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ORIGINAL ARTICLE

Delimitation of *Ophiostoma quercus* and its synonyms using multiple gene phylogenies

Joha W. Grobbelaar · Dilzara N. Aghayeva · Z. Wilhelm de Beer · Paulette Bloomer · Michael J. Wingfield · Brenda D. Wingfield

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Abstract *Ophiostoma quercus* is a morphologically variable species that causes sapstain on mostly hardwood hosts worldwide. Several species have been suggested as synonyms of *O. quercus* in the past, including *O. fagi, O. roboris*, *O. valachicum*, *O. kubanicum,* and *O. catonianum.* A recent collection of isolates resembling *O. quercus* from Azerbaijan provided the opportunity to reconsider the accuracy of these synonymies based on morphology and DNA sequence data. Four gene regions, the ribosomal internal transcribed spacer regions including the 5.8 s gene, part of the β-tubulin gene, translation elongation factor-1α, and histone gene, were used to determine the phylogenetic relationships between the various species and isolates of different origin. In all four resulting phylogenetic trees, isolates of *O. quercus*, *O. fagi*, and *O. roboris* formed a single, well-supported cluster, but with some internal variation. All the other species in the analyses, including *O. piceae* and *O. catonianum,* grouped distinctly with good node support. These results thus support the synonymy of *O. fagi* and *O. roboris* with *O. quercus*, and confirm that *O. piceae* and *O. catonianum* are distinct taxa. *Ophiostoma valachicum* and *O. kubanicum* could not be considered due to the absence of cultures, but based on published descriptions, we argue that *O. valachicum* should be regarded as a valid species in need of neotypification. *Ophiostoma kubanicum* was never validly described and should be excluded from the list of synonyms of *O. quercus*.

Taxonomic novelties *Pesotum roboris* (Georgescu, Teodoru and Badea) Grobbelaar, Z.W. de Beer and M.J. Wingf.; *Sporothrix roboris* (Georgescu, Teodoru and Badea) Grobbelaar, Z.W. de Beer and M.J. Wingf.

J. W. Grobbelaar · P. Bloomer · M. J. Wingfield · B. D. Wingfield (✉)
Department of Genetics, University of Pretoria,
Pretoria 0002, South Africa
e-mail: brenda.wingfield@fabi.up.ac.za

D. N. Aghayeva
Institute of Botany, ANAS,
Patamdar sh. 40,
Baku 370073, Republic of Azerbaijan

Z. W. de Beer
Department of Microbiology and Plant Pathology,
Forestry and Agricultural Biotechnology Institute (FABI),
University of Pretoria,
Pretoria 0002, South Africa

Introduction

The sapwood-staining fungi *Ophiostoma piceae* and *O. quercus* have had a confused taxonomic history. The former species was described in 1907 (Münch 1907) from sapstained pine and spruce in Germany while *O. quercus* was described in 1926 from oak in the former Yugoslavia (Georgevitch 1926, 1927) and has been implicated as a causal agent of oak decline in Central Europe (Cech et al. 1990). Hunt (1956) treated these species as synonyms and this remained the case for more than 30 years. However, a suite of studies including Morelet (1992), Przybyl and Morelet (1993), Brasier and Stephens (1993), Brasier and Kirk (1993), Halmschlager et al. (1994), Pipe et al. (1995) and Harrington et al. (2001) have treated these fungi in depth and they are now widely accepted to represent two distinct taxa. Most recently, DNA sequence analyses (Harrington et al. 2001) have confirmed that *O. piceae* is a predominantly conifer-infesting fungus, while *O. quercus* is mostly isolated from a variety of hardwood hosts.

A number of recent studies have shown that *O. quercus* is much more widely distributed on woody substrates than

previously recognized, with reports of the fungus coming from countries such as Australia (Harrington et al. 2001), Brazil and Japan (de Beer et al. 2003a), Canada (Brasier and Kirk 1993; Pipe et al. 1995; Kim et al. 1999), Chile (Zhou et al. 2004), Ecuador (Geldenhuis et al. 2004), Korea (Harrington et al. 2001), New Zealand (Thwaites et al. 2005), South Africa (de Beer et al. 2003a; Zhou et al. 2006; Kamgan Nkuekam et al. 2008a), Uganda (Kamgan Nkuekam et al. 2008b), Uruguay (Harrington et al. 2001), and the USA (Kim et al. 1999). These reports of the fungus have been from a large variety of non-native and native hardwood trees, and in some cases non-native plantation pines (de Beer et al. 2003a; Zhou et al. 2004, 2006; Thwaites et al. 2005). In terms of biology, the fungus appears to be a casual associate of many different bark beetles and other tree-wound visiting insect species (Kirisits 2004; Zhou et al. 2004, 2006; Romón et al. 2007; Kamgan Nkuekam et al. 2008a, b; Linnakoski et al. 2008).

During the period that *O. quercus* was treated as a synonym of *O. piceae*, several similar hardwood-infesting species were also listed as synonyms of *O. piceae*. These included *O. catonianum* (de Hoog 1974), *O. roboris* (de Hoog 1979), *O. fagi* (Hutchison and Reid 1988), *O. valachicum,* and *O. kubanicum* (Przybyl and de Hoog 1989). Considering the almost exclusive association of *O. piceae* with conifers, all of these species, other than *O. catonianum,* have been suggested to be synonyms of *O. quercus* (Brasier 1993; Brasier and Kirk 1989, 1993; Harrington et al. 2001). Harrington et al. (2001) included the ex-type isolate of *O. fagi* and an authentic isolate of *O. catonianum* (deposited at CBS by Goidánich) in their study. Based on nuclear ribosomal internal transcribed spacer (ITS) region sequences and mating compatibility, *O. fagi* was suggested as a synonym of *O. quercus,* but *O. catonianum* was shown to be a distinct taxon (Harrington et al. 2001). No cultures representing *O. valachicum*, *O. kubanicum*, and *O. roboris* were available for the study by Harrington et al. (2001). They thus listed these three species as questionable synonyms of *O. quercus*, and the status of these species remains unresolved today.

In view of the increasing number of reports of *O. quercus* from many countries of the world, it has become necessary to delimit the species' boundaries for this fungus. A collection of isolates resembling *O. quercus* and *O. roboris* from oak and chestnut in Azerbaijan prompted reconsideration of the synonyms of *O. quercus,* including the possible need to neo-typify *O. roboris*. Several recent studies of the genus *Ophiostoma* have shown that ITS sequences do not always distinguish between closely related fungal species, but that protein coding genes produce better resolution at the species level (Kim and Breuil 2001; Schroeder et al. 2001; Lim et al. 2004; de Meyer et al. 2008; Roets et al. 2008). The aim of this study was thus to identify the isolates tentatively treated as *O. quercus* from Azerbaijan, but also to reconsider the species' boundaries for taxa in the *O. piceae–O. quercus* complex using micromorphology together with phylogenetic analyses of DNA sequences derived from four nuclear gene regions.

