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FULL PAPER

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.

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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

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**

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

ITS internal transcribed spacer, *BT* β-tubulin, *EF* elongation factor-1α, *NA* not available

[a] CMW, culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

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 β-tubulin (BT) and elongation factor-1α (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.

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.

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

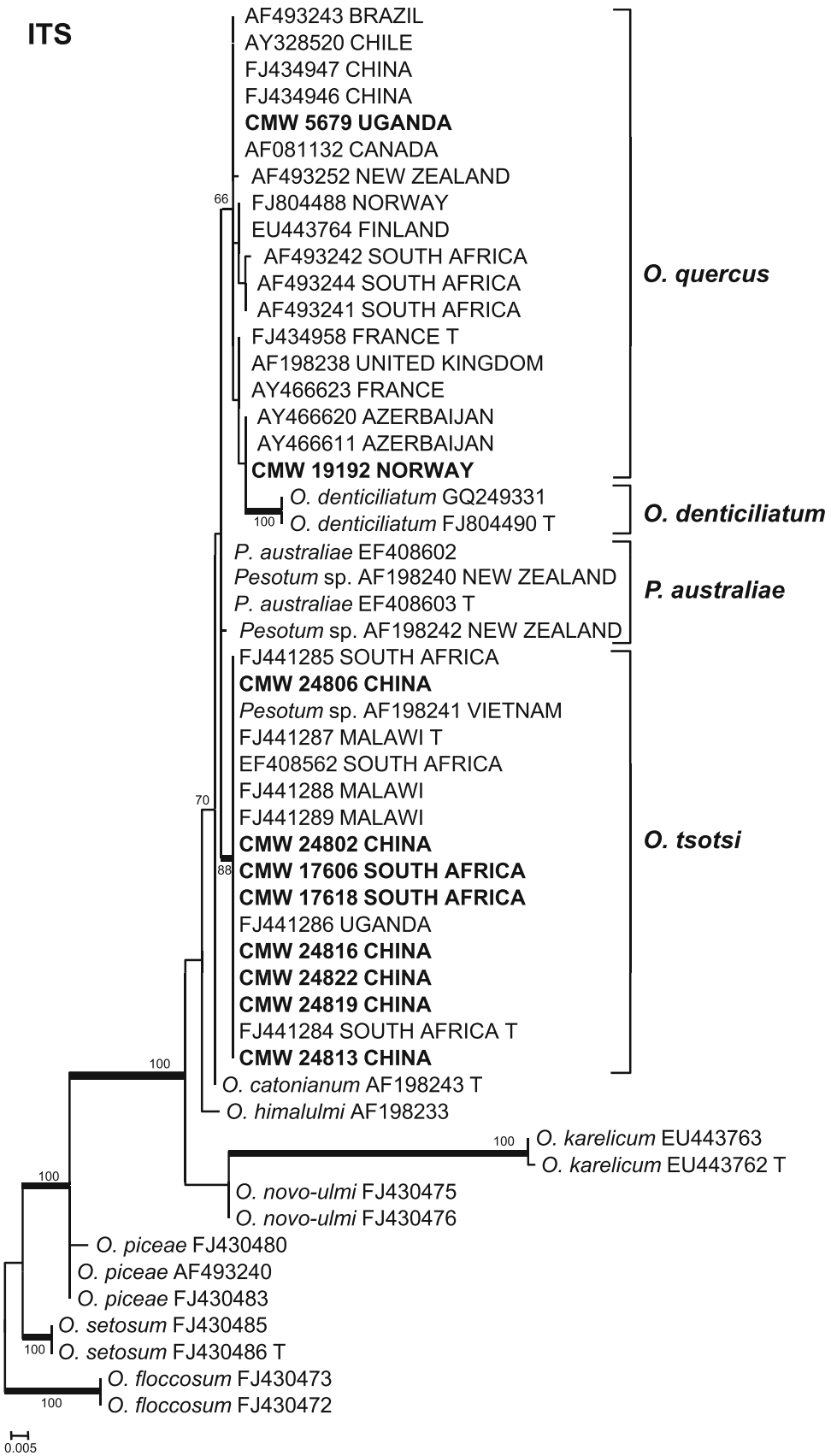

Fig. 1 Phylogram resulting from a maximum likelihood (ML) analysis of the internal transcribed spacer (*ITS*) sequences. ML bootstrap values (1000 replicates) above 70% are given at nodes. Branches with posterior probability support values (above 90%) obtained from Bayesian analyses are indicated with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

