Discovery of *Ophiostoma tsotsi* on *Eucalyptus* wood chips in China

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Abstract

Ophiostoma species such as *O. quercus* are the most frequent causal agents of sapstain of freshly felled hardwood timber and pulpwood. Many species are regarded as economically important agents of wood degradation. The aim of this study was to identify a collection of *Ophiostoma* isolates, resembling *O. quercus*, found on stained *Eucalyptus* pulpwood chips in China. DNA sequences of the internal transcribed spacer regions, including the 5.8S region, of the ribosomal DNA, and parts of the β-tubulin and elongation factor-1α genes, revealed that the isolates were not *O. quercus*. Surprisingly, they represented *O. tsotsi*, a wound-infesting fungus recently described from hardwoods in Africa. In addition, sequence data from an isolate from agarwood in Vietnam, identified in a previous study as belonging to an unknown *Pesotum* species, were also shown to represent *O. tsotsi*. A high level of genetic variability was observed among isolates of both *O. quercus* and *O. tsotsi*. This was unexpected and suggests that both species have been present in Asia for a significant amount of time.

Introduction

Eucalyptus spp. are becoming increasingly widely planted in plantations in many countries to produce a sustainable source of timber. This is largely due to their superb wood qualities, adaptability to a wide range of different environments, and their rapid growth (Turnbull 2000). They are planted extensively in Southeast Asia where the timber is mainly used for paper, oil, and pulp production. About 30% of China's 175 million ha of forests are commercial plantations, approximately 2 million ha of which consist of *Eucalyptus* and *Corymbia* species, hybrids, and clones (Anonymous 2006).

Diseases present one of the greatest threats to *Eucalyptus* plantation forestry, worldwide (Wingfield *et al*. 2008). In this regard, a number of known and novel forest pathogens have emerged from recent surveys on Eucalyptus in China (Butterworth and Lei 2005; Zhou *et al*. 2007, 2008). However, the pathogens listed in these surveys include only a single ophiostomatoid fungus and an uncharacterized *Ceratocystis* sp. (Zhou *et al*. 2008). This, despite the fact that in recent years numerous *Ceratocystis* and *Ophiostoma* species have been associated with disease and blue-stain on

commercial *Eucalyptus* trees, timber, and pulpwood (De Beer *et al*. 2003a, b; Roux *et al*. 2004; Van Wyk *et al*. 2007; Rodas *et al*. 2008). These fungal infections most often occur through wounds in the bark and sapwood of trees caused by commercial harvesting practices or animal damage (Roux and Wingfield 2009). The exposed sapwood is susceptible to colonization by ophiostomatoid fungi, vectored by a large variety of relatively non-specific insects (Seifert 1993).

One of the ophiostomatoid fungi most frequently isolated from exposed sapwood or *Eucalyptus* pulpwood chips is *Ophiostoma quercus* (Georgev.) Nannf. (De Beer *et al*. 2003a, b). This species is a ubiquitous sapstain fungus primarily occurring on hardwoods, and to a lesser extent on conifers, with a global distribution (Brasier and Kirk 1993; Harrington *et al*. 2001; Geldenhuis *et al*. 2004; Thwaites *et al*. 2004; Zhou et al. 2004; Kamgan *et al*. 2008; Linnakoski *et al*. 2008; Nkuekam et al. 2008). The first confirmed reports of *O. quercus* from east Asia were published only during the past decade (De Beer *et al*. 2003b; Lin *et al*. 2003; Kim *et al*. 2005; Chung *et al*. 2006; Masuya *et al*. 2009; Paciura *et al*. 2010). However, it has been suggested that isolates reported as *O. piceae* (Münch) Syd. & P. Syd. from several hardwood species in Japan by Nisikado and Yamauti (1935), possibly represented *O. quercus* (De Beer *et al*. 2003b).