Materials and methods

Isolates and herbarium specimens

Forty isolates were used for DNA sequence comparisons in this study (Table 1). These included 11 isolates resembling *O. quercus* and *O. roboris* from Azerbaijan. Isolates previously identified as *O. quercus* were also used, and these included five from France together with an ex-neotype culture of *O. quercus* (Morelet 1992), one from the UK (Brasier and Kirk 1993; Brasier and Stephens 1993; Pipe et al. 1995; Harrington et al. 2001), and three from South Africa (de Beer et al. 2003a). A single isolate from Azerbaijan, previously referred to as *O. roboris* (Guseinov 1984; Brasier and Kirk 1989) and subsequently treated as *O. quercus* (Webber and Brasier 1991; Brasier and Kirk 1993; Brasier and Stephens 1993; Pipe et al. 1995), was made available to us for inclusion in the study by Dr Clive Brasier (Forestry Commission Research Agency, Alice Holt Lodge, Farnham, Surrey, UK). The ex-type isolate of *O. fagi* and an authentic isolate of *O. catonianum* collected by Goidánich (1935) were obtained from the CBS. Our efforts to obtain type material for *O. roboris*, *O. kubanicum*, and *O. valachicum* were unsuccessful. However, with the assistance of Dr Vadim Mel'nik (Komarov Botanical Institut, St. Petersburg, Russia) we were able to obtain three dried culture herbarium specimens (Table 1) of *O. roboris* isolates used in inoculation tests by Potlajczuk (1957). The herbarium specimens of *O. roboris* consisted of three dried cultures on agar discs, one of which was labeled as *O. roboris*. The remaining two isolates had been obtained from re-isolations from *Acer* and *Betula* inoculated with the first isolate. A culture labeled as *O. kubanicum* (VKM-F 3181), received from the All Russian Collection of Micro-organisms in Moscow, was found to be contaminated with a *Fusarium* species and could not be salvaged. All attempts to obtain material of *O. valachicum* were unsuccessful, consistent with the experience of Przybyl and de Hoog (1989).

For phylogenetic comparisons, sequences of the above-mentioned isolates were compared with those of other species in the larger *O. piceae* complex (Harrington et al. 2001). These included *O. piceae, O. himal-ulmi, O. ulmi, O. novo-ulmi, O. floccosum, O. setosum*, and *Pesotum australiae.*

All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural

Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. In addition, representative isolates have been deposited at the CBS. Dried cultures of the isolates that produced perithecia (CMW 9256, CMW 9262) have been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM 57852, PREM 57851).

Morphology

For morphological comparisons, isolates from Azerbaijan and those of *O. quercus*, *O. catonianum*, *O. fagi*, and the '*O. roboris*' isolate of Guseinov (Table 1) were grown on MEA [20 g (Biolab)], 1,000 ml deionised water) and WA (20 g agar, 1,000 ml deionised water) amended with debarked oak twigs (40×5 mm). Fifty measurements were made for each taxonomically informative structure of the isolates that produced perithecia. Anamorphs of all isolates were studied, with 25 measurements made for each characteristic structure per isolate. Three-day-old slide cultures (Riddell 1950) of the mononematous anamorph structures were made and fixed in lactophenol for microscopy.

Scanning electron microscopy (SEM) was used to observe the anamorph structures. Small agar blocks were cut from the sporulating colonies with a sterilized scalpel and fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH=7.4). After three buffer rinses, the specimens were post-fixed in 1.0% aqueous osmium tetroxide for 30 min, dehydrated in a graded ethanol series starting at 30%, and dried with a Polaron Critical Point Drier. Specimens were coated with gold and examined using a JEOL JSM 6400 scanning electron microscope.

Growth studies

Growth of the same set of isolates used for morphological studies was compared on 2% MEA. Discs, 5 mm in diameter, were taken from the actively growing edge of colonies and placed at the centers of 90-mm Petri dishes. Three replicates were made for each isolate and these were incubated at temperatures ranging from 5 to 35°C, at 5°C intervals, for 10 days in the dark. Isolates were also grown at 32°C. Two measurements of colony diameter, perpendicular to each other, were taken each day from the second day of the trial. Six measurements were thus made for each isolate at each assessment. The entire experiment was repeated once.

DNA extraction, PCR and sequencing

Single conidium and ascospore cultures were prepared for all isolates and DNA extractions were performed as described by Aghayeva et al. (2004) using PrepMan Ultra Sample Preparation reagent (Applied Biosystems, Foster City, CA, USA). The ITS 1 and 2 regions of the ribosomal DNA operon, including the 5.8 S gene, were amplified using primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Part of the β-tubulin (BT) gene was amplified using primers Bt2A and Bt2B (Glass and Donaldson 1995). Bt2B was replaced in some cases with primer T10 (O'Donnell and Cigelnik, 1997) to obtain a longer fragment. Amplicons were also obtained from part of the translation elongation factor-1α (TEF-1α) gene, with primers EF1-728F and EF1-986R (Carbone and Kohn 1999) or primers EF1F and EF2R (Jacobs et al. 2004). Part of the histone gene (HIS) was amplified with primers H3-1A and H3-1B (Glass and Donaldson 1995).

The reaction mixture (50 µl final volume) contained five units of Expand *Taq* Polymerase (Roche Biochemicals, Mannheim, Germany), 1x PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 mM of each primer, and 1 µl DNA template. PCR reactions were performed in a thermal cycler (Hoffman-La Roche, Nutley, NJ, USA). PCR conditions for the ITS1/ITS2 regions were the same as those used by Aghayeva et al. (2004). The β-tubulin gene was amplified using an initial denaturation at 94°C for 1 min, followed by 30 cycles denaturation at 94°C for 1 min, primer annealing at 53–55°C (depending on the isolate) for 1 min, and elongation at 72°C for 1 min. A final extension step at 72°C for 5 min completed the program. The EF-1α was amplified using denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 1 min, primer annealing at 54–61°C (depending on the isolate) for 1 min 30 s and elongation at 72°C for 2 min. A final extension step was performed for 10 min at 72°C. The histone gene region amplified with a denaturation step at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 1 min 30 s, primer annealing at 60°C for 30 s, and elongation at 72°C for 1 min. This was followed by a final extension step for 8 min at 72°C.

PCR products were separated on a 1% (w/v) agarose gel stained with ethidium bromide and visualized under UV illumination. PCR fragments were purified using Sephadex G50 (Sigma-Aldrich, Chemie, Steinheim, Germany). Both strands of the PCR products were sequenced using amplification primers and the ABI PRISM® BigDye™ Terminator v 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). DNA sequencing reactions were analyzed on an ABI PRISM® 3100 Genetic Analyzer or an ABI PRISM™ 377 DNA sequencer.

Analyses of sequence data

Sequence data from the four genes were analyzed and contigs assembled using the Vector NTI Advance 10

Table 1 Isolates and herbarium specimens of *Ophiostoma* spp. considered in this study

