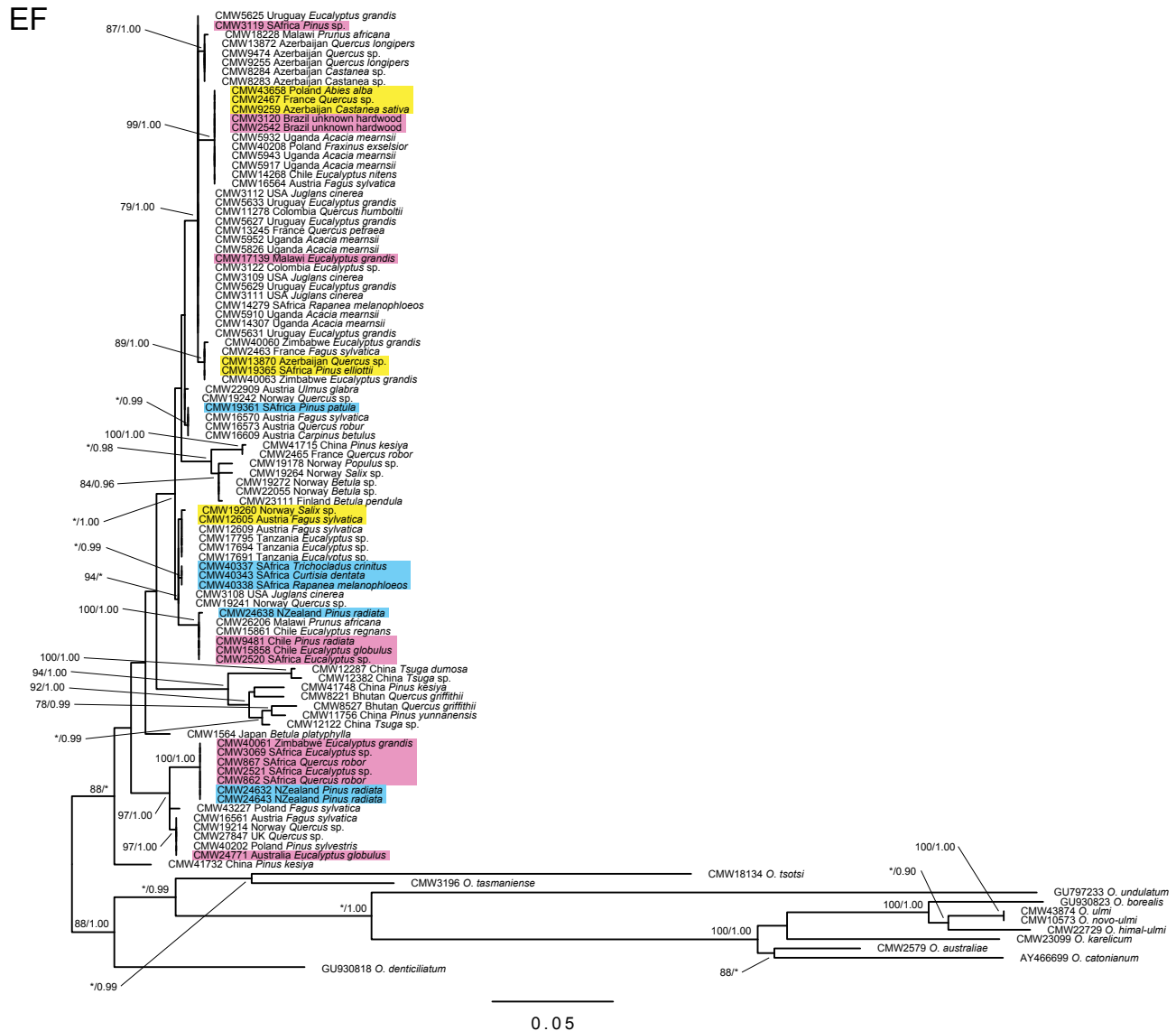
Fig. 1. (continued).

and prominently denticulate. Conidia were ellipsoidal to cylindrical, often with both ends pointed, and their shape and dimensions varied in individual isolates (Fig. 3 O).