As part of an ongoing survey of fungi infecting *Eucalyptus* and *Corymbia* species in China (Zhou *et al*. 2008), *Eucalyptus* pulpwood chips, collected in Guangdong province in the southern part of mainland China, were screened for the presence of ophiostomatoid fungi. A collection of cultures with *Pesotum* anamorphs reminiscent of the anamorph of *O. quercus* was isolated from the chips. The aim of this study was to determine the identity of these isolates, using culture morphology and DNA sequence comparisons of three gene regions that are regularly used to distinguish between *O. quercus* and closely related species (De Beer *et al*. 2003b; Linnakoski *et al*. 2008, 2009; Grobbelaar *et al*. 2009, 2010).

Materials and methods

Collection and isolation of fungi

Eucalyptus pulpwood chips were collected from a small commercial chipping factory in Leizhou, China. The wood chips were incubated in moist chambers at 25 C until fruiting structures appeared. Isolations were made and purified as described by Kamgan *et al*. (2008). For reference purposes, several isolates from Eucalyptus in South Africa, and other hosts in China and elsewhere were included (Table 1). All of the isolates sequenced in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) and a duplicate set is maintained in the China Eucalypt Research Centre (CERC).

Culture and anamorph morphology

Single spore cultures from germinating ascospores or conidia were prepared for all isolates obtained in this study. Isolates were grown on 2% malt extract agar (MEA; Biolab, Midrand, South Africa) at room temperature for 10 days. Culture morphology was compared to descriptions of those of *O. quercus* and closely related species. Fruiting structures were mounted in lactophenol and examined using a compound microscope.

DNA sequencing and phylogenetic analyses

Genomic DNA was extracted from actively growing fungal mycelium using the method described by Linnakoski *et al*. (2008). The internal transcribed spacer (ITS) regions, including the 5.8S region, of the ribosomal DNA, and parts of the b-tubulin (BT) and elongation factor-1a (EF) genes were amplified using the same primers and polymerase chain reaction (PCR) conditions as those

described by Grobbelaar *et al*. (2009). Contigs were assembled and sequences aligned in exactly the same manner as done by these authors. For reference purposes, published sequences of all three gene regions were obtained from GenBank for *O. quercus*, *O. tsotsi* Grobbelaar, Z.W. de Beer & M.J. Wingf., and other species closely related to *O. quercus*. No EF sequences were available for *O. karelicum* Linnak., Z.W. de Beer & M.J. Wingf. and *O. denticiliatum* Linnak., Z.W. de Beer & M.J. Wingf. that also form part of the hardwood group in the *O. piceae*-complex (Linnakoski *et al*. 2008, 2009). Phylogenetic relationships between isolates were examined using maximum likelihood (ML) and Bayesian inference (BI) as described by Grobbelaar *et al*. (2009). Appropriate substitution models were selected for the two types of analyses using the Akaike Information Criterion in Modeltest v. 3.7 (Posada and Crandall 1998) and MrModeltest v. 2.2 (http://www.abc.se/*nylander/), respectively. All trees were rooted against *O. floccosum* Math.-Käärik.

Table 1. *Ophiostoma* isolates from *Eucalyptus* in China, as well as reference isolates of *Ophiostoma*, for which DNA sequences were determined in the present study.

Teleomorph	CMW no.[a]	Host	Origin	Collector(s)	GenBank		
					ITS	BT	EF-1α
O. quercus	5679	*Acacia mearnsii*	Uganda	Roux	**HQ131894**	**HQ131893**	FJ441265
	19192	*Populus* sp.	Norway	Kamgan, Solheim	**HQ131895**	GQ249302	FJ441267
	12287	*Tsuga dumosa*	China	Zhou, De Beer	FJ434947	FJ455563	**HQ131904**
	12298	*Salix babylonica*	China	Zhou, De Beer	FJ434946	FJ455562	**HQ131905**
O. tsotsi	17573	*Terminalia serecia*	South Africa	Kamgan	EF408562	**FJ441255**	**HQ131906**
	17606	*Eucalyptus grandis*	South Africa	Kamgan	**HQ131896**	**FJ441256**	**HQ131907**
	17618	*E. grandis*	South Africa	Kamgan	**HQ131897**	**FJ441257**	**HQ131908**
	24802	*Eucalyptus* pulpwood	China	Wingfield, Zhou	**HQ131898**	**FJ441258**	**HQ131909**
	24806	*Eucalyptus* pulpwood	China	Wingfield, Zhou	**HQ131899**	**FJ441259**	**HQ131910**
	24813	*Eucalyptus* pulpwood	China	Wingfield, Zhou	**HQ131900**	**FJ441260**	**HQ131911**
	24816	*Eucalyptus* pulpwood	China	Wingfield, Zhou	**HQ131901**	**FJ441261**	NA
	24819	*Eucalyptus* pulpwood	China	Wingfield, Zhou	**HQ131902**	**FJ441262**	**HQ131912**
	24822	*Eucalyptus* pulpwood	China	Wingfield, Zhou	**HQ131903**	**FJ441263**	**HQ131913**
	24828	*Eucalyptus* pulpwood	China	Wingfield, Zhou	NA	**FJ441264**	**HQ131914**

