Ophiostomatoid fungi associated with the invasive pine-infesting bark beetle, *Dendroctonus valens*, in China

Min Lu[1,2], Zhou, X.D. [2,3], De Beer, Z.W.[2], Wingfield, M.J.[2] and Sun, J.-H.[1*]

[1]State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology (IOZ), Chinese Academy of Sciences (CAS), Beijing, China
[2]Tree Protection Co-operation Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa
[3]China Eucalypt Research Center (CERC), Chinese Academy of Forestry (CAF), Zhanjiang, Guangdong, China

Min Lu, Zhou, X.D., De Beer, Z.W., Wingfield, M.J. and Sun, J.-H. (2009). Ophiostomatoid fungi associated with the invasive pine-infesting bark beetle, *Dendroctonus valens*, in China. Fungal Diversity 38: 133-145.

Bark beetles (Coleoptera: Scolytinae) are common vectors of ophiostomatoid fungi, including several primary tree pathogens as well as important agents of sapstain. *Dendroctonus valens*, considered a secondary bark beetle in its native, North America, has caused unprecedented tree mortality in China. Several species of ophiostomatoid fungi are associated with the beetle in North America, but little research has been done on the fungi associated with this invasive bark beetle in China. The aim of this study was to isolate and characterize ophiostomatoid fungi associated with this *D. valens* in China. Ten ophiostomatoid fungi (*Leptographium procerum*, *L. pini-densiflorae*, *L. truncatum*, *Hyalorhinocladiella pinicola*, *Ophiostoma flocossum*, *O. ips*, *O. minus*, *O. piceae*, *O. abietinum* and an undescribed taxon close to *O. rectangulosporium*) were isolated from the bark beetle or its galleries and identified using morphology and DNA sequence comparisons. *Leptographium truncatum*, *L. pini-densiflorae*, *H. pinicola*, *O. flocossum*, and *O. minus* are reported from China for the first time. *Leptographium procerum* was the most frequently isolated species.

Key words: ophiostomatoid fungi, red turpentine beetle, invasive species, *Leptographium procerum*

Article Information
Received 30 December 2008
Accepted 4 March 2009
Published online 1 October 2009
*Corresponding author: J.-H. Sun; e-mail: sunjh@ioz.ac.cn

Introduction

Many bark beetle species (Coleoptera: Scolytinae) infest *Pinus* spp. (Wood and Bright, 1992). Most of these insects are considered secondary bark beetles in their native environment, but they can become problematic when their populations rise to unprecedented levels or when they are introduced into new areas such as those where *Pinus* spp. are planted as non-natives in intensively managed plantations (Wingfield and Swart, 1994).

Most conifer-infesting bark beetles vector a variety of fungi, including ophiostomatoid fungi (Six, 2003; Kirisits, 2004). This group includes several primary pathogens and many species that are agents of sapstain (Brasier, 1991; Seifert, 1993). Despite the many native species of conifer-infesting bark beetles in China, only nine species of ophiostomatoid fungi have been reported from this country (Table 1).

The red turpentine beetle, *Dendroctonus valens* is a common bark beetle on pines throughout its native range in North and Central America (Eaton and Lara, 1967), and is often associated with more aggressive bark beetle species. Tree mortality and outbreaks attributed to *D. valens* alone are rare in its native range (Smith, 1971). Several species of fungi, such as *L. terebrantis* and *O. ips*, have been reported as associates of *D. valens* in its native habitat (Table 2), but little is known regarding their ecology or relative importance. *Dendroctonus valens* was introduced into China in the early 1980's presumably when unprocessed logs were imported from the west coast of the United States (Cognato *et al*., 2005). Since 1999, *D. valens* has spread rapidly from Shanxi province to the adjacent provinces

Table 1. *Leptographium* and *Ophiostoma* species previously reported from China.