Species	Isolate no.[a]	Host	Origin[a]	Collector	GenBank Accession no.				Growth at 25°C[b]
					ITS	BT	TEF-1α	HIS	
O. catonianum	[c]CMW 11535; CBS 263.35	*Pyrus communis*	Italy	Goidánich	AF198243	AY466653	AY466699	AY466676	26.2
O. fagi (= *O. quercus*)	[d]CMW 11532; CBS 236.32	*Fagus* sp.	Germany	Loos	AF198237	AY466652	AY466698	AY466675	58.6
O. floccosum	CMW 12622; CBS 123601	*Pinus sylvestris*	Austria	Kirisits	FJ430472	FJ430502	FJ430487	FJ430517	NT
	CMW 12623	*P. sylvestris*	Austria	Kirisits	FJ430473	FJ430503	FJ430488	FJ430518	NT
O. himal-ulmi	CMW 22729; CBS 374.67	*Ulmus wallichiana*	India	Rebel	FJ430474	FJ430504	FJ430489	FJ430519	NT
O. novo-ulmi	CMW 186	Unknown	USA	Brown	FJ430475	FJ430505	FJ430490	FJ430520	NT
	CMW 1461	*U. procera*	USA	Brasier	FJ430476	FJ430506	FJ430491	FJ430521	NT
	CMW 1463	*U. procera*	USA	Brasier	FJ430477	FJ430507	FJ430492	FJ430522	NT
	CMW 10573; CBS 119476	*Picea abies*	Austria	Neumueller	FJ430478	FJ430508	FJ430493	FJ430523	NT
	CMW 25033; CBS 298.87	*Ulmus* sp.	Russia	Heybroek	FJ430479	FJ430509	FJ430494	FJ430524	NT
O. piceae	CMW 12615	*Larix decidua*	Austria	Kirisits	FJ430480	FJ430510	FJ430495	FJ430525	NT
	CMW 12617	*L. decidua*	Austria	Kirisits	FJ430481	FJ430511	FJ430496	FJ430526	NT
	CMW 2468; 0.95; CBS 123600	*P. abies*	France	Morelet	FJ430482	FJ430512	FJ430497	FJ430527	NT
	CMW 2751	*Larix* sp.	USA	Wingfield	FJ430483	FJ430513	FJ430498	FJ430528	NT
	CMW 3099	*Picea* sp.	Canada	Wingfield	FJ430484	FJ430514	FJ430499	FJ430529	NT
O. quercus	CMW 2462; CBS 115764; 0.97	*Fagus sylvatica*	France	Morelet	AY466623	AY466643	AY466689	AY466666	49.4
	CMW 2463; CBS 116453; 0.96	*F. sylvatica*	France	Morelet	AF493239	AY466644	AY466690	AY466667	52.9
	CMW 2464; CBS 115763; 0.99	*F. sylvatica*	France	Morelet	AY466624	AY466645	AY466691	AY466668	50.3
	CMW 2465; CBS 117912; 0.98	*Q. robur*	France	Morelet	AY466625	AY466646	AY466692	AY466669	54.4
	[e]CMW 2467; CBS 117913; 0.80	*Quercus* sp.	France	Morelet	AY466626	AY466647	AY466693	AY466670	53.6
	CMW 2520; CBS 116321	*Eucalyptus* sp.	SA	De Beer	AF493241	AY466648	AY466694	AY466671	43.9
	CMW 2534; CBS 117914	*E. grandis*	SA	Kemp	AF493242	AY466649	AY466695	AY466672	41.1
	CMW 3119; CBS 115871	*Pinus* sp.	SA	De Beer	AF493244	AY466650	AY466696	AY466673	44.2
	CMW 7650; CBS 102352; H1042	*Quercus* sp.	UK	Webber	AF198238	AY466651	AY466697	AY466674	49.6
	CMW 8283; CBS 118111	*Castanea sativa*	AZ	Aghayeva	AY466611	AY466631	AY466677	AY466654	52.4
	CMW 9255; CBS 115798	*Quercus longipes*	AZ	Aghayeva	AY466612	AY466632	AY466678	AY466655	50.4
	CMW 9256; CBS 115796	*C. sativa*	AZ	Aghayeva	AY466613	AY466633	AY466679	AY466656	43.8
	CMW 9257; CBS 115799	*C. sativa*	AZ	Aghayeva	AY466614	AY466634	AY466680	AY466657	50.5
	CMW 9258; CBS 115864	*C. sativa*	AZ	Aghayeva	AY466615	AY466635	AY466681	AY466658	NT
	CMW 9259; CBS 115800	*C. sativa*	AZ	Aghayeva	AY466616	AY466636	AY466682	AY466659	NT
	CMW 9262; CBS 115797	*C. sativa*	AZ	Aghayeva	AY466617	AY466637	AY466683	AY466660	52.5
	CMW 9263; CBS 115864	*C. sativa*	AZ	Aghayeva	AY466618	AY466638	AY466684	AY466661	NT
	CMW 9267; CBS 115795	*Quercus* sp.	AZ	Aghayeva	AY466619	AY466639	AY466685	AY466662	57.4
	CMW 9474; CBS 115794	*Quercus* sp.	AZ	Aghayeva	AY466620	AY466640	AY466686	AY466663	44.4

	CMW 9475; CBS 115863	*Q. longipes*	AZ	Aghayeva	AY466621	AY466641	AY466687	AY466664	52.8
"*O. roboris*" (= *O. quercus*)	CMW 12618; CBS 115950; H2003	*Q. longipes*	AZ	Guseinov	AY466622	AY466642	AY466688	AY466665	42.1
	[f] LEP	*Quercus* sp.	Russia	Potlajchuk	–	–	–	–	NT
	[f] LEP	[g] *Acer* sp.	Russia	Potlajchuk	–	–	–	–	NT
	[f] LEP	[g] *Betula* sp.	Russia	Potlajchuk	–	–	–	–	NT
O. setosum	CMW 12378	*Tsuga* sp.	China	Zhou	FJ430485	FJ430515	FJ430500	FJ430530	NT
	CMW 16534; CBS 123602	*Picea glauca*	Canada	Uzunovic	FJ430486	FJ430516	FJ430501	FJ430531	NT
P. australiae	CMW 6589; CBS 121026	*Acacia mearnsii*	Australia	Wingfield	EF408602	EF408605	–	–	NT
	[h] CMW 6606; CBS 121025	*A. mearnsii*	Australia	Wingfield	EF408603	EF408606	–	–	NT

[a] *CMW* Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, *CBS* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, *AZ* Azerbaijan, *SA* South Africa, *UK* United Kingdom

[b] All measurements of colony diameters on MEA in mm after 10 days. *NT* Not tested

[c] Authentic culture (Goidánich 1935)

[d] Ex-type culture (Loos 1932)

[e] Ex-neotype culture (Morelet 1992)

[f] *LEP* Herbarium specimens from the All-Russian Institute of Plant Protection, St. Petersburg, Russia. Specimens were not numbered

[g] Dried cultures that were re-isolated from *Acer* and *Betula* that were inoculated with the first isolate (Potlajczuk 1957)

[h] Ex-type culture (Kamgan et al. 2008b)

software (Invitrogen). The assembled forward and reverse contigs were aligned in MAFFT v 5.731, option E-INS (Katoh et al. 2005; Morrison 2006), then checked manually for inconsistencies. Gaps were treated as 5th characters. Exon positions were identified using the available sequences from GenBank. GenBank accession numbers for all sequences generated in this study, as well as some ITS sequences from previous studies that were obtained from GenBank, are listed in Table 1. Relationships between the isolates using sequences of the four gene regions (ITS, β-tubulin, EF-1α, and HIS) were determined by maximum likelihood (ML) and Bayesian Inference (BI). *Ophiostoma setosum* was used as the outgroup taxon in all cases as it is closely related to *O. quercus* but clusters in the 'coniferous group' within the *O. piceae* complex, thus avoiding confusion that might have arisen by choosing a fungus that could possibly have been a synonym of *O. quercus*.

The most appropriate model(s) of sequence evolution for the relevant genes were chosen using the Akaike information criterion (AIC) as selected from a set of 56 hierarchically nested contenders in Modeltest v 3.7 (Posada and Crandall 1998) for each of the four genes. The ML analysis was conducted using the best model results in PhyML (Guindon and Gascuel 2003). Nonparametric bootstrap analyses (Felsenstein 1985) employed one heuristic search for the 1,000 bootstrap replicates under maximum likelihood to estimate the reliability of nodes.

The BI analysis was performed in MrBayes v 3.1.2 (Ronquist and Huelsenbeck 2003) using the best fitting model selected by the AIC test in MrModeltest v 2.2 (Nylander 2004). Model parameters were derived from the default prior distributions. The MrBayes analysis was comprised of four independent runs of 1,000,000 generations using duplicate Monte Carlo Markov chain searches with four chains (one cold, three hot to improve mixing) and a sampling frequency of every 100 generations. Stationarity for LnL and nucleotide substitution parameters was determined and, consequently, the relevant numbers of trees sampled for each gene region were discarded as burn-in before the consensus topology and posterior probabilities were calculated.