3.3. Phylogenetic analyses

The ITS1-ITS2 alignment had 622 bases (71 were variable), while the alignments of the βt, CAL, and EF gene regions had 452 bases (144 variable), 545 bases (197 variable), and 927 bases (392 variable) respectively. The general time reversible (GTR) + gamma model was the best supported model for all datasets.

The *O. quercus* isolates did not form a monophyletic clade in the ITS1-ITS2 tree (Fig. S1), as several other species (*O. australiae*, *O. borealis*, *O. catonianum*, *O. himal-ulmi*, *O. tasmaniense*, *O. tsotsi*, and *O. undulatum*) were present within the clade that contained all of the *O. quercus* strains. However, most nodes in the ITS1-ITS2 tree did not have strong statistical support (>75 % ML, >0.9 Bayesian), suggesting that the marker had insufficient resolution to determine phylogenetic patterns. This observation is consistent with previous

studies of *O. quercus* (Grobbelaar et al., 2009). For this reason, ITS1-ITS2 was not considered in the remaining analyses.

In the βt, EF and CAL trees (Fig. 1), the *O. quercus* isolates formed a monophyletic clade, while the other species from the *O. ulmi* species complex formed a clear outgroup. The phylogenies based on these gene regions were incongruent based on the topologies of the three trees. In addition, the isolates did not form monophyletic clades based on isolate origin or host taxonomy in any of the trees.