Results

Culture and anamorph morphology

There was substantial variability in culture and anamorph morphology among the isolates from China and elsewhere, but all corresponded broadly to culture descriptions for *O. quercus* (Morelet 1992; Halmschlager *et al*. 1994; Harrington *et al*. 2001), *Pesotum australiae* Kamgan-Nkuekam, K. Jacobs & M.J. Wingf. (Nkuekam *et al*. 2008), *O. denticiliatum* (Linnakoski *et al*. 2009), and *O. tsotsi* (Grobbelaar *et al*. 2010). However, none of the Chinese isolates produced a teleomorph and none of the isolates could be conclusively assigned to any of the above mentioned four species based on phenotypic characters.

DNA sequencing and phylogenetic analyses

Amplicons from the partial ITS, BT, and EF gene regions, respectively, consisted of approximately 580, 315, and 330 base pairs, and the aligned data sets included 53, 48, and 41 isolates. For all three data sets the generalised time reversible (GTR) substitution model was selected as the most appropriate, with varying values for the proportion of invariable sites and gamma distribution rates. Results from the ML and BI analyses yielded concordant topologies with respect to the composition of the clades for all three gene regions.

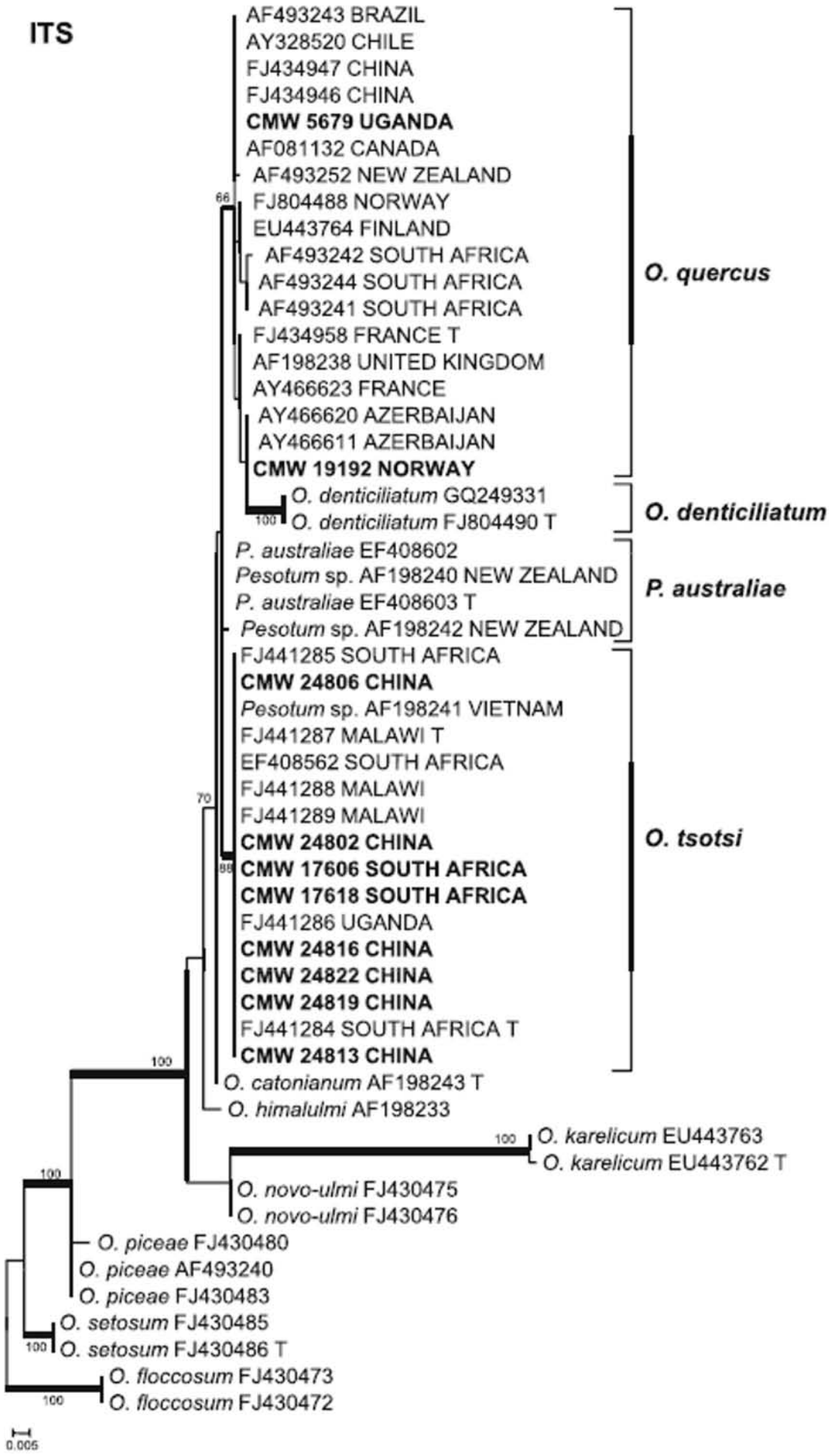
ITS
AF493243 BRAZIL
AY328520 CHILE
FJ434947 CHINA
FJ434946 CHINA
CMW 5679 UGANDA
AF081132 CANADA
AF493252 NEW ZEALAND
FJ804488 NORWAY
EU443764 FINLAND
AF493242 SOUTH AFRICA