Results

Morphology

Isolates of *O. quercus*, *O. fagi*, *O. catonianum*, and the '*O. roboris'* isolate (CMW 12618), varied in culture morphology and color. The colony morphology and colour of 10-day-old cultures of all isolates were similar, but differences became more pronounced after synnemata or perithecia had formed. The formation of rings or sectors of growth in

culture was not consistent in different cultures of the same isolate and this feature was not considered taxonomically informative.

Only two isolates (CMW 9256, CMW 9262) formed perithecia in culture (Figs. 1 and 2). Morphological features of these isolates are summarized in Table 2. The most obvious differences in the sexual state of the two isolates were the neck length and ascospore shape (Figs. 1 and 2).

Most isolates, including the Guseinov '*O. roboris*' isolate (CMW 12618), produced both *Pesotum* and *Sporothrix* anamorphs in culture. The herbarium specimens from Russia, labeled as *O. roboris*, contained *Pesotum* and *Sporothrix* structures similar to those of the *O. quercus* isolates, and perithecia were absent. Isolate CMW 9262 from Azerbaijan produced, in addition to the typical *Pesotum* and *Sporothrix* anamorphs, a third anamorph structure (Fig. 2g). This structure that is best described as *Hyalorhinocladiella*-like was less common than the other two types in both the original and single conidium cultures and was not observed in any of the other isolates.

The ex-type isolate of *O. fagi* obtained from CBS formed *Pesotum* and *Sporothrix* states on MEA cultures stored at 4°C. Although most of the synnemata were fused, mature compacted synnemata similar to those of *O. quercus* were also observed. The ex-type culture of *O. catonianum* (CMW 11535) formed only a *Sporothrix* anamorph in culture, with no synnematous structures present.

Growth studies

All isolates started to grow on the second day at 15°C and above. At 10°C, growth started on the third day and at 5°C growth was only observed on the sixth day. For most of the isolates, optimum growth was observed at 25°C (Table 1). Growth of the *O. quercus* isolates at 25°C after 10 days varied between 41 and 57 mm. In the same period, the *O. fagi* isolate grew to 58.6 mm, the '*O. roboris'* isolate (CMW 12618) to 42.1 mm, and CMW 9262 (with the *Hyalorhinocladiella*-like anamorph) to 52.5 mm. The ex-type isolate of *O. catonianum* grew slower reaching an average colony diameter of 26.2 mm in 10 days at 25 °C.

Analyses of sequence data

All four datasets consisted of sequences obtained from 40 isolates grouping within the *O. piceae* complex. Aligned DNA sequence from ITS gene region yielded 592 characters, including gaps, while the BT gene region alignment had 331 characters, the TEF-1α gene region 495 characters, and the HIS region 294 nucleotides and no gaps. The phylogenetic information content varied across the four genes with most of the variability seen within the introns of the BT, HIS, and ITS gene regions. However, the TEF-1α gene displayed a large amount of variation throughout the sequence and included numerous indels.

Results of the maximum likelihood and Bayesian analyses yielded trees with concordant topologies and similar levels of node support for all four genes. Stationarity was achieved after 100,000 generations across all four genes. We preferred not to combine the data for the four gene regions as each phylogeny represents a unique evolutionary history. Results are, therefore, presented independently of each other (Fig. 3a–d).

In all four gene regions, *O. floccosum*, *O. setosum*, *O. piceae*, *O. novo-ulmi*, *O. himal-ulmi*, *Pesotum australiae*, and *O. catonianum* were separated from each other in distinct clusters (Fig. 3a–d). The rest of the isolates grouped in a single, well-supported lineage that included the ex-neotype of *O. quercus* (CMW 2467) from France, an isolate from the UK (CMW 7650), some South African isolates, the ex-type isolate of *O. fagi* (CMW 11532 from Germany), and an isolate from Azerbaijan previously treated as *O. roboris* (CMW 12618). This lineage also included all the other isolates from Azerbaijan, including the isolate (CMW 9262) producing the mononematous *Hyalorhinocladiella*-like anamorph.

Analyses of sequences for the BT gene and TEF-1α showed the most strongly supported terminal nodes of all four genes studied, with posterior probabilities close to 1.00 at almost all the internal nodes. The TEF-1α gene showed the most resolution and best defines the relationships between the 40 sequences analysed. Interestingly, all the hardwood inhabiting species were more closely related to each other than to species found predominantly on other tree species (indicated as HW in Fig. 3a–d).

Discussion

The four gene genealogies presented in this study show that *O. quercus* isolates represent a well-supported monophyletic group (group Q, Fig. 3a–d). In these analyses, *O. quercus* was typified by the ex-neotype isolate of the species (CMW 2467) from France (Morelet 1992; Przybyl and Morelet 1993). Group Q included other authentic *O. quercus* isolates used in previous studies, from France (Morelet 1992), the UK (Brasier and Kirk 1993; Brasier and Stephens 1993; Pipe et al. 1995; Harrington et al. 2001), and South Africa (de Beer et al. 2003a), as well as isolates obtained in this study from various hardwood hosts in Azerbaijan. Although several sub-groups were present within group Q in analyses for all four gene regions, these were not consistent between the various gene regions and did not have statistical support. However, these sub-groups