3.4. Network analyses

The concatenated alignment had 1753 bases (303 were variable). In the global networks, there were no clear geographical patterns in the arrangement of the isolates (Fig. 4, Fig. S2). A lack of clear geographical structure was also observed in the continent-specific networks, except for South America, where isolates clustered according to the countries from which they were sampled (Fig. S3). There were only two haplotypes from the combined
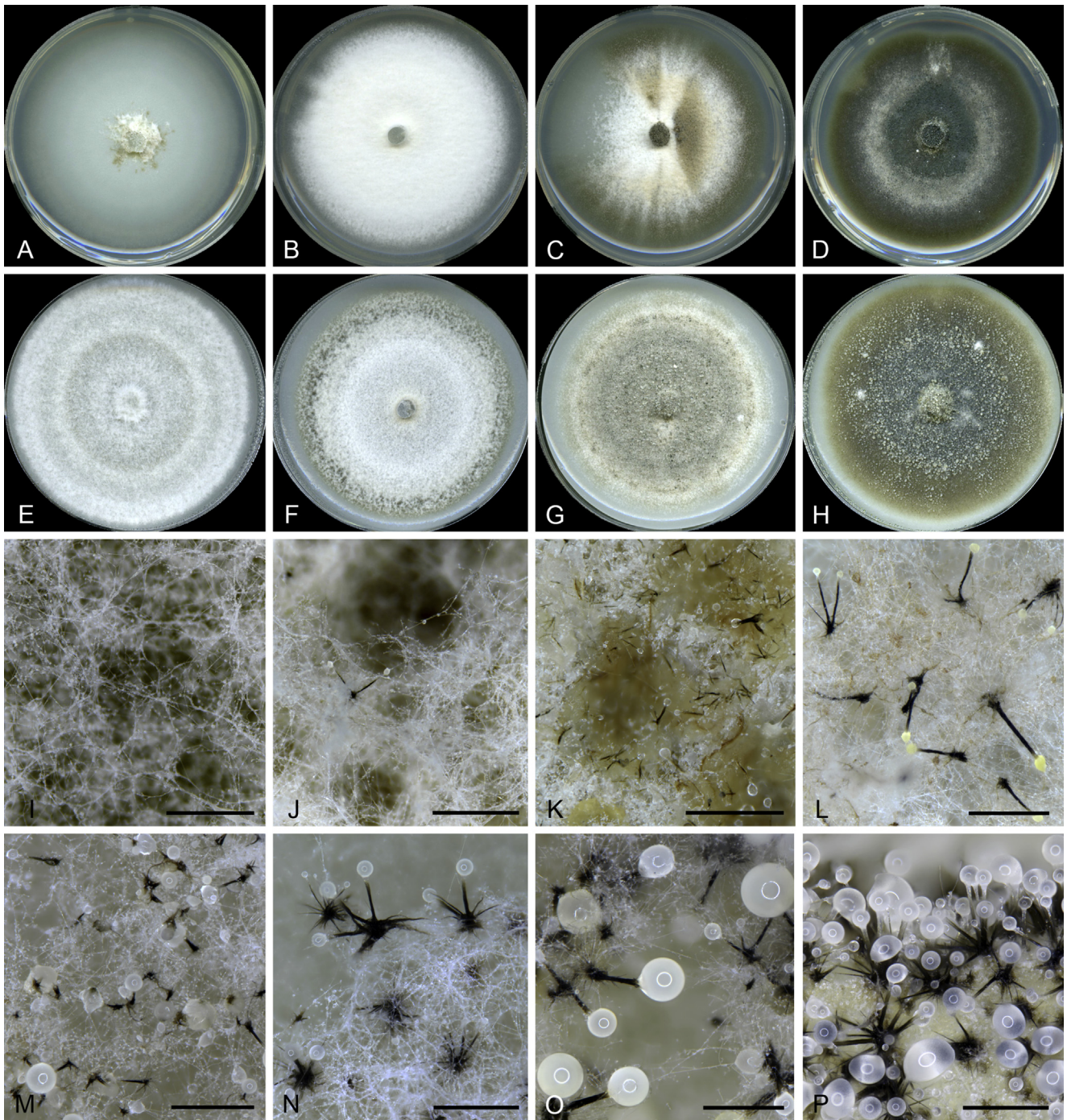
Fig. 2. Culture characteristics of *Ophiostoma quercus*. (A–H). Cultures grown in the dark for 14 d at 25 °C. (A–D). on 2 % MEA. (E–H). on oat agar. (I–P). Close-up of the cultures on 2 % malt extract agar grown under near UV light for 20–24 d at room temperature. Culture codes and countries of origin for each culture photographed are listed in Table S1. Scale bars: J–P = 500 μm.

dataset that were present from multiple continents. In this case, one haplotype was present in both Africa and South America, and another was present in both Asia and Europe (Fig. 4).

3.5. Genetic diversity

In total, 73 haplotypes were observed within the concatenated dataset (Fig. 4; Table 2). The greatest number of haplotypes observed was in isolates from Europe, followed by Africa, Asia, South America, North America, and Oceania. Genetic diversity was highest in Asia and lowest in North America based on expected heterozygosity, numbers of polymorphic loci, and mean allelic diversity. Isolates from each continent had a low level of clonality, with Africa having the highest, and North America having the lowest level.

