Mycopathologia
DOI 10.1007/s11046-009-9267-8

Ophiostoma tsotsi sp. nov., A Wound-infesting Fungus of Hardwood Trees in Africa

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

Received: 8 June 2009 / Accepted: 4 December 2009

Abstract Polymorphic sequence-characterised marker assays from a recent diversity study on the Ascomycete fungus *Ophiostoma quercus* reported that some isolates from Africa were genetically distinct from *O. quercus*. In the present study, these African isolates were compared with authentic *O. quercus* isolates by evaluating morphological characters, growth in culture, mating compatibility and DNA sequence data. The isolates from Africa were morphologically similar to *O. quercus,* presenting *Pesotum* and *Sporothrix* synanamorphs in culture. Phylogenetic analyses of the ribosomal internal transcribed spacer regions 1 and 2, β-tubulin and translation elongation factor 1-α gene regions confirmed that the African group represents a distinct species within the hardwood lineage of the *O. piceae* complex, closely related to *O. ulmi* and *O. himal-ulmi*. Mating studies between *O. quercus* and the African isolates showed that isolates mated predominantly with those of their own group, although there were rare cases of fertile crosses between the groups. Isolates residing in the African lineage are described here as a new species, *O. tsotsi* sp. nov.

Keywords *Ophiostoma quercus* · Blue-stain fungi · *Pesotum* · Ophiostomatales

Introduction

"Ex Africa semper aliquid novi—From Africa there is always something new".

Pliny the Elder (AD 23—AD 79).

The international timber trade between countries from various continents is continuously expanding, and with that the risk of the introduction of new pests and pathogens [1]. The most prevalent of these are wood-infesting bark beetles and their associated fungi, most often belonging to the Ophiostomatales, an order in the Ascomycetes. Three teleomorph genera are currently recognised in the Ophiostomatales: *Ceratocystiopsis, Grosmannia* and *Ophiostoma* [2]. The majority of the *ca.* 230 known species in the order cause stain of conifer and angiosperm wood which significantly reduces commercial value [3]. Additionally, included within this order of fungi are numerous tree pathogens such as the Dutch elm disease (DED)-causing fungi, renowned for the

Electronic supplementary material The online version of this article (doi:10.1007/s11046-009-9267-8) contains supplementary material, which is available to authorized users.

J. W. Grobbelaar · P. Bloomer · M. J. Wingfield · B. D. Wingfield
Department of Genetics, University of Pretoria, Pretoria 0002, South Africa

Z. W. de Beer (✉)
Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa
e-mail: wilhelm.debeer@fabi.up.ac.za

Published online: 07 January 2010

devastation they have caused to elm forests throughout the northern hemisphere [4, 5].

Related to the DED fungi is *Ophiostoma quercus,* a ubiquitous species reported most frequently from recent inventories of bark beetle associates [6–11], fungi isolated from wounds on trees [12–15], and stained wood products [16–21]. Understanding the population biology and origin of *O. quercus* will greatly assist in risk assessments and the formulation of appropriate quarantine measures.

Recently, our research group developed polymorphic sequence-characterised markers as a first step towards the exploration of genetic diversity in various populations of *O. quercus* [22]. These markers were tested on a collection of *O. quercus*-like isolates from Africa. The results revealed a high number of polymorphisms in the repeat and flanking sequences, generating many haplotypes. Further analyses of the data showed two distinct groups within the collection of what was believed to represent only *O. quercus* isolates. One group included the ex-neotype isolate and other authentic isolates of *O. quercus* and thus represented this species. The second group consisted only of isolates originating from sub-Saharan Africa. The aim of the present study was to compare fungal morphology and DNA sequences and use mating compatibility studies to resolve the taxonomic status of the two distinct groups of *O. quercus* isolates.

Materials and Methods

Fungal Isolates

Ophiostoma quercus-like isolates were collected from wounds on trees and cut timber of various types in Malawi, South Africa and Uganda (Table 1). Isolations were made as described by Kamgan Nkuekam et al. [13]. Single spore cultures were prepared for all isolates from germinating conidia or ascospores. All isolates used are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI). Herbarium specimens were deposited in the National Collection of Fungi, Pretoria, South Africa (PREM), and representative cultures including those used in mating compatibility tests have been deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

DNA Extraction, PCR Amplification and Sequencing

DNA was extracted from cultures using PrepMan Ultra (Applied Biosystems Inc., Foster City, CA, USA) and diluted 1 part in 10 parts deionised water. Primer pairs ITS1F [23] and ITS4 [24] were used to amplify the internal transcribed spacer (ITS) regions 1 and 2, including the ribosomal 5.8S gene. For the β-tubulin region, the primers T10 [25] and Bt2b [26] were used, and for the partial translation elongation factor 1-α (TEF1-α), primers EF1F and EF2R were used [27]. Conditions for the PCR and sequencing reactions were described by Grobbelaar et al. [15]. Sequenced products were purified, and fragments run on an ABI PRISM 3100 automated sequencer (Applied Biosystems Inc., Foster City, CA, USA).

Phylogenetic Analyses

Sequence data were analysed, and contigs assembled using the Vector NTI Advance 10 software (Invitrogen Corporation, Carlsbad, CA, USA). For comparisons, published sequences of related species from GenBank (accession numbers in Figs. 1, 2) were downloaded. The data sets were aligned using the E-INS option in MAFFT v. 5.731 [28]. Data for the three gene regions were analysed separately, because data were not available from GenBank for similar reference isolates.