AF493244 SOUTH AFRICA
AF493241 SOUTH AFRICA
FJ434958 FRANCE T
AF198238 UNITED KINGDOM
AY466623 FRANCE
AY466620 AZERBAIJAN
AY466611 AZERBAIJAN
CMW 19192 NORWAY
O. quercus
O. denticiliatum GQ249331
O. denticiliatum FJ804490 T
O. denticiliatum
P. australiae EF408602
Pesotum sp. AF198240 NEW ZEALAND
P. australiae EF408603 T
Pesotum sp. AF198242 NEW ZEALAND
P. australiae
FJ441285 SOUTH AFRICA
CMW 24806 CHINA
Pesotum sp. AF198241 VIETNAM
FJ441287 MALAWI T
EF408562 SOUTH AFRICA
FJ441288 MALAWI
FJ441289 MALAWI
CMW 24802 CHINA
CMW 17606 SOUTH AFRICA
CMW 17618 SOUTH AFRICA
FJ441286 UGANDA
CMW 24816 CHINA
CMW 24822 CHINA
CMW 24819 CHINA
FJ441284 SOUTH AFRICA T
CMW 24813 CHINA
O. tsotsi
O. catonianum AF198243 T
O. himalulmi AF198233
O. karelicum EU443763
O. karelicum EU443762 T
O. novo-ulmi FJ430475
O. novo-ulmi FJ430476
O. piceae FJ430480
O. piceae AF493240
O. piceae FJ430483
O. setosum FJ430485
O. setosum FJ430486 T
O. floccosum FJ430473
O. floccosum FJ430472
66
100
70
98
100
100
100
100
100
100
0.005

Figure 1. Phylogram resulting from a maximum likelihood (ML) analysis of the internal transcribed spacer (ITS) sequences. ML bootstrap values (1000 replicates) above 70% are given at nodes. Branches with posterior probability support values (above 90%) obtained from Bayesian analyses are indicated with bold lines. Isolate numbers for sequences obtained in the present study are printed in bold type. T indicates ex-type isolates of species.