4. Discussion

No clear geographical pattern was evident based on the global genetic diversity of *O. quercus*. There was also no evidence of
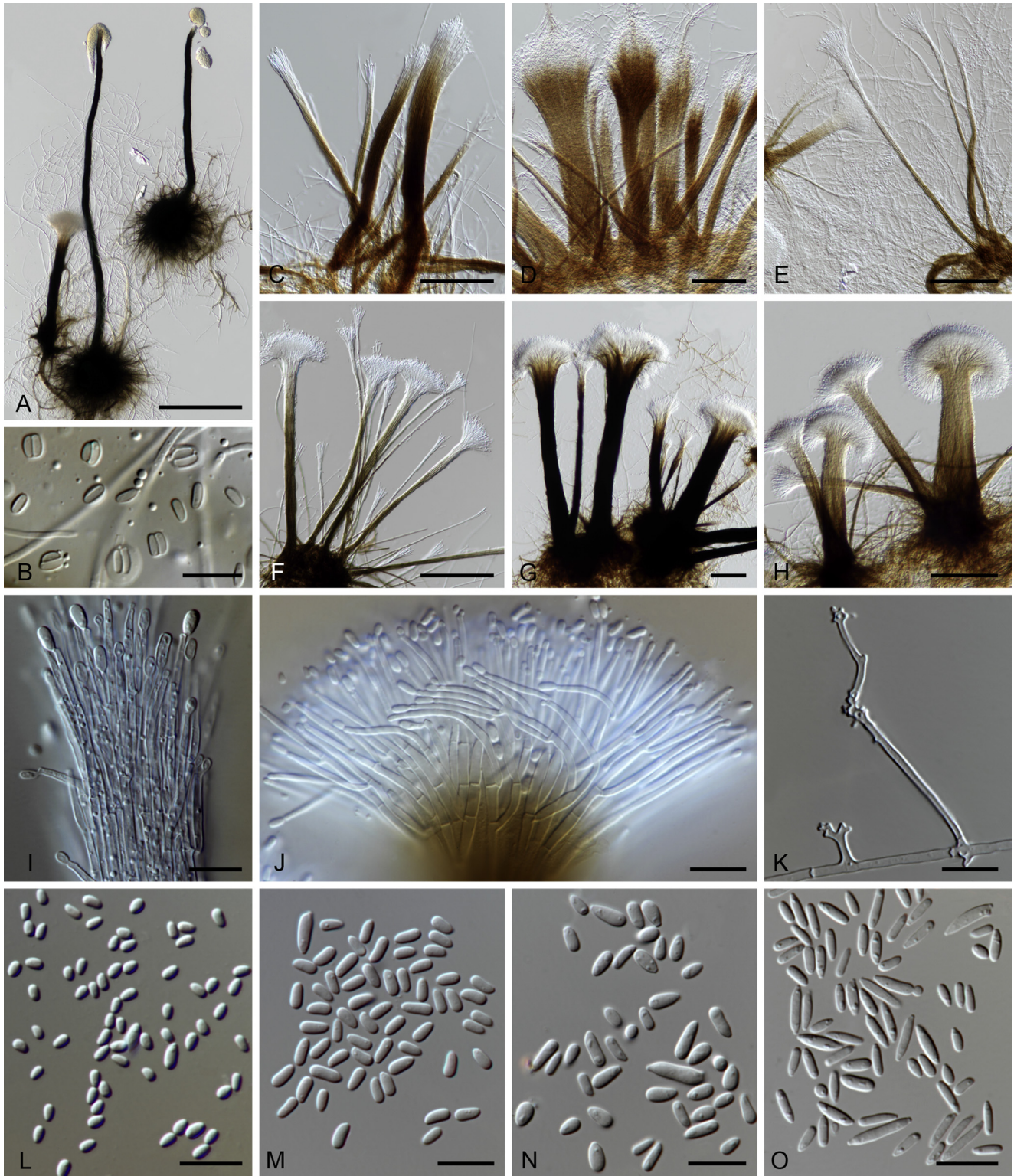
Fig. 3. Micrographs of *Ophiostoma quercus*. (A). Ascomata with a long ostiolar neck with slimy droplets of ascospores at the tip of the neck. (B). Ascospores. (C–H). Synnematous conidiophores of pesotum-like asexual state. (I, J). Conidiogenous apparatus and conidiogenous cells of pesotum-like asexual state. (K). Conidiophores and conidiogenous cells of sporothrix-like asexual state. (L). (M). Conidia of pesotum-like asexual state. (N). A mixture of conidia of two asexual states. (O). Conidia of sporothrix-like asexual state. Culture codes and countries of origin for each culture photographed are listed in Table S1. Scale bars: A = 250 μm; C–H = 100 μm; B, I–O = 10 μm.