Three different methods of phylogenetic analysis were used for each data set. These included Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI). The ITS and β-tubulin data sets were rooted against the sequences of type species of the genus *Ophiostoma*, *O. piliferum*. In the case of TEF1-α, phylogenetic analyses were performed without an out-group, as no sequence data were available for an appropriate root.

Un-weighted MP analyses were calculated using PAUP v. 4.0 beta 10 (Sinauer Associates, Sunderland, MA). The heuristic search algorithm was utilised to find the most parsimonious trees using tree bisection-reconnection (TBR) branch-swapping. One thousand bootstrap replicates [29] were performed to calculate confidence levels.

For ML, the most appropriate substitution models were selected using the Akaike Information Criterion in Modeltest v. 3.7 [30] for each of the three gene

Table 1 *Ophiostoma* isolates used in this study

Species	CMW no.	Other numbers	GenBank numbers			Host	Origin	Collector
			ITS	β-tubulin	TEF1-α			
O. floccosum	12622	CBS 123601	FJ430472	FJ430502	FJ430487	*Pinus sylvestris*	Austria	T Kirisits
	12623		FJ430473	FJ430503	FJ430488	*P. sylvestris*	Austria	T Kirisits
O. himal-ulmi	22729	CBS 374.67	FJ430474	FJ430504	FJ430489	*Ulmus* sp.	India	H Rebel
O. novo-ulmi	1461		FJ430476	FJ430506	FJ430491	*U. procera*	USA	C Brasier
	10573	CBS 119476	FJ430478	FJ430508	FJ430493	*Picea abies*	Austria	A Neumueller
	25033	CBS 298.87	FJ430479	FJ430509	FJ430494	*Ulmus* sp.	Russia	HM Heybroek
O. piceae	2468	CBS 123600	FJ430482	FJ430512	FJ430497	*P. abies*	France	M Morelet
	3099		FJ430484	FJ430514	FJ430499	*Picea* sp.	Canada	MJ Wingfield
	12615		FJ430480	FJ430510	FJ430495	*Larix decidua*	Austria	T Kirisits
	12617		FJ430481	FJ430511	FJ430496	*L. decidua*	Austria	T Kirisits
O. quercus	2465[a]	CBS 117912	AY466625	AY466646	AY466692	*Quercus* sp.	France	M Morelet
	2467	CBS 117913	AY466626	AY466647	AY466693	*Quercus* sp.	France	M Morelet
	2521		**FJ441283**	**FJ441275**	–	*Eucalyptus* sp.	South Africa	ZW de Beer
	3119[a]	CBS 115871	AY493244	AY466650	AY466696	*Pinus* sp.	South Africa	ZW de Beer
	5679		–	–	**FJ441265**	*Acacia mearnsii*	Tanzania	J Roux
	15930		–	–	**FJ441266**	*A. mearnsii*	Tanzania	R Heath
	19192		–	–	**FJ441267**	*Populus* sp.	Norway	G Kamgan, H Solheim, J Grobbelaar
	19251		–	–	**FJ441268**	*Quercus* sp.	Norway	G Kamgan, H Solheim, J Grobbelaar
	19256[a]		–	**FJ441276**	–	*Quercus* sp.	Norway	G Kamgan, H Solheim, J Grobbelaar
O. setosum	16534	CBS 123602	**FJ430486**	**FJ430516**	**FJ430501**	*Picea glauca*	Canada	A Uzunovic
O. tsotsi sp. nov.	3116		**FJ441285**	**FJ441278**	**FJ441270**	*A. mearnsii*	South Africa	ZW de Beer
	3117[b]	CBS 122287; PREM 59820	**FJ441284**	**FJ441277**	**FJ441269**	*Eucalyptus* sp.	South Africa	ZW de Beer
	5943[a]	PREM 60015	**FJ441286**	**FJ441279**	**FJ441271**	*A. mearnsii*	Uganda	J Roux
	15239[a,b]	CBS 122288; PREM 59820	**FJ441287**	**FJ441280**	**FJ441272**	*E. grandis*	Malawi	R Heath
	16779	PREM 60016	**FJ441288**	**FJ441281**	**FJ441273**	*E. grandis*	Malawi	J Roux
	18134[a]	CBS 123599	**FJ441289**	**FJ441282**	**FJ441274**	*Julbenardia globiflora*	Malawi	J Roux

Accession numbers to sequences obtained in the present study are printed in bold type

CMW Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, *CBS* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, *PREM* Herbarium of the National Collection of Fungi in Pretoria, South Africa