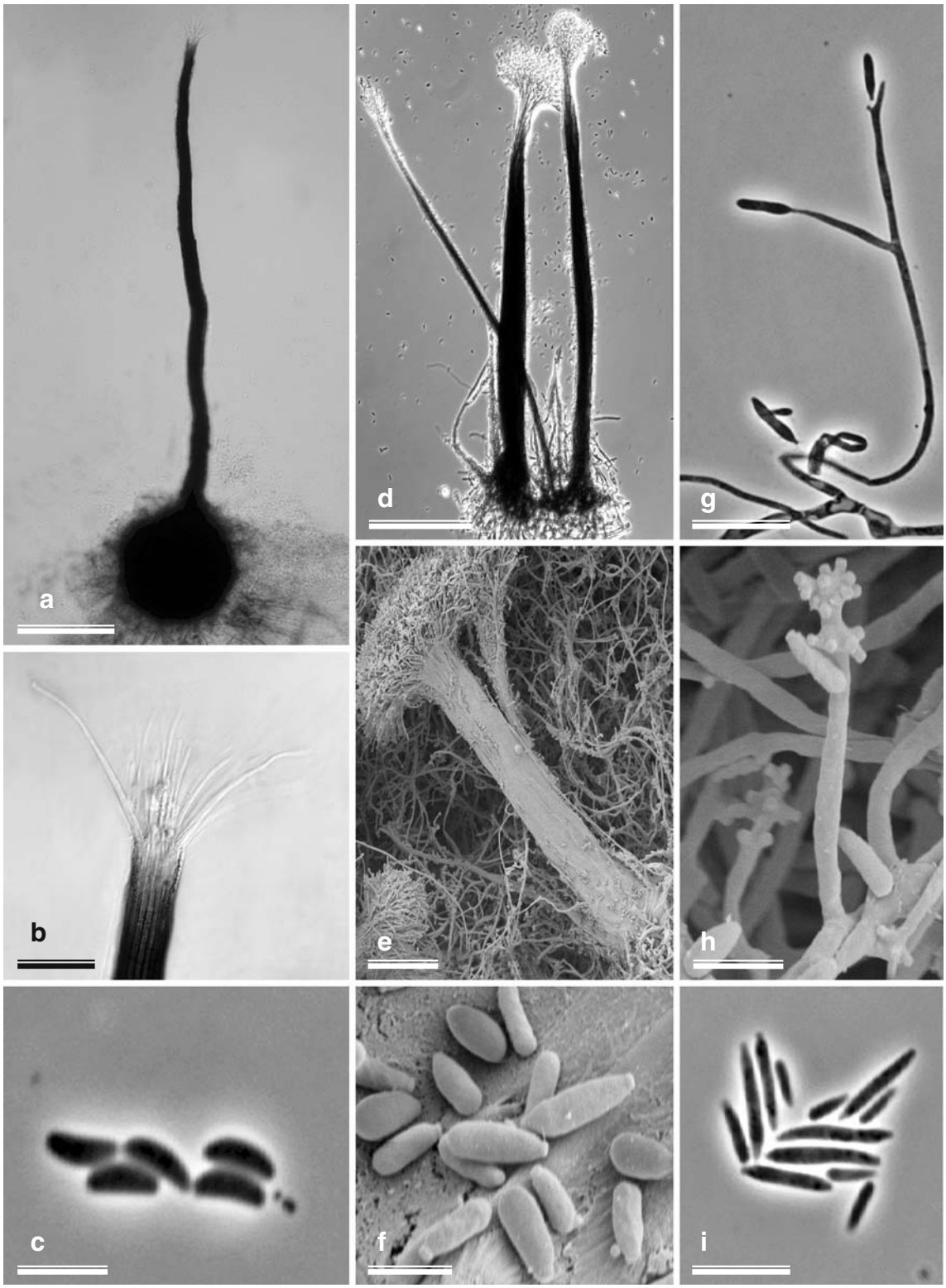


Fig. 1 Morphological characteristics of an isolate of *Ophiostoma quercus* from Azerbaijan (CMW 9256). **a** Perithecium. **b** Ostiolar hyphae. **c** Allantoid ascospores in side view. **d** *Pesotum* type synnematous conidiophore. **e** Scanning electron micrograph (SEM) of a synnematous conidiophore (*Pesotum* type). **f** SEM of conidia of *Pesotum* anamorph. **g** Conidiogenous cells of *Sporothrix* anamorph. **h** SEM of *Sporothrix* anamorph. **i** Conidia of *Sporothrix* anamorph. *Scale bars*: **a,d,e**=100 μm, **b,g–i**=10 μm, **c,f**=5 μm

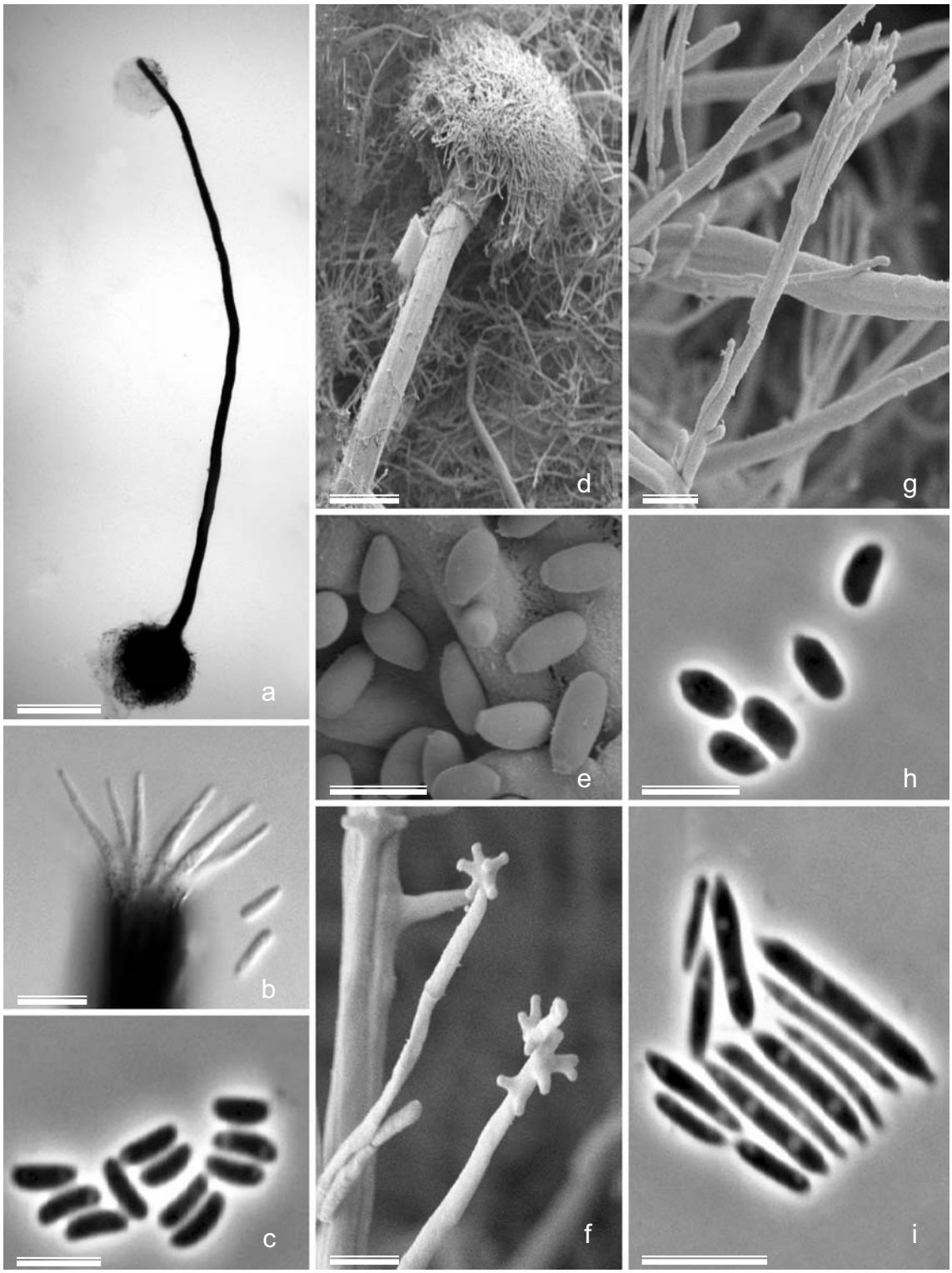


Fig. 2 Morphological characteristics of the so-called *Ophiostoma roboris* isolate from Azerbaijan (CMW 9262). **a** Perithecium. **b** Ostiolar hyphae. **c** Reniform ascospores in side view. **d** Scanning electron micrograph (SEM) of a synnematous conidiophore (*Pesotum* type). **e** SEM of conidia of *Pesotum*. **f** SEM of *Sporothrix* anamorph. **g** SEM of *Hyalorhinocladiella* anamorph. **h** Conidia of *Hyalorhinocladiella* anamorph. **i** Conidia of *Sporothrix* anamorph. *Scale bars*: **a, d**=100 μm, **b,g,i**=10 μm, **c,f,h**=5 μm

are indicative of substantial genetic variation within the species.

The culture morphology and growth in culture of the *O. quercus* isolates, including those from Azerbaijan, showed considerable variability, similar to that reported in previous studies (Morelet 1992; Brasier and Stephens 1993; Halmschlager et al. 1994). All these isolates produced typical *Pesotum* and *Sporothrix* anamorphs in culture. Two of the isolates from Azerbaijan also formed teleomorphs in culture (Figs. 1 and 2). Although the averages calculated for the perithecial and synnematal dimensions of these two isolates (CMW 9256 and CMW 9262) differed from each other, ranges of both isolates reside within the published ranges for *O. quercus* (Table 2). The one Azerbaijan isolate (CMW 9262) differed from the other isolates and produced unusual anamorph structures that can best be described as *Hyalorhinocladiella*-like.