geographical structure for any continent except South America. Although the fungus is found globally, most likely due to human activities, we were unable to unequivocally discern continents where the fungus might have originated. Overall, these patterns, combined with the high genetic diversities observed for each continent, suggest that introductions of *O. quercus* occur globally, regularly, and potentially with large numbers of propagules. It is even possible that new genotypes of *O. quercus* are emerging in the
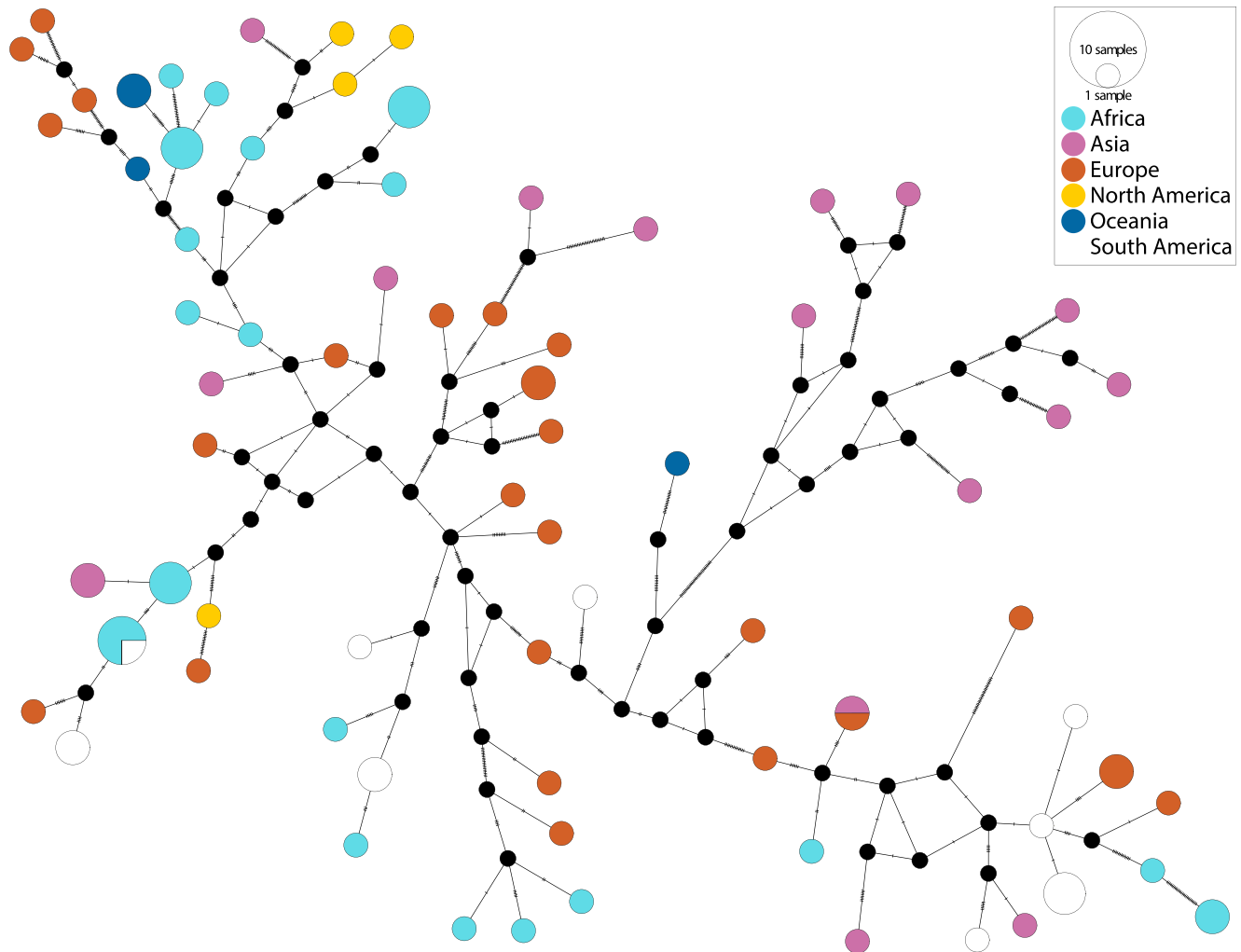
Fig. 4. Median-joining network of *O. quercus* isolates collected globally, based on the concatenated (βt, EF, and CAL) dataset. Circle sizes are proportional to the number of isolates that share a haplotype. Colours represent continent of origin (see legend). (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Genetic diversities of *O. quercus* isolates within each continent.