[a] Isolates used in the growth studies

[b] Isolates mated to obtain the holotype

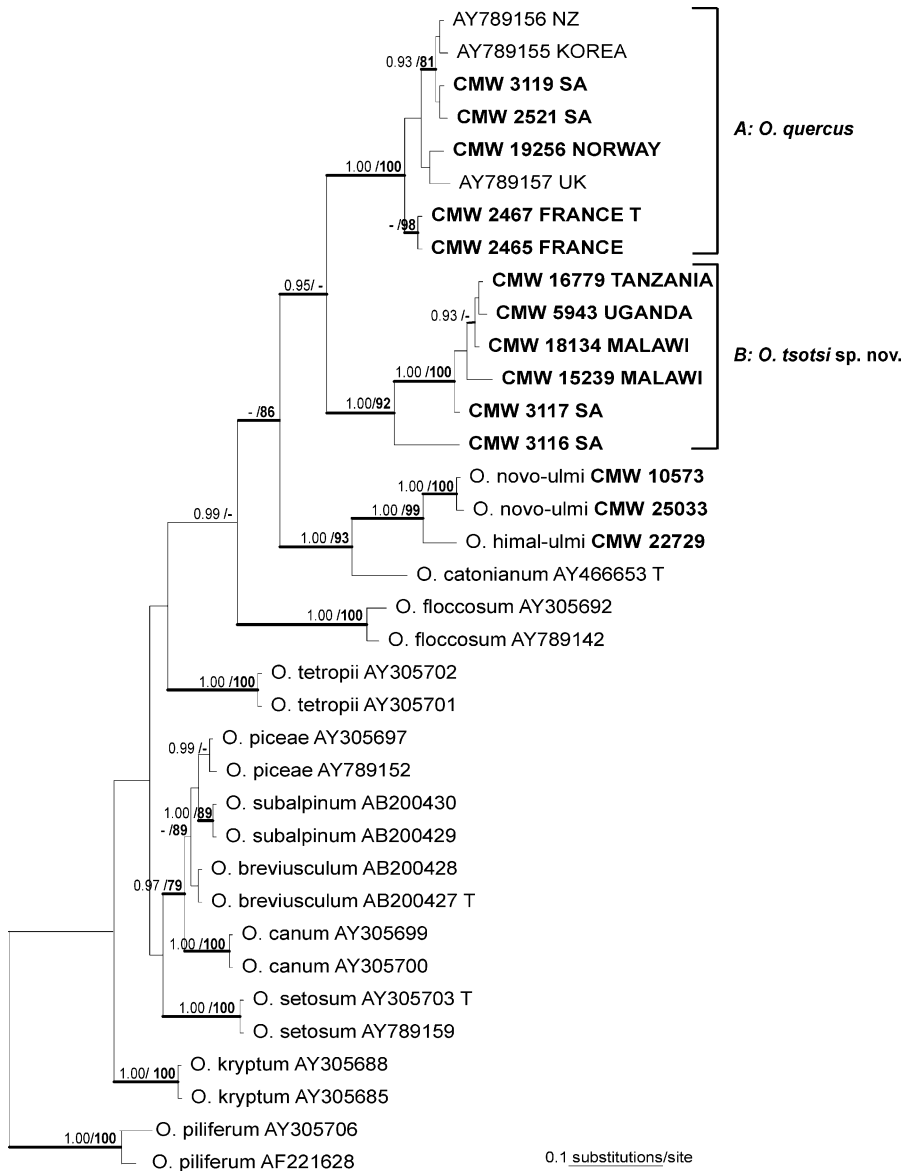

Fig. 1 Phylogram resulting from a Bayesian Monte Carlo Markov chain (MCMC) analysis of the β-tubulin sequences. For each node, posterior probability (pp) support values are given first followed by the ML bootstrap (bs) values (1,000 replicates). Scores of 0.90 pp or 70 bs and less are indicated with '–'. Bootstrap support values above 75% for Maximum parsimony are indicated with bold lines at branching points. Isolate numbers of sequences obtained in this study are printed in bold type. T = ex-type isolates

regions. ML analyses were conducted in PAUP. The reliability of nodes was estimated using 1,000 bootstrap replicates.

Bayesian analyses were performed in MrBayes v. 3.1.2 [31] using the best fit model selected by the AIC test in MrModeltest v. 2.2 [http://www.abc.se/~nylander/]. The Bayesian analyses comprised four independent runs of one million 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. The first 1,000 trees sampled were discarded as burn-in.

Growth in Culture

Three *O. quercus* isolates and three from the African group were selected (Table 1) for comparative growth studies. Mycelium-covered agar plugs (7 mm diameter) were transferred from the leading edge of one-week-old actively growing colonies to 90 mm diameter petri dishes containing 2% malt extract agar (MEA, Biolab, Midrand, South Africa). Three plates per isolate were incubated in the dark at each temperature, ranging from 5° to 35°C at 5°C intervals. For the purposes of measuring colony

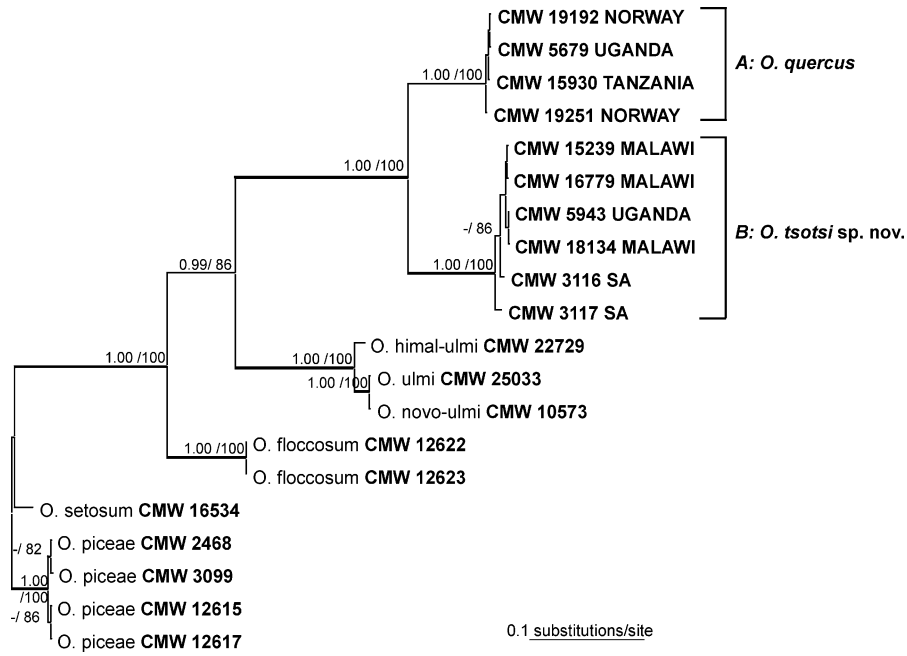

Fig. 2 Phylogram resulting from a Bayesian Monte Carlo Markov chain (MCMC) analysis of the TEF1-α sequences. For each node, posterior probability (pp) support values are given first followed by the ML bootstrap (bs) values. Scores of 0.90 pp or 70 bs and less were excluded and are indicated with '–'. Bootstrap support values above 75% for Maximum parsimony are indicated with bold lines at branching points. Isolate numbers of sequences obtained in this study are printed in bold type. *T* = ex-type isolates

diameter, two measurements at right angles to each other were made every other day for 10 days, starting on day two. The mean diameter was calculated in mm (±SD) for each isolate.