In the ITS tree (Fig. 1) *O. quercus*, *O. denticiliatum*, *P. australiae*, and *O. tsotsi* grouped together in a weakly supported monophyletic lineage. Within this lineage, only the lineages containing *O. denticiliatum* and *O. tsotsi* had significant statistical support. The Chinese isolates from *Eucalyptus* all grouped with the African isolates of *O. tsotsi*. A single isolate from *Aquilaria crassna* (agarwood) in Vietnam, labelled by Harrington *et al*. (2001) as an unknown *Pesotum* species, also grouped with the *O. tsotsi* isolates. Two other *Pesotum* isolates, respectively from *Pinus* and *Nothofagus* in New Zealand (Harrington *et al*. 2001), had identical sequences and grouped with *Pesotum australiae*, although these four isolates did not form a monophyletic lineage with statistical support. The lineage containing *O. quercus* isolates showed considerable variation between isolates, and did not have strong statistical support.

The BT tree (Fig. 2) showed better resolution between *O. quercus*, *O. denticiliatum*, *P. australiae*, and *O. tsotsi*, with good statistical support for all four lineages. Although the Chinese isolates from *Eucalyptus* did not all have identical sequences, all the isolates grouped clearly with the African *O. tsotsi* isolates. The BT sequences of both *O. quercus* and *O. tsotsi* exhibited substantial variation among isolates.

Isolates of *O. quercus*, *O. tsotsi*, and *P. australiae* formed three well-supported lineages based on the EF data (Fig. 3). The isolates from *Eucalyptus* in China all grouped with *O. tsotsi*. In the present study, EF sequences were also produced for two *O. quercus* isolates from *Tsuga* in China, which Paciura *et al*. (2010) identified based on ITS and BT sequences. These two isolates formed a sub-clade within the larger, well-supported *O. quercus* group. Both *O. quercus* and *O. tsotsi* lineages exhibited substantial variation in EF sequences among isolates.

Discussion

In this study, *Ophiostoma tsotsi* was discovered on wood of exotic *Eucalyptus* trees in China. This is the first time the fungus has been reported outside of Africa. Furthermore, analyses of ITS sequence data suggested that a previously collected *Pesotum* isolate from agarwood in Vietnam also represents *O. tsotsi*. Sequence data for both the BT and EF gene regions of *O. tsotsi* showed substantial variability within this species and the closely related *O. quercus*.

Ophiostoma tsotsi is phylogenetically closely related to and morphologically virtually indistinguishable from *O. quercus* (Grobbelaar *et al*. 2010). Very limited knowledge is available for this fungus, but it seems that its host range and distribution overlap with those of *O. quercus* (Harrington *et al*. 2001; De Beer *et al*. 2003b; Grobbelaar *et al*. 2010). *Ophiostoma quercus* primarily occurs on hardwoods and for many years it was considered a synonym of *O. piceae* (Münch) Syd. & P. Syd. (Hunt 1956), which mainly occurs on conifers (Harrington *et al*. 2001). Mating compatibility (Morelet 1992; Brasier and Kirk 1993), growth studies (Brasier and Stephens 1993), and DNA-based techniques (Halmschlager *et al*. 1994; Pipe *et al*. 1995; Kim *et al*. 1999; Harrington *et al*. 2001; De Beer *et al*. 2003b), have confirmed that *O. piceae* and *O. quercus* are distinct species. Based on these results, De Beer *et al*. (2003b) suggested that many reports of '*O. piceae*' on hardwoods, in the almost 40-year-period during which *O. quercus* was treated as a synonym of *O. piceae*, might have actually represented *O. quercus*. However, in recent studies several novel cryptic species similar to *O. quercus* have been described from hardwoods, including *O. tsotsi*, *Pesotum australiae*, and *O. denticiliatum* (Kamgan *et al*. 2008; Linnakoski *et al*. 2009;