Continent	N	MLG	N:MLG	He (s.d.)	# polymorphic loci[A]	Ā (s.d.)
Africa	28	19	1.474	0.03077 (0.09812)	195	1.124 (0.368)
Asia	17	16	1.063	0.04485 (0.12404)	277	1.180 (0.443)
Europe	26	24	1.083	0.03300 (0.10728)	211	1.140 (0.406)
North America	4	4	1.000	0.00884 (0.06592)	31	1.018 (0.132)
Oceana	4	3	1.333	0.03832 (0.13645)	130	1.076 (0.271)
South America	13	9	1.444	0.02129 (0.09411)	99	1.059 (0.245)
Worldwide	92	73	1.260	0.03554 (0.09917)	385	1.273 (0.569)

N — number of isolates, MLG — number of multilocus genotypes, He — expected heterozygosity, Ā — mean allelic diversity (across all loci), s.d. — standard deviation. [A] measured out of 1753 total nucleotides.

exotic ranges of the fungus, and are being introduced back to the source continent(s). This may have contributed to the ambiguity in the data regarding the movement of the fungus.

O. quercus was most diverse in Asia and Europe based on most of the diversity metrics. This appears to support the hypothesis that the fungus originated in Europe (Brasier and Kirk, 1993; Harrington et al., 2001), or more broadly Eurasia, as species are frequently most diverse in their native ranges (Lee, 2002; Sakai et al., 2001). It should, however, be noted that genetic diversities of exotic populations can be high relative to native populations. Such situations emerge where the exotic populations were

established from multiple introduction events from multiple locations within the native range of a species (Roman and Darling, 2007). In addition, the genetic diversities of *O. quercus* in Africa, North America, Oceania, and South America were high, even if they were lower than those observed for Europe and Asia. Observed diversities may have also been impacted by sample size effects, as considerably fewer isolates were available for some continents. Host diversity could also have influenced the observed fungal diversities, because cultures were isolated from at least seven host species in Asia and 12 in Europe, compared with one in North America and two in Oceania.

Some support for the hypothesis that *O. quercus* originated in Eurasia arises from the fact that Asia may be the origin of the fungi that cause Dutch elm disease (Brasier, 1990). In addition, several of the other species in the *O. ulmi* species complex are commonly found in Asia (*O. tsotsi*, Chang et al., 2017), Europe (e.g., *O. borealis*, Kamgan Nkuekam et al., 2010; *O. catonianum*, Harrington et al., 2001; *O. karelicum*, Linnakoski et al., 2008), or both Europe and Asia (*O. denticiliatum*, Huang and Chen, 2013; Linnakoski et al., 2009). This is consistent with what has been observed with other species complexes in the *Ophiostomatales*, as species within a given species complex tend to originate from the same continent. For example, Asia appears to be the centre of diversity for the *Leptographium procerum* species complex (Linnakoski et al., 2012; Yin et al., 2015). It should be noted that some species have been found only in Oceania. Examples include *O. australiae* (Kamgan Nkuekam et al., 2008), as well as *O. tasmaniense* and *O. undulatum* (Kamgan Nkuekam et al., 2011), although this continent is geographically close to Eurasia.

Interestingly, there was very low clonality observed for isolates on any continent. Exotic fungi frequently undergo rapid expansion via clonal propagation after they are introduced to a new location (Gladieux et al., 2014). However, even for the continents where *O. quercus* was most clonal (Africa and South America), the ratio of isolates to MLG was low (<2.000, where each MLG would have two representatives on average). The low levels of clonality, the high levels of genetic diversity, and the observed incongruence between the genetic markers suggests that the fungus evolves rapidly due to frequent out-crossing. This could explain why *O. quercus* is able to colonize an unusually wide diversity of host tree species, including angiosperms and gymnosperms.