Mating Compatibility

Nine authentic *O. quercus* isolates and one from the African lineage were selected (Table 2) to determine whether the individuals of these two distinct groups were capable of mating with each other. Single spore cultures were transferred to 2% MEA and incubated for 14 days in the dark at 25°C. Agar blocks (ca. 4 mm × 4 mm) covered in mycelium of two different isolates (as indicated in Table 2) were placed adjacent to each other on plates containing WA (20 g agar, 1,000 ml distilled water), supplemented with debarked, autoclaved oak or eucalyptus twigs (ca. 5 mm × 5 mm × 25 mm). As a control, each isolate was also paired against itself. All isolates were crossed against two *O. quercus* tester strains of opposite mating types, either CMW 2520 or CMW 2521, from a previous study [17]. Plates were incubated at 22°C in the dark for 2–4 weeks and inspected weekly under a dissecting microscope for the presence of ascomata.

Crosses were considered positive when ascomata produced droplets of viable ascospores and where no ascomata formed on control plates. Positive crosses were scored with a (+), and mating types of either "a" or "b" were assigned based on the positive crosses with the tester strains. Crosses that produced

Table 2 Results of mating compatibility studies between nine isolates of *O. quercus* and an isolate representing the African group (number underlined)

Mating type →		a					b				
↓	Isolate no (CMW) → ↓	3119	19256	2534	18136	*2521	17600	19231	8221	*2520	5943
a	3119 South Africa	-	-	-	-	-	+	+	+	+	+
	19256 Norway		-	-	-	-	+	+	+	+	SP
	2534 South Africa			-	-	-	+	+	+	-	+
	18136 Malawi				-	-	+	+	+	-	(+)
	2521* South Africa					-	+	+	+	+	+
b	17600 South Africa						-	-	-	-	-
	19231 Norway							-	-	-	-
	8221 Bhutan								-	-	SP
	2520* South Africa									-	-
	5943 Uganda										-

Mating types (a or b) were assigned based on crosses with two *O. quercus* tester strains (*) developed by De Beer et al. [17]

\+ Positive cross: perithecia with ascospores formed

(+) Positive cross, but only a few perithecia formed

− Negative cross: no perithecia formed

SP sterile perithecia contained no ascospores

no ascomata were scored as negative (−). Crosses that produced ascomata without ascospores were labelled as sterile (S). In positive crosses between isolates of the *O. quercus* and African groups, drops of single spores were lifted from the ascomata with a sterile needle, suspended in sterile distilled water, shaken and spread onto 2% MEA plates. After 12–48 h of incubation, plates were inspected microscopically for germinating ascospores to determine the viability of the F1 progeny. Subsequently, F2 and F3 progenies were also cultured to confirm whether the isolates maintained viability.

Morphology

Ascomata from a positive cross between isolates residing in the African group, as well as anamorph structures were removed from the wood surface, mounted on glass slides in lactophenol and studied using a Zeiss Axiovision 2 Plus light microscope (Carl Zeiss, Jena, Germany). Twenty-five of each of the morphological structures were measured. The means and standard deviations were calculated and are presented as: (minimum-) mean minus standard deviation–mean–mean plus standard deviation (-maximum).

Scanning electron microscopy (SEM) was used for detailed examination of the ascomata and ascospores of *O. quercus*, the isolates of the African group, as well as a hybrid obtained in vitro between the two groups of isolates. The material was prepared and studied as described by Grobbelaar et al. [15].

Results

PCR and Sequence Analyses

Amplicons of approximately 620, 435 and 711 bp in length were produced for the ITS, β-tubulin and the TEF1-α gene regions, respectively. GenBank accession numbers are presented in Table 1.

Phylogenetic analyses

Analyses of the ITS data set (supplementary Fig. 1) showed two clusters with insufficient statistical support within what was previously considered to be *O. quercus*. The topologies of the MP consensus tree and the phylograms obtained from ML and Bayesian analyses were similar. MP analyses yielded 211 parsimony-informative characters, a consistency index (CI) of 0.87 and a retention index (RI) of 0.93 with four trees being retained.

Bayesian and ML analyses of the β-tubulin data (Fig. 1) generated phylograms that separated the isolates in this study into two well-supported lineages, respectively, representing *O. quercus* (A) and the African isolates (B). MP resulted in 116 parsimony-informative characters, a CI = 0.66 and RI = 0.87. The MP cladogram (bootstrap values in Fig. 1) also separated the taxa in two well-supported clades.

All phylogenetic analyses applied to the TEF1-α sequences confirmed the existence of two lineages (Fig. 2 a, b) with strong bootstrap support and posterior probabilities. MP analysis yielded 431 informative characters, a CI = 0.76 and RI = 0.89.

The datasets all supported the inclusion of the African lineage into the hardwood clade of the *O. piceae* complex. The African lineage is closely related to, but clearly distinct from *O. novo-ulmi, O. ulmi* and *O. himal-ulmi*.