Ophiostoma fagi was described from *Fagus sylvatica* in Germany by Loos (1932). During the period that *O. quercus* was treated a synonym of *O. piceae*, Upadhyay (1981), Hutchinson and Reid (1988), and Przybyl and de Hoog (1989) listed *O. fagi* as a synonym of *O. piceae*. Harrington et al. (2001) suggested that *O. fagi* should be treated as a synonym of *O. quercus* based on ITS sequence data and sexual compatibility using the ex-type isolate of *O. fagi*. We included the same isolate (CMW 11532) in the present study. In phylograms obtained from all four gene regions, this species clustered within the *O. quercus* group (Q). We thus support the synonymy of *O. fagi* with *O. quercus* as proposed by Harrington et al. (2001). This is in contrast to the report of Melin and Nannfeldt (1934) who stated that the synnematal anamorph of this isolate had been lost, and placed *O. fagi* in the '*pilifera* group' of *Ophiostoma* that contained species with only mononematous anamorphs.

Ophiostoma roboris was originally described from Romania (Georgescu et al. 1948) and, together with *O. quercus*, was considered a synonym of *O. piceae* by de Hoog (1979) and Kowalski and Butin (1989). The synonymy was questioned by Przybyl and de Hoog (1989) based on a lack of authentic material and differences in anamorph morphology. Guseinov (1984) described an isolate from oak in Azerbaijan as *O. roboris* based on morphological similarities with the descriptions by Georgescu et al. (1948). In both publications (Georgescu et al. 1948, Guseinov 1984), a synnematal ('*Graphium*') anamorph was described that is currently recognized as the *Pesotum* anamorph. The typical *Sporothrix* synanamorph that is usually associated with species in the *O. piceae* complex was often described as 'of the *Cladosporium*- or *Cephalosporium*-type' in older publications (e.g., Loos 1932). Georgescu et al. (1948), Guseinov (1984), and Georgiev (1986), however, described the synanamorph of *O. roboris* as a *Hyalodendron roboris*. They distinguished it from the '*Cladosporium*'- or '*Cephalosporium*-type' anamorphs of related species, because it formed a mononematous conidiophore that lacked the pronounced denticles characteristic of the form genus *Sporothrix*. Considering the illustrations of Georgescu et al. (1948, Figs. 32–33), this mononematous anamorph broadly resembles the *Hyalorhinocladiella*-like anamorph observed in one of the isolates in the present study. Kowalski and Butin (1989) presented a similar illustration for the anamorph of *O. quercus* (as '*Ceratocystis piceae*'), and also described this as an *Hyalodendron*-type. However, the genus *Hyalodendron* is restricted to anamorphs of Basidiomycetes (de Hoog 1979) and is thus not available for *Ophiostoma* anamorphs.

The *'O. roboris'* isolate described by Guseinov (1984), was successfully crossed with authentic *O. quercus* isolates by Brasier and Kirk (1989, 1993), who then suggested *O. roboris* might be a synonym of *O. quercus*. The same isolate (CMW 12618) was included in the present study and could not be distinguished from *O. quercus* isolates based on morphology or sequence data comparisons (Fig. 3a–d). Our results, therefore, confirm those of previous studies (Brasier and Kirk 1989, 1993; Webber and Brasier 1991; Brasier and Stephens 1993; Pipe et al. 1995; Lin et al. 2003) suggesting that this isolate should be treated as *O. quercus*. However, we did not observe the characteristic *Hyalorhinocladiella*-like ('*Hyalodendron*') anamorph described by Guseinov (1984) in this particular isolate (CMW 12618). One of our other isolates (CMW 9262) from Azerbaijan formed an anamorph that corresponded with the line drawings of the '*Hyalodendron roboris*' anamorph in the original description of *O. roboris* (Georgescu et al. 1948, pp. 208–209). Based on the morphological similarities, this isolate could have been considered to represent a neotype for *O. roboris*, but it also grouped within the *O. quercus* group (Q) in all four phylogenies. We thus conclude that *O. roboris* should be treated a synonym of *O. quercus*.

The presence of the *Hyalorhinocladiella*-like ('*Hyalodendron*') anamorph in a species like *O. quercus* is not surprising. In the original definition for the anamorph genus *Pesotum,* Crane and Schoknecht (1973) included a continuum of structures between the classical synnematous structure at the one extreme, and a typical mononematous, denticulate *Sporothrix* conidiophore, at the other. Several intermediate structures and sizes had been recognized, and contributed to the confusing use of generic names such as *Cladosporium*, *Cephalosporium*, *Hyalodendron*, and *Rhinotrichum* to describe *Ophiostoma* anamorphs in many previous publications (Przybyl and de Hoog 1989; Benade et al. 1997, 1998). Following Harrington et al. (2001), we accept the broad definition of *Pesotum* by Crane and

Table 2 Morphological characters of *Ophiostoma quercus* and its synonyms

Country →	*O. quercus* (Georgevitch 1926, 1927) Slavonija (Croatia)	*O. quercus* (Lehmann 1932) Germany	*O. fagi* (Loos 1932) Germany	*O. roboris* (Georgescu et al. 1948) Romania	*O. quercus* neotype (Morelet 1992) France	Present study (typical *O. quercus* morphotype) Azerbaijan	Present study (typical *O. roboris* morphotype) Azerbaijan
Isolate no.	–	–	CMW 11532	–	CMW 2467	CMW 9256	CMW 9262
Perithecia	–	Black	Black, in groups	Black, spherical	Black	Black	Black
Base diameter	(129)150–240	115–130	135–220	(95)128×136(160)	80–185	(125)234(217)	(110)146(191)
Neck							
Length	970–990	630–850	860–1,920	476–1,500(2,000)	1,000–2,000	306–736	869–2,036
Width at base	(22)–27	21–24	–	22.8–30.4	–	19–60	24–30
Width at apex	14	8–10	–	7.6–11.4	–	(9)11–18(24)	(9)11–16(20)
Ornamental hyphae	–	–	Absent or very short, curved outward	–	–	Brown, dense	Dark brown dense
	–	–	–	–	–	22–114×1.6–3.9	17–40×1.3–2.5
Ostiolar hyphae							
Color	–	–	–	Hyaline	Hyaline	Hyaline	Hyaline
Shape	–	–	–	3-septate	Divergent	Divergent	Divergent
Size	<31.5×5	23–26	12–58×1–2	1–20(38)×1	<32	27–48×1–2	31–42×1.4–2
Ascospores							
Shape	Reniform	Reniform	Ellipsoidal, curved	Reniform	Reniform	Allantoid	Reniform
Color	–	–	–	Hyaline	Hyaline	Hyaline	Hyaline
Size	4×2	4×2	3.5–4.7×0.7–1.2	3.2–3.5×0.9–1	2.8–4.3×1.4–2	2.6–4.4×0.9–1.5	3.4–4.8×1.5–2.4
Anamorph 1	[a]*Graphium*	[a]*Graphium*	[a]*Graphium*	[a]*G. roboris*	[a]*Graphium*	*Pesotum*	*Pesotum*
Synnemata							
Color	Brown	–	Black	Brown	Dark brown–black	Brown, hyaline at apex	Brown, lighter at apex
Size	260–285(619)	(300)425(600)	420(625) × 8–11(23)	336–1,000×7.6–240	300–1,200	335–692×(9)17–57(77)	294–916×(14)22–99 (133)
Conidia							
Shape	Elliptical to oval	Elliptical	Cylindrical	Unicellular, pear shaped	Ellipsoid to ovoid	Oblong–obovoid, base truncate	Ellipsoidal, base obtuse
Colour	Hyaline	–	–	Hyaline	Hyaline	Hyaline	Hyaline
Size	4–2	3.6×2.3	3–5×1.2–2.2	3.2–3.8×1.2	2.8–4.7×1.4–2	2.5–4.1×1–1.6	3.5–4.8×1.5–2.4