Another unexpected observation was the production of perithecia by one of the cultures (CMW 5631). *O. quercus* is a heterothallic fungus (Wilken et al., 2012), and typically requires interactions between cultures with different mating types (MAT1-1 and MAT1-2) for perithecia production. The production of sexual spores by the culture suggests that some isolates of *O. quercus* are homothallic, or may be able to undergo unisexual reproduction (Wilson et al., 2015). Further studies are needed to test these hypotheses, and to determine how frequently *O. quercus* isolates produce sexual spores without interacting with an isolate having the opposite mating type.

The exceptionally high genetic and morphological diversity in *O. quercus* observed in this study is unusual for the tree-associated and insect-dispersed fungi in the *Ophiostomatales*. Most species in this Order have little or no population-level variation in the ITS, βt, EF and CAL markers. In some cases individual single nucleotide polymorphisms (SNPs) are sufficient to differentiate between species (e.g., the *Grosmannia serpens*, *L. procerum*, and *Sporothrix schenckii* species complexes; de Meyer et al., 2008; Duong et al., 2012; Yin et al., 2015). In addition, culture and microscopic characteristics are typically consistent within ophiostomatoid species (De Beer and Wingfield, 2013). However, *O. tsotsi*, which is also in the *O. ulmi* species complex, is similar to *O. quercus* in that it is highly diverse based on the few isolates that have been studied (Grobbelaar et al., 2010), and capable of colonizing both conifers and hardwoods (Chang et al., 2017; Grobbelaar et al., 2010). *Ophiostoma piceae*, which is closely related to *O. quercus* but not in the *O. ulmi* species complex, is another fungus that has exceptionally high genetic diversity (De Beer et al., 2003; Gagné et al., 2001; Harrington et al., 2001), although it has been isolated only from conifers. Interestingly, all three fungal species have substantial morphological variation, are widely (in the case of *O. quercus* and *O. piceae* globally) distributed, and can infect a wide diversity of host tree species (De Beer et al., 2003; Grobbelaar et al., 2010; Przybyl and De Hoog, 1989), suggesting that similar factors may

have influenced their distribution and evolution. Alternatively, *O. quercus* could have originated as a number of genetically-distinct but closely-related species that hybridized after being brought into contact with each other due to human activities.

Although the global distribution of *O. quercus* may be fairly unique for the *Ophiostomatales*, other plant-associated fungi have similar global distributions with no known source populations. For example, some plant pathogens in the *Botryosphaeriales*, such as *Botryosphaeria dothidea* (Marsberg et al., 2017), *Lasiodiplodia theobromae* (Mehl et al., 2017; Santos et al., 2017), and *Neofusicoccum parvum* (Sakalidis et al., 2013), are globally widespread, but it is unknown where the fungi are native or aliens. Both *O. quercus* and the globally distributed fungi in the *Botryosphaeriales* have limitations as to how far their propagules can spread. *B. dothidea*, *L. theobromae*, and *N. parvum* are spread between hosts via wind and rain. In contrast, *O. quercus* typically requires arthropod vectors for distribution. Although the mechanisms of dispersal might differ, these fungi all have global ranges, highlighting the impact of human activities on their current geographic distributions.

This study represents the most thorough examination of the global diversity of *O. quercus*. Although we were unable to conclusively determine where the fungus might have originated, it was possible to confirm that it is hyper-diverse wherever it is found. More thorough sampling of *O. quercus* from different continents, the application of additional markers (e.g., SNPs), and the use of recently developed scenario testing software would improve our understanding of the global diversity of *O. quercus*. Such data would also make it possible to more clearly understand the pathways and history of movement of the fungus.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.funbio.2018.05.005.

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