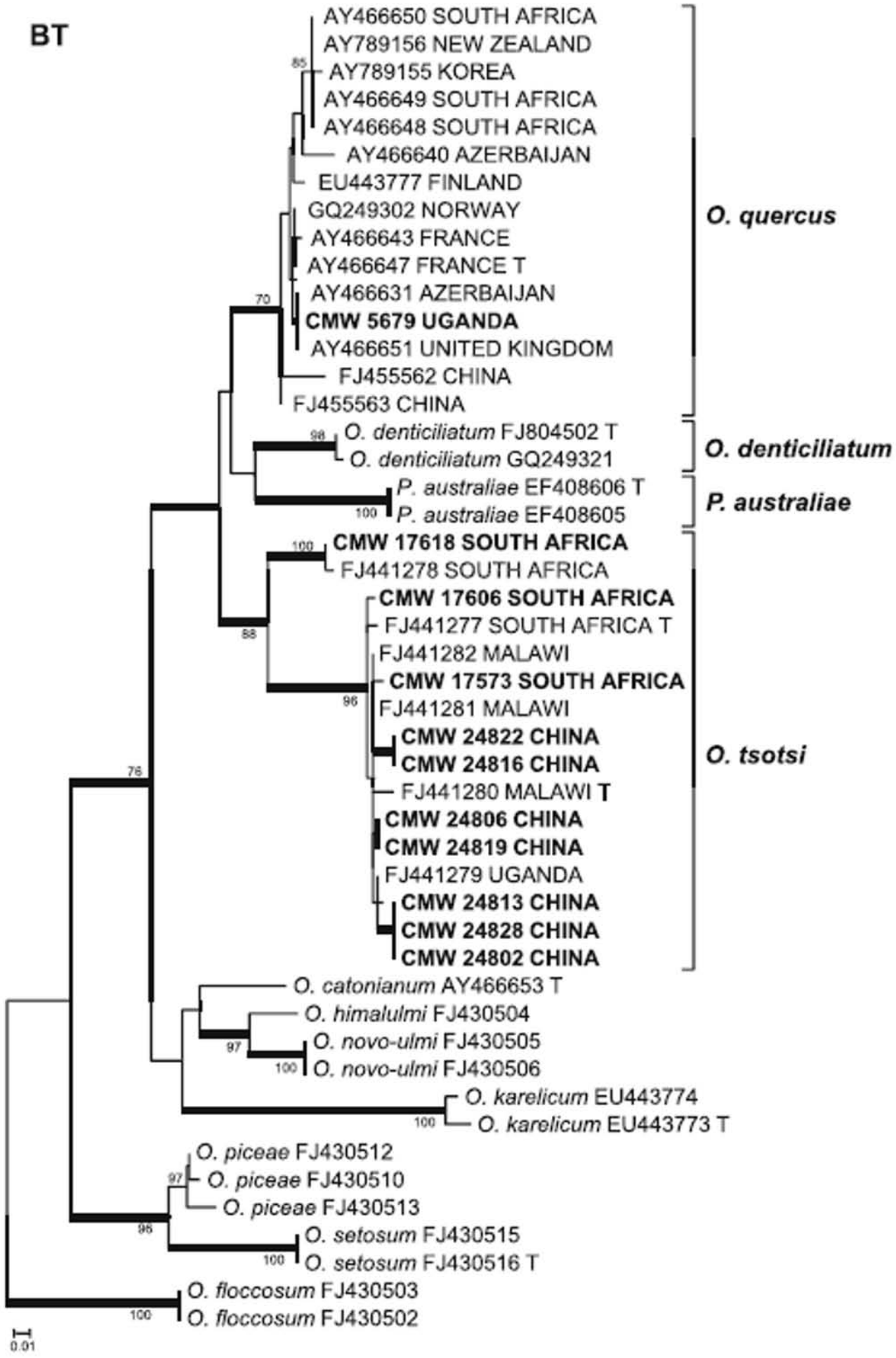
BT
AY466650 SOUTH AFRICA
AY789156 NEW ZEALAND
AY789155 KOREA
AY466649 SOUTH AFRICA
AY466648 SOUTH AFRICA
AY466640 AZERBAIJAN
EU443777 FINLAND
GQ249302 NORWAY
AY466643 FRANCE
AY466647 FRANCE T
AY466631 AZERBAIJAN
CMW 5679 UGANDA
AY466651 UNITED KINGDOM
FJ455562 CHINA
FJ455563 CHINA
O. quercus
O. denticiliatum FJ804502 T
O. denticiliatum GQ249321
O. denticiliatum
P. australiae EF408606 T
P. australiae EF408605
P. australiae
CMW 17618 SOUTH AFRICA
FJ441278 SOUTH AFRICA
CMW 17606 SOUTH AFRICA
FJ441277 SOUTH AFRICA T
FJ441282 MALAWI
CMW 17573 SOUTH AFRICA
FJ441281 MALAWI
CMW 24822 CHINA
CMW 24816 CHINA
FJ441280 MALAWI T
CMW 24806 CHINA
CMW 24819 CHINA
FJ441279 UGANDA
CMW 24813 CHINA
CMW 24828 CHINA
CMW 24802 CHINA
O. tsotsi
O. catonianum AY466653 T
O. himalulmi FJ430504
O. novo-ulmi FJ430505
O. novo-ulmi FJ430506
O. karelicum EU443774
O. karelicum EU443773 T
O. piceae FJ430512
O. piceae FJ430510
O. piceae FJ430513
O. setosum FJ430515
O. setosum FJ430516 T
O. floccosum FJ430503
O. floccosum FJ430502
0.01