Growth in Culture

Results of growth studies showed a trend where African isolates grew at a reduced rate when compared to *O. quercus* isolates at all temperatures below 30°C. At 30°C (Fig. 3), the mean colony diameter of African isolates was 45 mm (±8.3 mm) and that of *O. quercus* 34 mm (±6.8 mm). None of the isolates grew at 35°C.

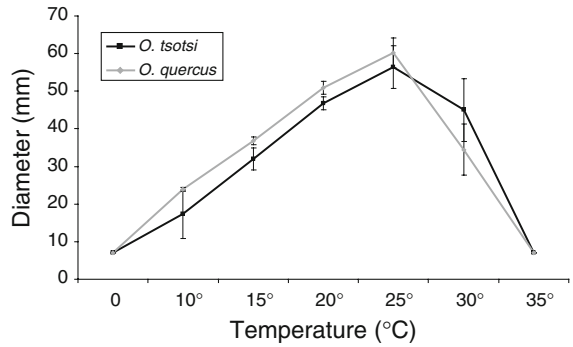

Fig. 3 Comparison of mean growth (±standard deviation) of *O. quercus* and *O. tsotsi* on MEA after 10 d at different temperatures. Three isolates were tested per species

Mating Compatibility

Most crosses between the *O. quercus* tester strains and other *O. quercus* isolates were fully fertile (Table 2). All isolates behaved in a heterothallic fashion and could clearly be segregated into two mating types based on their reactions with the tester strains. None of the control crosses produced ascomata. Positive crosses between *O. quercus* isolates and an African isolate (CMW 5943) occurred but were infrequent and resulted in sterile perithecia or only a few perithecia. Culture morphology of the progeny from positive crosses between *O. quercus* isolates and the African isolates was highly variable, similar to that of the parent isolates.

Morphology

The African isolates showed considerable variation in culture morphology, as was observed for *O. quercus* isolates. *Pesotum* and *Sporothrix* synanamorphs of the African isolates (Fig. 4) were similar to those of *O. quercus*. Ascomata of the African isolates were also similar to those of *O. quercus*, but the ascospore morphology differed slightly (Fig. 4). The ascospores of *O. quercus* were distinctly reniform in side view [32, 33], while those of the African isolates were orange segment shaped and less distinctly curved.

Taxonomy

Differences in morphology and β-tubulin and TEF1-α DNA sequences revealed that the African group of isolates treated in this study represents a species distinct from *O. quercus*. The fungus is thus described here as a novel taxon:

Ophiostoma tsotsi Grobbelaar, Z.W. de Beer & M.J. Wingf. sp. nov. MycoBank MB515219 (Fig. 4).

Etymology: tsotsi, from the Sesotho language meaning "to con", describing the cryptic nature of the African fungus that was not recognised as distinct from *O. quercus* [http://en.wikipedia.org/wiki/Tsotsitaal].

Ascomata: Bases nigrae vel brunneae, globosae. Colla atra, recta vel parum curvata. Hyphae ostiolares divergentes. Asci non visi. Ascosporae hyalinae, unicellulares, forma segmenti citri, in massa luteola in apice colli. Anamorpha Pesotum: in cultura dominans. Synnemata brunnea vel nigra, basi cum rhizoideis brunneis septatis; conidia hyalina unicellularia elliptica in guttula mucosa eburnea portata. Anamorpha Sporothrix micronema; cellulae conidiogenae proxime ex hyphis orientes, conidia hyalina laevia.

Holotype: PREM 59820, consisting of a dried culture, representing a cross between isolate CMW 3117 = CBS 122287 from *Eucalyptus* wood in South Africa collected by Z.W. de Beer and isolate CMW 15239 = CBS 122288 from *Eucalyptus grandis* in Malawi, isolated by R.N. Heath.

Additional specimens: Anamorph cultures PREM 60015, PREM 60016 and CBS 123599.

Teleomorph: Ascomata (Fig. 4a) emerging after 20 d on sterilised oak twigs or partially embedded in media when isolates of opposite mating type are paired. *Bases* black or brown, globose, (76.4–)79.4–100.1–120.8(–156) µm diameter longitudinally, (72.2–)91.1–119.2–147.3(–180.1) µm diameter transversely; ornamented with non-septate hyphae (16.7–)26.7–47.6–68.5(–94.6) µm long, (1.0–)1.3–2.0–2.7(–3.54) µm wide at apex. *Ascomatal necks* smooth, dark, light at apex, straight or slightly curved (351.8–)737–1047.7–1358.4(–1647) µm long, (17.6–)21.6–26.3–31(–36.1) µm wide at base, (8.5–)9.1–11.6–14.1(–14.9) µm at the apex. *Ostiolar hyphae* (Fig. 4b) hyaline, divergent, non-septate (1.1–)13.2–21–28.8(–33.81) µm × (0.6–)1–1.3–1.6(–1.6) µm. *Asci* not observed. *Ascospores* (Fig. 4c) hyaline, one celled, narrowly ellipsoidal, orange segment shaped in side view (2.8–)3.2–3.7–4.2(–4.6) µm × (1.5–)1.6–1.8–2(–2.5) µm, accumulated in a light yellow mass at the tip of the neck.