Fungal species	Host	Insect	References
Leptographium elegans	*Chamaecyparis formosensis*	-	Wingfield *et al.*, 1994
L. procerum	*Pinus tabuliformis*	*Dendroctonus valens*	Lu *et al.*, 2008
L. yunnanense	*Pinus yunnanensis*	*Tomicus piniperda*	Zhou *et al.*, 2000
L. sinoprocerum	*Pinus tabuliformis*	*Dendroctonus valens*	Lu *et al.*, 2008
Ophiostoma abietinum	*Pinus yunnanensis*	*Tomicus sp.*	Zhou *et al.*, 2008
O. ips	*Pinus yunnanensis*	*Tomicus sp.*	Zhou *et al.*, 2008
O. piceae	*Larix olgensis*	*Ips subelongatus*	Zhou *et al.*, 2008
O. quercus	*Cunninghamia lanceolata*	*Crypturgus* spp.	Lin *et al.*, 2003
	Pinus yunnanensis	*Tomicus sp.*	Zhou *et al.*, 2008
	Tsuga dunosa	Weevil	Zhou *et al.*, 2008
O. setosum	*Abies* sp.	-	Zhou *et al.*, 2008
	Tsuga dunosa	Weevil	Zhou *et al.*, 2008

Table 2. Fungi previously reported from *Dendroctonus valens* in North America.

Species	Host	Origin	Reference
Ceratocystis collifera	*Pinus teocote*	Mexico	Marmolejo & Butin, 1990
[a]*Graphium* sp.	*Pinus ponderosa*	California	Owen *et al.*, 1987
[b]*Grosmannia clavigera*	Not known	Oregon	Six *et al.*, 2003
[c]*Grosmannia europhioides*	Not known		*Wright and Cain, 1961
[c]*Grosmannia piceaperda*	Not known		*Rumbold, 1931
Leptographium procerum	*P. sylvestris*	Minnesota	Wingfield, 1983
	P. resinosa, P. banksiana	Wisconsin	Wingfield, 1983; Klepzig *et al.*, 1995
	P. strobus	Vermont	[d]Jacobs *et al.*, 2004
L. terebrantis	*P. ponderosa*	California	Harrington and Cobb, 1983; Owen *et al.*, 1987
	P. sylvestris	Minnesota	Wingfield, 1983
	P. resinosa, P. banksiana	Wisconsin	Wingfield, 1983; Klepzig *et al.*, 1995
	Not known	Oregon	Six *et al.*, 2003
L. wageneri	*P. ponderosa*	California	Goheen and Cobb, 1978
L. wageneri var. *ponderosum*	*P. ponderosa; P. jeffreyi*	California	Schweigkofler *et al.*, 2005
L. wingfieldii	*P. resinosa, P. strobus*	Vermont	Jacobs *et al*., 2004
Ophiostoma ips	*P. ponderosa*	California	Owen *et al.*, 1987
	P. resinosa	Wisconsin	Klepzig *et al.*, 1995
[c]*O. Piliferum*	Not known		Perry, 1991

[a]Possibly a *Pesotum* anamorph of an *Ophiostoma* species.
[b]The identity of the only *G. clavigera* isolate (C813) isolate reported from *D. valens* remains uncertain. Although morphologically similar to *G. clavigera*, it groups in some analyses with *G. clavigera* and in others with *L. terebrantis* (Six *et al*., 2003).
[c]Species listed as associates of *D. valens* by Perry (1991), but none of the original references cited by Perry (*) mention *D. valens,* rendering these associations questionable.
[d]*L. procerum* isolates were misidentified as *L. terebrantis*.

of Hebei, Henan, and Shaanxi where it has infested over 500,000 ha of pine forest, causing extensive tree mortality. More than 10 million *Pinus tabuliformis* trees have been killed, as well as other pine species, including *P. bungeana* (Yan *et al.*, 2005). This devastation has stimulated research into various aspects of the biology of the host-beetle-fungus relationships, including the chemical ecology of this invasive pest (Zhang *et al.*, 2006; Lu *et al.*, 2007). In addition, a recent study by Lu *et al.* (2008) described a new fungal species, *L. sinoprocerum* Lu, Decock & Mariate associated with *D. valens* in China. During the course of 2006, we isolated ophiostomatoid fungi collected both from *D. valens* and their breeding galleries from China. The aim of this study was to identify these fungi using comparisons of morphology and DNA sequence data.