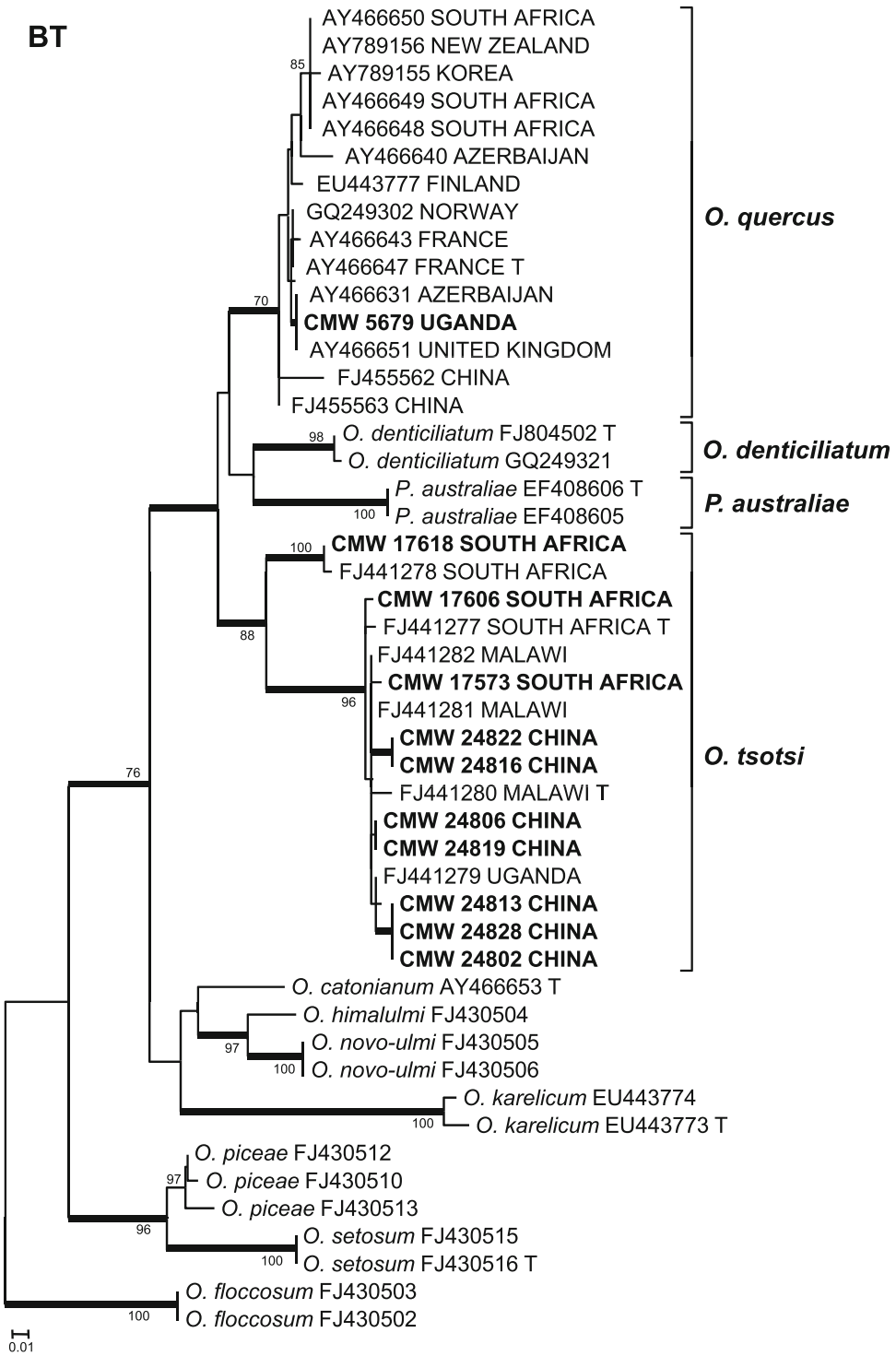

Fig. 2 Phylogram resulting from a maximum likelihood (ML) analysis of the β-tubulin (*BT*) sequences. ML bootstrap values (1000 replicates) above 70% are given at nodes. Branches with posterior probability support values (above 90%) obtained from Bayesian analyses are indicated with *bold lines*. Isolate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