Anamorph 2	–	–	[b]*Cladosporium*-type	[d]*Hyalodendron roboris*	*Sporothrix*	*Sporothrix*	*Sporothrix*
Conidia size	–	–	6–15×2.5–4	21–45×2.2–2.5	4–20×1.4–2.8	14–95(233)×1.1–2.5	44–156(209)×1.2–3.4
Anamorph 3	–	–	[c]*Cephalosporium*-type			Not present	*Hyalorhinocladiella*-like
						Fig. 1 a–i	Fig. 2 a–i

Isolates sequenced, and measurements done in this study are printed in blue. All measurements in µm

[a] Synnematous anamorphs of *Ophiostoma* are currently placed in the genus *Pesotum* (Ophiostomatales), rather than *Graphium* (Microascales), based on phylogeny (Okada et al. 1998; Harrington et al. 2001)

[b] The genus *Cladosporium* is characterized by dark colored conidia, and associated with *Davidiella* teleomorphs (Capnodiales) (Crous et al. 2007), and is thus not available for *Ophiostoma* anamorphs

[c] *Cephalosporium* is a synonym of *Acremonium* (Gams 1968), of which the type species is classified in the Hypocreales (Glenn et al. 1996). *Cephalosporium* anamorphs of *Ophiostoma* were transferred to *Sporothrix* by De Hoog (1974)

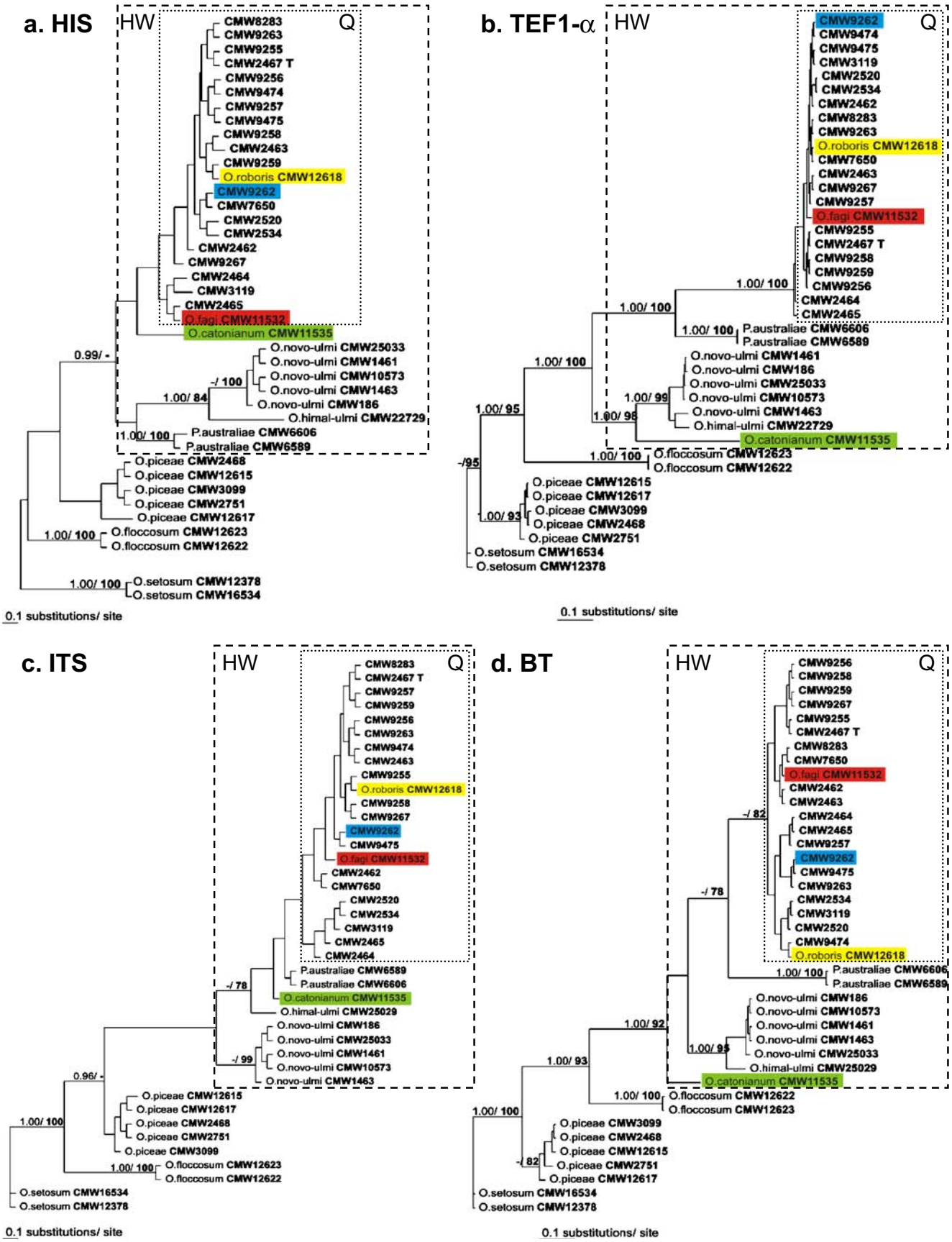
[d] The type species of *Hyalodendron* is a basidiomycete (De Hoog 1979). *Hyalodendron* is thus not available to anamorphs of *Ophiostoma*

Schoknecht (1973), which includes the *Hyalorhinocladiella*-like structures described for a species like *O. roboris*.

Treating *O. roboris* as a synonym of *O. quercus* creates a technical taxonomic problem. The *Pesotum* and *Sporothrix* synanamorphs of *O. quercus* had never been supplied with binary names, while those of *O. roboris* were described as *Graphium roboris* and *Hyalodendron roboris,* respectively. However, neither of the latter genera are available for anamorphs of *Ophiostoma* (de Hoog 1979; Okada et al. 1998). Furthermore, Article 59 of the ICBN (McNeill et al. 2006) allows the epithets of these two form species to be available for the anamorphs of *O. quercus*. The practice of assigning binary names to anamorphs, especially when the teleomorph is known and described, is becoming outdated. However, to avoid the use of an inappropriate anamorph genus name in future nomenclators listing *Ophiostoma* species, new combinations have been provided for the two *O. roboris* synanamorphs.

Ophiostoma catonianum has been listed as a synonym of *O. piceae* by several authors (de Hoog 1974; Upadhyay 1981; Hutchison and Reid 1988; Przybyl and de Hoog 1989). However, Morelet (1992) and Okada et al. (1998) treated *O. catonianum* as a synonym of *O. quercus* because the species was described from *Pyrus,* a hardwood host (Goidánich 1935). The authentic isolate (CMW 11535) from the original study included in the present study consistently grouped in a lineage distinct from *O. quercus* in all four gene regions analyzed, and it clearly represents a distinct species. Goidánich (1935) described the synanamorphs of *O. catonianum* as *Hyalodendron pirinum* and *Graphium pirinum*. Morelet (1992) and Okada et al. (1998) made the necessary new combinations, namely *Sporothrix pirinum* and *Pesotum pirinum*, respectively, but treated these as the anamorphs of *O. quercus.* Harrington et al. (2001), however, distinguished between *O. quercus* and *O. catonianum* based on ITS sequence data, and correctly listed *S. pirinum* and *P. pirinum* as the anamorphs of *O. catonianum*. According to the original description, the species has shorter perithecial necks and longer ostiolar hyphae than *O. quercus*, and also had a homothallic mating system rather than the heterothallic system of other species in the *O. piceae* complex (Goidánich 1935, Harrington et al. 2001).