Materials and Methods

Isolation of fungi

Specimens of *D. valens* were collected from felled *P. tabuliformis* trees in two provinces of China between May and October

2006. In Shaanxi province, 162 beetles were collected in the Yaopin Forest Station (N 35° 46′, E 109° 16′; average, elevation 1000 m). In Shanxi province, 186 beetle specimens were collected in the Qinyuan County (N 35° 18′, E 110° 21′; average elevation 1100 m), and 167 beetles at the Tunlanchuan Forest Station (N 37° 48′, E 111° 44′; average elevation 1400 m). At each sampling site, five pines (at least 20 m far from each other) randomly selected for beetle sampling were felled one year before sampling. In order to compare the fungi in the galleries with those isolated directly from the beetles, thirty galleries were randomly selected for fungal sampling in May 2006 from the five pines felled at the Tunlanchuan Forest Station.

All bark beetle specimens were kept separately, and frozen at -70°C for twenty minutes before fungi were isolated. Bark beetles were squashed directly onto the surface of a medium selective for *Ophiostoma* spp. (20g Biolab malt extract, 20g Biolab agar and 1000ml deionised water, amended with 0.05% cycloheximide and 0.04% streptomycin). Galleries were examined using a dissection microscope and spore masses were transferred to the selective medium following the techniques used by Zhou *et al*. (2001). All cultures 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.

Morphological studies

Pure cultures were sorted into groups based on culture morphology. Anamorph structures were mounted in lactic acid on glass slides and examined microscopically. To induce the production of perithecia, anamorphic isolates were grown on 1.5% oatmeal agar (15g oat powder, 20g Biolab agar and 1000ml deionised water) and 2% WA medium (20g Biolab agar and 1000 ml distilled water) with sterilized pine twigs at 25°C in the dark.

DNA extraction

Single hyphal tip cultures were prepared from representative cultures randomly selected for DNA sequencing (Tables 3 and 4). Each culture was grown in 50ml malt extract broth (30g Biolab malt extract, and 1000ml distilled water) at 25°C in the dark for 10 days. Mycelium was then harvested by filtration through Whatman no. 1 filter paper and freeze dried. Freeze-dried mycelium was ground into a fine powder. DNA was extracted from the resulting powder using PrepMan Ultra Sample reagent (Applied Biosystems, CA, USA) following the manufacturer's protocols.

DNA Sequencing

Three gene regions, most often used in *Leptographium* and *Grosmannia* species delineation (Jacobs *et al*., 2004; Massoumi Alamouti *et al*., 2006; Lu *et al*., 2008), were amplified for *Leptographium*-like isolates. These were part of the nuclear ribosomal DNA operon including the internal transcribed spacer (ITS) 2 region and part of the large subunit (LSU), as well as partial β-tubulin and elongation factor 1α (EF-1α) genes. Primer pairs used in the respective PCR reactions were ITS3 and LR3 (White *et al.*, 1990), Bt2a and Bt2b (Glass and Donaldson, 1995), and EF1F and EF2R (Jacobs *et al.*, 2004).

For *Ophiostoma* species, the ITS 1 and 2 regions including the 5.8S gene of the rDNA operon, as well as part of the β-tubulin gene, are most often used for species delimitation (Aghayeva *et al*., 2004; Roets *et al.*, 2006; Linnakoski *et al*., 2008). These regions were amplified for the relevant isolates from this study. Primers used were ITS1F (Gardes and Bruns, 1993) and ITS4 (White *et al.*, 1990) for the ITS regions, and Bt2a and Bt2b (Glass and Donaldson, 1995) for the β-tubulin gene.