Pesotum synanamorph: synnemata (Fig. 4d, e) brown or black (394.7–)438.2–658.3–878.4(–1049.7) µm long (28.3–)29.3–32.9–36.5(–37.7) µm wide at base and (21–)26.2–33.4–40.6(–47.9) µm wide at apex, attached to substrate with brown, septate rhizoids (53.8–)50.1–90–129.90(–211.7) µm long and (1.5–)1.8–2.2–2.6(–3.1) µm wide; conidiogenous cells (0.8–)0.9–1.2–1.5(–2) × (4.9–)6.5–12.9–19.3(–29.6) µm; conidia (Fig. 4f) hyaline, smooth, one celled, elliptical (1.1–)1.3–1.5–1.7(–1.8) × (2.7–)3.1–3.4–3.7(–4.1) µm, aggregating into an ivory-coloured mucilaginous spore drop.

Sporothrix synanamorph (Fig. 4g, h, i): micronematous, conidiogenous cells arising directly from hyphae with multiple denticles (7.7–)23.1–27–53(–78.6) × (0.9–)1.5–2.1–2.4(–2.6) µm; conidia hyaline, smooth (3–)5.3–13–20.7(–24) × (0.5–)0.9–2–2.9(–3.4) µm.

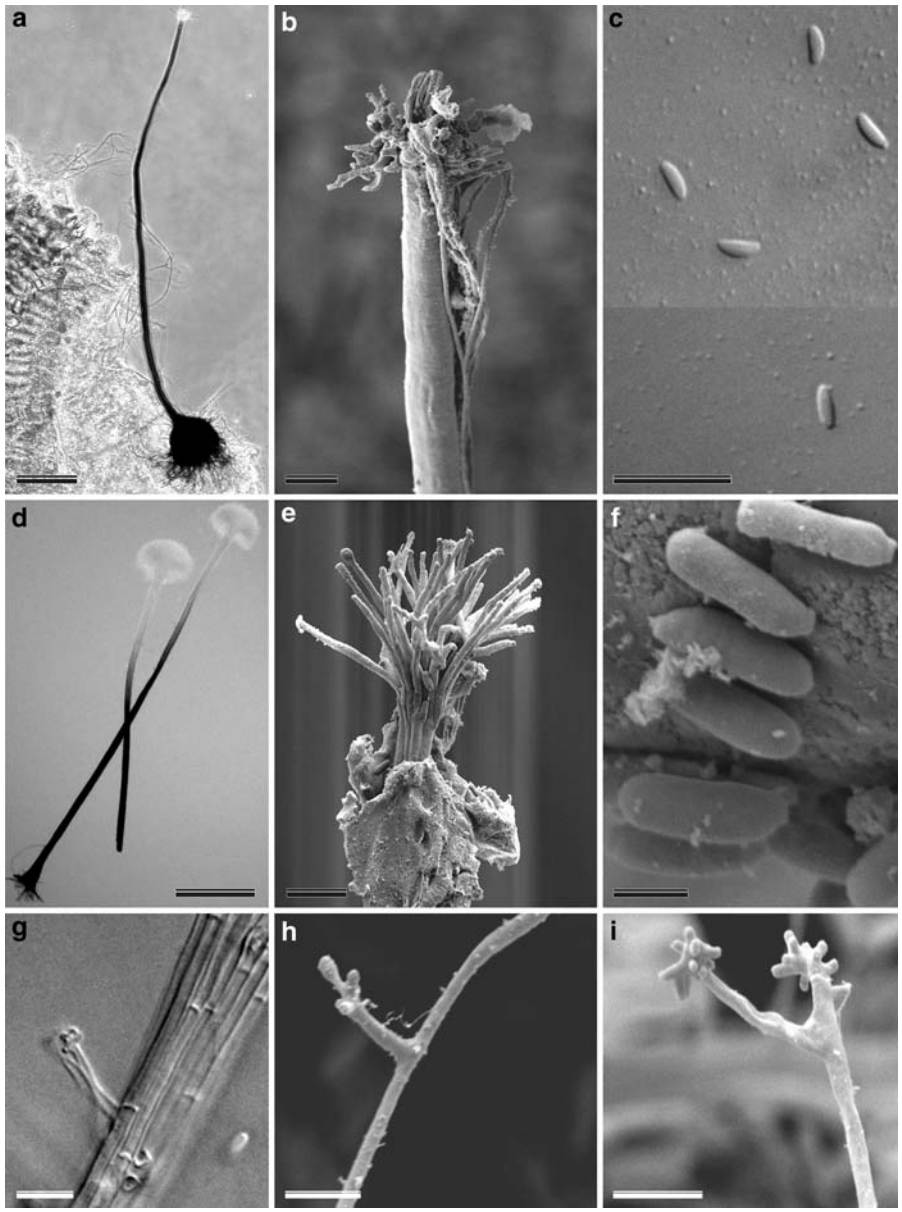

Fig. 4 Morphological characteristics of *Ophiostoma tsotsi* (from holotype) (CMW 3117 × CMW 15239). Teleomorph: **a** perithecium (bar = 100 μm), **b** scanning electron micrograph (SEM) of ostiolar hyphae at apex of perithecial neck (bar = 10 μm) and **c** ascospores, side view above and face view below (bar = 10 μm). *Pesotum* synanamorph: **d** synnemata (bar = 100 μm), **e** synnematal apex with conidiogenous cells (SEM, bar = 10 μm) and **f** conidia (SEM, bar = 5 μm). *Sporothrix* synanamorph: **g** denticulate conidiogenous cell (bar = 100 μm), **h** conidiogenous cell with emerging conidia (SEM, bar = 10 μm) and **i** conidiogenous cells with characteristic denticles (SEM, bar = 10 μm)

Colonies reaching 56.4 mm diameter on 2% MEA in 10 days at 25°C in the dark. *Pesotum* synanamorph dominant in culture. Mycelium hyaline, superficial on agar.