Ophiostoma valachicum was treated as a *nomen dubium* by Upadhyay (1981), but Przybyl and de Hoog (1989) and Harrington et al. (2001) considered it as a possible synonym of *O. piceae* and *O. quercus*, respectively. No material exists for this species, but it was originally isolated from oak in Romania and validly published in the same paper as *O. roboris* (Georgescu et al. 1948). The original morphological description mentions only *Rhinotrichum valachicum* as anamorph (Georgescu et al. 1948), but no synnematous anamorph that would be expected if it were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O.roboris CMW12618
CMW9262
CMW7650
CMW2520
CMW2534
CMW2462
CMW9267
CMW2464
CMW3119
CMW2465
O.fagi CMW11532
O.catonianum CMW11535
O.novo-ulmi CMW25033
O.novo-ulmi CMW1461
O.novo-ulmi CMW10573
O.novo-ulmi CMW1463
O.novo-ulmi CMW186
O.himal-ulmi CMW22729
P.australiae CMW6606
P.australiae CMW6589
O.piceae CMW2468
O.piceae CMW12615
O.piceae CMW3099
O.piceae CMW2751
O.piceae CMW12617
O.floccosum CMW12623
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O.floccosum CMW12622
O.floccosum CMW12623
O.piceae CMW3099
O.piceae CMW2468
O.piceae CMW12615
O.piceae CMW2751
O.piceae CMW12617
O.setosum CMW16534
O.setosum CMW12378
-/ 82
-/ 78
1.00/ 92
1.00/ 93
1.00/ 95
1.00/ 100
1.00/ 100
-/ 82
0.1 substitutions/ site

◀ **Fig. 3** Phylograms resulting from Bayesian analyses of: **a** the partial Histone (*HIS*) gene, **b** translation elongation factor-1α (*TEF-1α*), **c** the rDNA internal subscribed spacers (*ITS*) regions 1 and 2, and **d** the Bt2 region of the β-tubulin (*BT*) gene. The box delimiting *Ophiostoma quercus* is indicated with *Q*, and the lineage representing hardwood species as *HW*. Species previously considered synonyms of *O. quercus* are indicated in *shaded boxes*, with the Azerbaijan isolate presenting the *O. roboris* morphotype (CMW 9262) in *dark grey*. The ex-neotype isolate of *O. quercus* (CMW 2467) are indicated with *T*. The posterior probability support values are given first followed by the ML bootstrap values (1,000 replicates) for each node respectively (scores of 0.95 pp or 70 bs and less have not been included and are indicated with -)

closely related to *O. quercus*. Although Sczerbin-Parfenenko (1953) confirmed that no other conidial stages are known for *O. valachicum*, Potlajczuk and Schekunova (1985) and Georgiev (1986) mentioned a *Graphium* anamorph in addition to the *Rhinotrichum* anamorph for isolates of Russian and Bulgarian origin, respectively. The genus *Rhinotrichum* was not validly published (Hawksworth et al. 1983), and is thus not available for use. Furthermore, Przybyl and de Hoog (1989) suggested the *Rhinotrichum*-type anamorphs of *Ophiostoma* spp. are simply one of three manifestations of the genus *Sporothrix,* a conclusion with which we concur. The absence of a synnematous anamorph from the original description, white colonies, crescent-shaped ascospores, and curved conidia (Georgescu et al. 1948, this study) distinguishes *O. valachicum* from species in the *O. piceae* complex. We thus recognize *O. valachicum* as a distinct and valid species. Neotypification of this species would make it possible to determine its phylogenetic placement, which would most likely be in the *O. stenoceras–S. schenckii* complex as defined by de Beer et al. (2003b).

Ophiostoma kubanicum was described from oak in the former Soviet Union (Sczerbin-Parfenenko 1953), but was invalidly published as no Latin description was given (Article 36.1, McNeill et al. 2006). Furthermore, no authentic material of this species is available for study, making validation impossible. For this reason, we suggest that it should be excluded from future treatments of the genus *Ophiostoma*.

The delimitation of *O. quercus* and clarification of its synonyms in the present study have paved the way for future studies that will focus on population-level questions pertaining to this species. *Ophiostoma quercus* isolates consistently display considerable phenotypic variation as well as variation in DNA sequences. The capacity to colonize wounds on a wide range of hosts, its association with many different insect vectors, and its worldwide distribution raises intriguing questions regarding its origin and ecology. A clear definition of the species boundaries for this fungus as presented in this study will make it possible to resolve some of those questions.

Taxonomy

Phylogenetic analyses of sequences for four gene regions were used to delimit *Ophiostoma quercus* sensu stricto and to clarify the status of those species that have in the past been listed as synonyms of this species. These data, together with morphological characteristics extracted from descriptions of *O. quercus* and its synonyms led to the conclusion that *O. fagi* and *O. roboris* are valid synonyms of *O. quercus*. In contrast, *O. catonianum* and *O. valachicum* should be considered as distinct taxa. We further argue that *O. kubanicum* was not validly published and that it should not be treated as a synonym of *O. quercus*.

***Ophiostoma quercus* (Georgev.) Nannf.**, in Melin and Nannf., Svenska Skogsvårsför. Tidskr. 32: 408. 1934.

Basionym Ceratostomella quercus Georgev., Compt. Rend. Hebd. Séances Acad. Sci. 183: 759. 1926. [as '*Querci*'] (*non Ceratostomella quercus* A.C. Santos and Sousa da Câmara, Agronomia Lusitania 17: 136. 1955., *nom. inval*.)

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= *Ceratostomella fagi* W. Loos, Arch. Mikrobiol. 3: 376. 1932.

≡ *Ophiostoma fagi* (W. Loos) Nannf., in Melin and Nannf., Svenska Skogsfor. Tidskr. 32: 408. 1934.

≡ *Ceratocystis fagi* (W. Loos) C. Moreau, Rev. Myc. (Paris) Suppl. Col. 17: 22. 1952.

= *Ophiostoma roboris* Georgescu and Teodoru, in Georgescu, Teodoru and Badea, Anal. Inst. Cerc. Exp. For. Rom., Ser 1. 11: 207. 1948.

≡ *Ceratocystis roboris* (Georgescu and Teodoru) Potl., in Potlajczuk and Schekunova, Nov. Sist. Niz. Rast. 22: 154. 1985.

Anamorph ***Pesotum roboris* (Georgescu, Teodoru and Badea) Grobbelaar, Z.W. de Beer and M.J. Wingf. comb. nov.**

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Synanamorph ***Sporothrix roboris* (Georgescu, Teodoru and Badea) Grobbelaar, Z.W. de Beer and M.J. Wingf. comb. nov.**

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Anamorph Sporothrix (Przybyl and de Hoog 1989).

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Nomen invalidum

***Ophiostoma kubanicum* Sczerbin-Parfenenko**, Rak. Sos. Bol. List. Porod (Moscow) p. 49. 1953.

= *Ceratocystis kubanica* (Sczerbin-Parfenenko) Potlajczuk, Nov. Sist. Niz. Rast. 22: 153. 1985.

Anamorph Graphium kubanicum Sczerbin-Parfenenko, Rak. Sos. Bol. List. Porod (Moscow) p. 51. 1953.

Synanamorph Sporothrix (Przybyl and de Hoog 1989).

≡ *Verticillium kubanicum* Sczerbin-Parfenenko, Rak. Sos. Bol. List. Porod (Moscow) p. 51. 1953.

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