Template DNA was amplified in a 50 µL PCR reaction volume, consisting of 1 µL of DNA solution (100-200ng μL^{-1}), 0.5 µL of Expand High Fidelity PCR System enzyme mix (2.5 U) (Roche Molecular Biochemicals, Alameda, CA), 5 µL of Expand HF buffer (10×) without $MgCl_2$, 5 µL of $MgCl_2$ (25 mM), and 1 µL of each primer (10 mM). PCR reactions were performed on an Eppendorf Mastercycler® Personal (PerkinElmer, Hamburg, Germany). The PCR conditions were as follows: 95°C for 2 minutes, followed by 40 cycles, where each cycle included 30 seconds at 95°C, 30 seconds at 54-60°C, and 1 minute at 72°C. A final elongation step was conducted for 8 minutes at 72°C. A negative control, using water without DNA, was included with each PCR. PCR products were visualized under UV illumi-

Table 3. *Leptographium terebrantis* and *L. procerum* isolates from origins other than China, used for comparative purposes in this study. Accession numbers for sequences obtained in this study are printed in italics. Numbers for isolates obtained from *D. valens* are underlined.

Isolate no.		Host	Insect	[e]Origin	Collector	GenBank No.		
[a]CMW	[b]Other					ITS	β-tubulin	EF-1α
Leptographium procerum								
13[A]	CBS516.63	*P. resinosa*		USA, NY	Kendrick	-	EU296783	EU296790
45	CBS118580	*P. sylvestris*		USA, MN	Wingfield	EU785399	EU785366	EU785415
[c]10216		*P. strobes*	*D. valens*	USA, VT	Bergdahl	AY553385	AY534933	AY536179
[c]10217		*P. strobes*	*D. valens*	USA, VT	Bergdahl	AY553386	AY534934	AY536180
Leptographium terebrantis								
11		*P. banksiana*		USA, MN	Wingfield	EU785386	EU785348	EU785403
47		*Pinus* sp.	*D. valens*	USA, CA	Harrington	EU785376	EU785353	EU785404
663[T]	CBS337.70	*P. taeda*	*D. terebrans*	USA, LA	Barras	EU785383	EU785349	EU785412
717	C20	*Pinus* sp.	*D. valens*	USA, CA	Harrington	EU785375	EU785357	EU785413
718	C45	*Pinus* sp.	*D. valens*	USA, CA	Harrington	EU785377	EU785355	EU785405
1763	-	*P. ponderosa*	*D. valens*	USA, ID	Bertagnole	EU785378	EU785347	EU785407
1764	-	*P. ponderosa*	*D. valens*	USA, ID	Bertagnole	EU785379	EU785356	EU785408
1765	-	*P. ponderosa*	*H. porosus*	USA, ID	Bertagnole	EU785380	EU785351	EU785409
1767	C846	*P. contorta*		Canada	Yamaoka	EU785381	EU785350	EU785411
1820	C1303	*P. contorta*		Canada	Yamaoka	EU785382	EU785358	EU785414
1823	C1288	*P. contorta*		Canada	Yamaoka	EU785384	EU785352	EU785410
[d]2814	CBS115209	*P. sylvestris*		USA, MN	Wingfield	EU785385	EU785354	EU785406
-	C418	*P. ponderosa*	*D. brevicomis*	USA, CA	Harrington	AY544607	AY263191	AY544630
-	UAMH9722	*P. contorta*		Canada, BC	Reid	AY544606	AY263192	AY544629
-	AU98Pr2155	sapwood		Canada, BC	Uzunovic	AY544608	AY544622	AY544631
-	AU1561213	sapwood		Canada, BC	Uzunovic	AY544609	AY544623	AY544632

[a]CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[b]CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; C = Culture collection of T. C. Harrington, Department of Plant Pathology, Iowa State University, Iowa, USA; UAMH = University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden, Canada; AU = Culture collection of C. Breuil, University of British Columbia, Canada.

[c]As *L. terebrantis* in Jacobs *et al*. (2004).

[d]CMW 2814 is a duplicate of CMW 9. According to Jacobs *et al*. (2004) CMW 9 had the same sequence as CMW 10216 and 10217. In this study we determined sequences using CMW 2814, which were distinct from those of CMW 10216 and 10217.

[e]States and provinces abbreviated as follows: BC = British Colombia; CA = California; ID = Idaho; LA = Louisiana; MN = Minnesota; NY = New York; VT = Vermont.