Sexuality: heterothallic.

Host range: Associated with wounds on native and exotic hardwood tree species in Southern Africa infested by bark beetles, flies and nitidulid beetles.

Distribution: Presently known from sub-Saharan African countries, but most likely occurs elsewhere on *Eucalyptus* spp. and *Acacia mearnsii*.

Discussion

Ophiostoma quercus is a well-known species, commonly found on angiosperm wood in many parts of the world. In this study, it was shown that isolates from Africa resembling *O. quercus* represent a distinct phylogenetic lineage for which the new name *O. tsotsi* has been provided. The first evidence for the existence of a cryptic species amongst *O. quercus* isolates emerged from a genetic sampling using polymorphic markers [22] designed for *O. quercus* isolates from Southern Africa. Results revealed the differences between the genotypes, separating the isolates into two groups, the unique nature of which was confirmed in this study.

Phylogenetic analyses for β-tubulin and TEF1-α gene regions confirmed that *O. quercus*-like isolates from sub-Saharan Africa represented a species distinct from *O. quercus*. The new species, *O. tsotsi,* groups closely to *O. quercus* and the Dutch elm disease pathogens, *O. ulmi* and *O. himal-ulmi,* within the hardwood clade of the *O. piceae* complex.

The morphology of *O. tsotsi* is similar to that of *O. quercus.* Phenotypically, isolates of both species can be highly variable in culture. However, ascospores of *O. tsotsi* differ slightly in shape from those of *O. quercus.* In addition, cultures of *O. tsotsi* grew more slowly than those of *O. quercus* at 25°C, but faster than the latter at 30°C. Although these morphological characteristics appear stable, speciation does not necessarily involve morphological changes. Therefore, genetic diversity and the use of molecular sequence data are especially important to delineate closely related species [34, 35] such as those in this study.

In mating studies between *O. tsotsi* and authentic *O. quercus* isolates, isolates predominantly mated with those of their own group. However, a rare case of interfertility was observed where one isolate from each of the two taxa mated, but the resulting ascospore progeny had reduced germination capacity. Incomplete reproductive isolation has been reported for other examples of fungal speciation [36–38]. Partial interfertility sometimes occurs between distinct species [39], but usually produces fewer ascospores with a decreased ascospore germination percentage than that seen at intraspecific level [40].

Ophiostoma tsotsi, discovered in this study, commonly occurs on cut timber throughout Southern Africa. Interestingly, this new taxon is frequently found in association with *O. quercus*, and it is not surprising that it remained hidden from recognition as a discrete entity. *O. quercus* is considered to be a species of northern hemisphere origin [41], and its presence on lumber in Africa suggests that it has been introduced into this region. This could easily have occurred with wood and wood products transported from the northern hemisphere to Africa. Although movement of wood products from Africa to northern hemisphere countries would have been less common, it is also possible that *O. tsotsi* has a distribution more extensive than is currently known. Population genetic studies on *O. quercus* and *O. tsotsi* will contribute to a deeper understanding of the distribution and origin of these fungi. In addition, the biology, pathogenicity and ecological role of *O. tsotsi* are unknown. In view of its relatedness to the Dutch elm disease-causing fungi, these aspects of its ecology deserve serious consideration.

Acknowledgments We thank Jolanda Roux, Ronald Heath and other colleagues who assisted with the collection of isolates. We acknowledge our African collaborators: Gerald Meke from the Forestry Research Institute of Malawi, Aza Mbaga from the Tanganyika Wattle Company, Tanzania, the Forestry Department and Makerere University, Uganda. We are also grateful to Dr. Seonju Marincowitz for assisting with light microscopy, Mr Alan Hall for assistance with scanning electron microscopy and Dr. Hugh Glen for providing the Latin diagnosis. Funding for this study was provided by the National Research Foundation (NRF), the Tree Protection Co-operative Programme (TPCP), the THRIP initiative of the Department of Trade and Industry (DTI) and the Department of Science and Technology (DST)/NRF Centre of Excellence in Tree Health Biotechnology, South Africa.

References

1. Piel F, Gilbert M, De Cannière C, Grégoire J-C. Coniferous round wood imports from Russia and Baltic countries to Belgium. A pathway analysis for assessing risks of exotic pest insect introductions. Divers Distrib. 2008;14:318–28.
2. Zipfel RD, De Beer ZW, Jacobs K, Wingfield BD, Wingfield MJ. Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Stud Mycol. 2006;55:75–97.