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

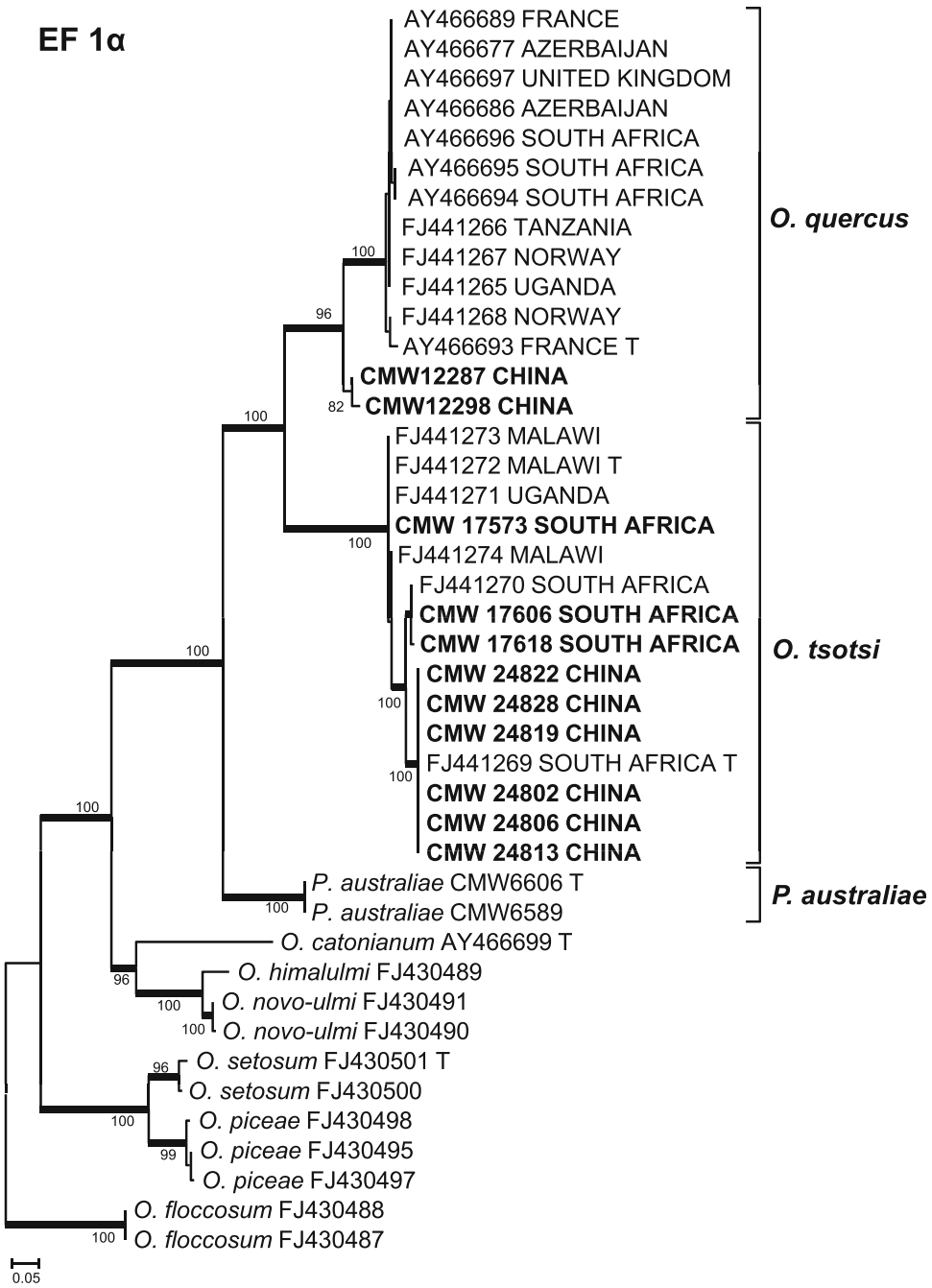

Fig. 3 Phylogram resulting from a maximum likelihood (ML) analysis of the elongation factor-1α (*EF 1α*) sequences. ML bootstrap values (1000 replicates) above 70% are given at nodes. Branches with posterior probability support values (above 90%) obtained from Bayesian analyses are indicated with *bold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

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; 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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