Figure 2. Phylogram resulting from a maximum likelihood (ML) analysis of the b-tubulin (BT) sequences. ML bootstrap values (1000 replicates) above 70% are given at nodes. Branches with posterior probability support values (above 90%) obtained from Bayesian analyses are indicated with bold lines. Isolate numbers for sequences obtained in the present study are printed in bold type. T indicates ex-type isolates of species.

Grobbelaar *et al*. 2010). These studies and our results in the present study show that caution should be taken not to assume that all fungi from hardwoods that are morphologically similar to this species actually represent *O. quercus*.

Prior to this study, *O. tsotsi* was known only from Africa, where it is found on both exotic Eucalyptus and native hardwoods (Grobbelaar *et al*. 2010). Discovery of the fungus on exotic *Eucalyptus* in China might give the impression that the fungus was introduced into China. However, the isolate from native agarwood in Vietnam (Harrington *et al*. 2001) alters our perceptions regarding a possible African origin for the fungus. It is entirely possible that the fungus has been in Southeast Asia for a long time and might even be endemic to this region. The genetic variability among isolates from both Africa and China is indicative of widespread sexual recombination in both regions. More extensive sampling from native hardwoods, including eucalypts, in Australasia and Southeast Asia, and exotic eucalypt plantations in areas such as Africa and South America would be required to provide conclusive answers to questions concerning the origin of *O. tsotsi*. In this regard, the influence of host specialization and a very long history of human movement of timber across and between continents would also need to be considered.

Ophiostoma tsotsi has been isolated from fresh, exposed wounds in the cambium of living trees (Grobbelaar *et al*. 2010), and in the present study from stained pulpwood. At present, nothing is known regarding its pathogenicity. Like *O. quercus*, it is probably not a serious tree pathogen (Geldenhuis *et al*. 2004). It is more likely an insect-vectored fungus that is a primary colonist of freshly exposed sapwood and the causal agent of sapstain on felled timber. However, *O. tsotsi* groups within the hardwood clade of the *O. piceae*-complex (Harrington et al. 2001; Grobbelaar *et al*. 2009), relatively close to *O. novo-ulmi* Brasier and *O. ulmi* (Buisman) Nannf., the devastating tree pathogens responsible for Dutch elm disease pandemics during the last century. Thus, the possibility that *O. tsotsi* might pose a threat to living trees should not be overlooked. Apart from its pathogenicity, numerous unanswered questions remain regarding the biology and ecological role of this fungus. These questions should be addressed to ensure an accurate assessment of the risks posed by the possible introduction of *O. tsotsi* into new environments through the import and export of timber and pulpwood chips.