3. Seifert KA, Wingfield MJ, Kendrick WB. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: Wingfield MJ, Seifert KA, Webber J, editors. *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. St. Paul, Minnesota: APS Press; 1993. pp. 141–51.
4. Brasier CM. China and the origins of Dutch elm disease: an appraisal. Plant Pathol. 1990;39:5–16.
5. Brasier CM. *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. Mycopathol. 1991;115:151–61.
6. Lin TC, Huang JW, Hsieh WH. Identification of ophiostomatoid fungi associated with Chinese fir wilt in Taiwan [in Chinese]. Plant Pathol Bull. 2003;12:33–42.
7. Zhou XD, De Beer ZW, Ahumada R, Wingfield BD, Wingfield MJ. *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. Fungal Divers. 2004;15:261–74.
8. Zhou X, De Beer ZW, Wingfield MJ. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. Stud Mycol. 2006;55:269–77.
9. Carlier F-X, Decock C, Jacobs K, Maraite H. *Ophiostoma arduennense* sp. nov. (Ophiostomatales, Ascomycota) from *Fagus sylvatica* in southern Belgium. Mycol Res. 2006;110:801–10.
10. Romón P, Zhou X, Iturrondobeitia JC, Wingfield MJ, Goldarazena A. *Ophiostoma* species (Ascomycetes: Ophiostomatales) associated with bark beetles (Coleoptera: Scolytinae) colonizing *Pinus radiata* in northern Spain. Can J Microbiol. 2007;53:756–67.
11. Linnakoski R, De Beer ZW, Rousi M, Niemelä P, Pappinen A, Wingfeld MJ. Fungi including *Ophiostoma karelicum* sp. nov., associated with *Scolytus ratzeburgi* infesting birch in Finland and Russia. Mycol Res. 2008;112:1475–88.
12. Geldenhuis MM, Roux J, Montenegro F, De Beer ZW, Wingfield MJ, Wingfield BD. Identification and pathogenicity of *Graphium* and *Pesotum* species from machete wounds on *Schizolobium parahybum* in Ecuador. Fungal Divers. 2004;15:135–49.
13. Kamgan Nkuekam G, Jacobs K, De Beer ZW, Wingfield MJ, Roux J. *Ceratocystis* and *Ophiostoma* species including three new taxa, associated with wounds on native South African trees. Fungal Divers. 2008;29:37–59.
14. Kamgan Nkuekam G, Jacobs K, De Beer ZW, Wingfield MJ, Roux J. *Pesotum australi* sp. nov. and *Ophiostoma quercus* associated with *Acacia mearnsii* trees in Australia and Uganda, respectively. Australas Plant Pathol. 2008;37:406–16.
15. Grobbelaar J, Aghayeva D, De Beer ZW, Bloomer P, Wingfield M, Wingfield B. Delimitation of *Ophiostoma quercus* and its synonyms using multiple gene phylogenies. Mycol Prog. 2009;8:221–36.
16. De Beer ZW, Wingfield BD, Wingfield MJ. The occurrence of *Ophiostoma piliferum*-like fungi on pulpwood chips and other wood sources in South Africa. S A J Sci. 2003;99:34–6.
17. De Beer ZW, Wingfield BD, Wingfield MJ. The *Ophiostoma piceae* complex in the southern hemisphere: a phylogenetic study. Mycol Res. 2003;107:469–76.
18. Kim G-H, Kim J-J, Lim YW, Breuil C. Ophiostomatoid fungi isolated from *Pinus radiata* logs imported from New Zealand to Korea. Can J Bot. 2005;83:272–8.
19. Kim G-H, Kim J-J, Breuil C. Sap-staining fungi from logs and boards of two commercially important pines in Korea. Holzforschung. 2007;61:333–6.
20. Thwaites JM, Farrell RL, Hata K, Carter P, Lausberg M. Sapstain fungi on *Pinus radiata* logs—from New Zealand forest to export in Japan. J Wood Sci. 2004;50:459–65.
21. Thwaites JM, Farrell RL, Duncan SM, Reay SD, Blanchette RA, Hadar E, et al. Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. N Z J Bot. 2005;43:653–63.
22. Grobbelaar JW, Barnes I, Cortinas M-N, Bloomer P, Wingfeld MJ, Wingfeld BD. Development and characterization of polymorphic markers for the sap-stain fungus *Ophiostoma quercus*. Mol Ecol Resour. 2008;9:399–401.
23. Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol Ecol. 1993;2:113–8.
24. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and application. San Diego: Academic Press; 1990. p. 315–22.
25. O'Donnell K, Cigelnik E. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol Phylogenet Evol. 1997;7:103–16.
26. Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous *Ascomycetes*. Appl Environ Microbiol. 1995;61:1323–30.
27. Jacobs K, Bergdahl DR, Wingfield MJ, Halik S, Seifert KA, Bright DE, et al. *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. Mycol Res. 2004;108:411–8.
28. Katoh K, Toh H. Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform. 2008;9:286–98.
29. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evol. 1985;39:783–91.
30. Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. Bioinformatics. 1998;14:817–8.
31. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003;19:1572–4.
32. Upadhyay HP. A monograph of *Ceratocystis* and *Ceratocystiopsis*. Athens: University of Georgia Press; 1981.
33. Kowalski T, Butin H. Taxonomie bekannter und neuer *Ceratocystis*-Arten an Eiche (*Quercus robur* L). J Phytopathol. 1989;124:236–48.
34. Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, Winker K, et al. Cryptic species as a window on diversity and conservation. Trends Ecol Evol. 2007;22:148–55.
35. Noor MAF, Feder JL. Speciation genetics: evolving approaches. Nat Rev Gen. 2006;7:851–61.
36. Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, et al. Phylogenetic species recognition and

species concepts in fungi. Fungal Genet Biol. 2000;31: 21–32.
37. Engelbrecht CJB, Harrington TC. Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. Mycologia. 2005;97:57–69.
38. Kohn LM. Mechanisms of fungal speciation. Ann Rev Phytopathol. 2005;43:279–308.
39. Brasier CM. The genetic system as a fungal taxonomic tool: gene flow, molecular variation and sibling species in the *'Ophiostoma piceae—O. ulmi'* complex and its taxonomic and ecological significance. In: Wingfield MJ, Seifert KA, Webber J, editors. *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. St. Paul, Minnesota: APS Press; 1993. pp. 77–92.
40. Harrington TC, McNew DL. Partial interfertility among the *Ceratocystis* species on conifers. Fungal Genet Biol. 1998;25:44–53.
41. Harrington TC, McNew D, Steimel J, Hofstra D, Farrell R. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch Elm disease fungi. Mycologia. 2001;93:111–36.