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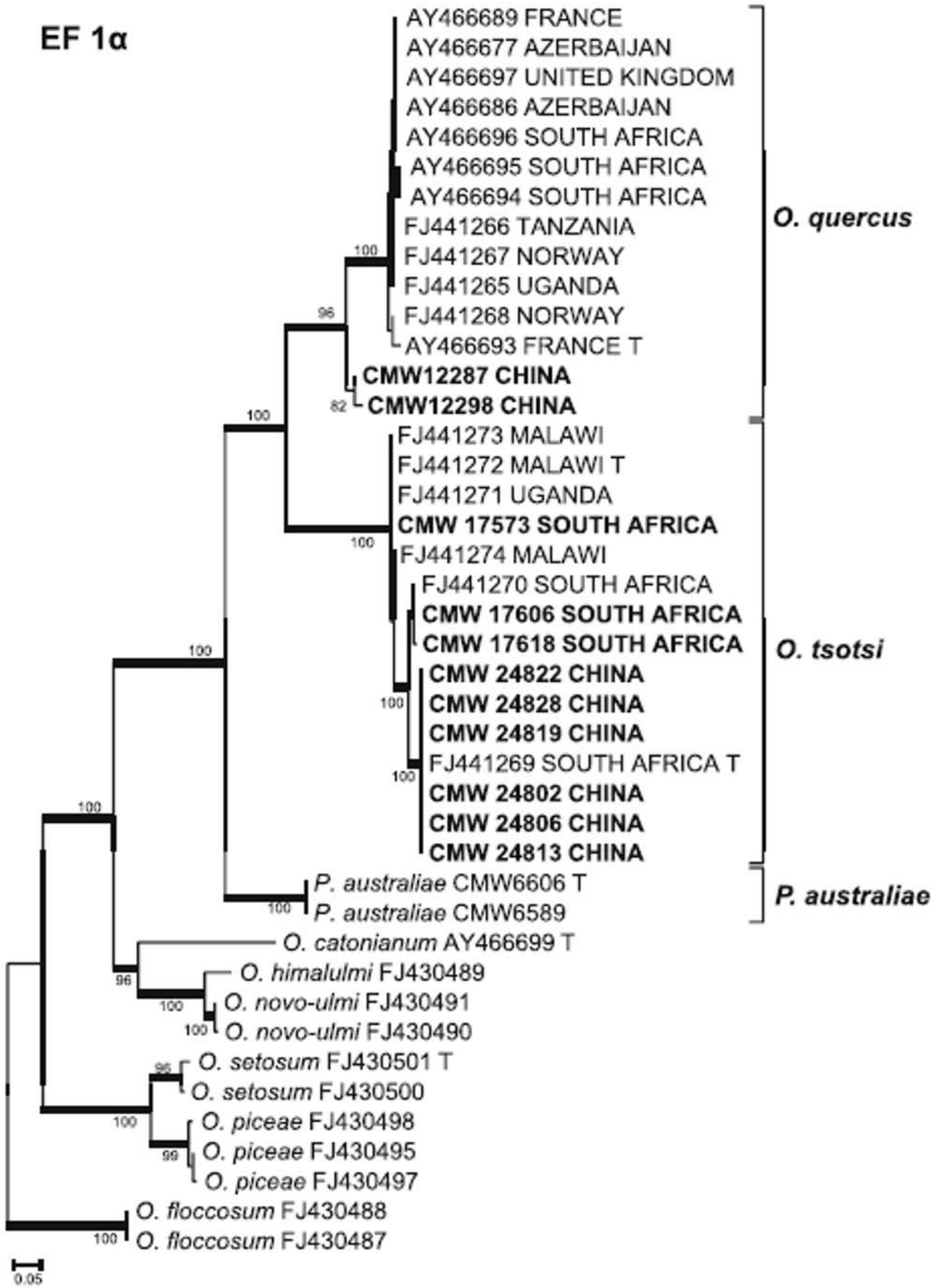

Figure 3. Phylogram resulting from a maximum likelihood (ML) analysis of the elongation factor-1a (EF 1a) sequences. ML bootstrap values (1000 replicates) above 70% are given at nodes. Branches with posterior probability support values (above 90%) obtained from Bayesian analyses are indicated with bold lines. Isolate numbers for sequences obtained in the present study are

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