[A]Authentic culture from original collection by Kendrick (1962). [T]Ex-type culture.

nation on a 1% agarose gel stained with ethidium bromide (10mg mL^{-1}). Amplification products were purified using the High Pure PCR Product Purification Kit (Boehringer, Mannheim, Germany).

Sequencing reactions were carried out with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer Applied BioSystems) following the manufacturer's instructions. Sequencing was performed on an ABI PRISM 377 Autosequencer (Perkin-Elmer Applied BioSystems). PCR products were sequenced with the same primers (2 mM) used for PCR. Consensus sequences were assembled using Vector NTI Advance™ 9.0. Data sets were compiled in MEGA 4 (Tamura *et al.*, 2007). Reference sequences were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Alignments were done online using MAFFT 6 (Katoh and Toh, 2008), and these were checked manually and edited where necessary in MEGA 4.

Sequence analyses

Three different phylogenetic analyses were conducted. Maximum Parsimony (MP) and Maximum Likelihood (ML) were done in PAUP 4.0b10 (Sinauer Associates Inc., Sunderland, MA, USA), and Bayesian

Table 4. Morphological groups of ophiostomatoid isolates obtained from *D. valens* and their galleries on *P. tabuliformis* in China during the course of this study.

Group	Anamorph in culture	Isolate No.	Genbank No.			Species identified based on sequences
			ITS	β-tubulin	EF-1α	
A	*Leptographium*	[a]CMW 25569	EU785387	EU785359	EU785423	*L. procerum*
		CMW 25614	EU785391	EU785368	EU785422	
		CMW 25618	EU785396	EU785364	EU785420	
		CMW 25627	EU785393	EU785360	EU785418	
		CMW 25639	EU785389	EU785361	EU785416	
		CMW 25648	EU785395	EU785363	EU785421	
		CMW 25662	EU785390	EU785362	EU785417	
		CMW 25670	EU785394	EU785365	EU785419	
		CMW 25675	EU785392	EU785369	EU785424	
		CMW 25683	EU785388	EU785367	EU785425	
B	*Leptographium*	CMW 25600	EU785397	EU785370	EU785426	*L. pini-densiflorae*
		CMW 25611	EU785398	EU785371	EU785427	
C	*Leptographium*	CMW 25684	EU785374	EU785346	EU785400	*L. truncatum*
D	*Hyalorhinocladiella*	CMW 25613	EU785372	EU785344	EU785402	*H. pinicola*
		CMW 25602	EU785373	EU785345	EU785401	
E	*Hyalorhinocladiella*	CMW 26254	EU785442	EU785430		*O. minus* (European variety)
F	*Pesotum*	CMW 26264	EU785447	EU785435		*O. piceae*
		CMW 26266	EU785448	EU785436		
G	*Pesotum*	CMW 25802	EU785440	EU785428		*O. floccosum*
		CMW 26270	EU785441	EU785429		
H	*Pesotum*	CMW 26255	EU785443	EU785431		*O. ips*
		CMW 25782	EU785444	EU785432		
I	*Pesotum*	CMW 26258	EU785449	EU785437		*O. rectangulosporium*-like
		CMW 26259	EU785450	EU785438		
		CMW 26261	EU785451	EU785439		
J	*Sporothrix*	CMW 26262	EU785446	EU785434		*O. abietinum*
		CMW 26269	EU785445	EU785433		

[a] CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

inference using the Markov chain Monte Carlo (MCMC) algorithm was done in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003).

MP analyses were conducted using only parsimony informative characters. Heuristic searches were done with the following settings: tree bisection-reconnection (TBR) branch swapping, 100 replicates of random sequence addition, and multrees in effect. Confidence support was estimated using 1000 bootstrap replicates.

For ML analyses, the best fit substitution models for each data set were established using the Akaike Information Criterion (AIC) in Modeltest 3.7 (Posada and Crandall, 1998). A maximum number of 1000 trees were retained, and nodal support was determined by nonparametric bootstrapping using 1000 replicates.

Bayesian analyses were performed on each of the matrices using the best fit substitution model obtained with AIC in MrModeltest 2.2 (http://www.abc.se/~nylander/). Five million generations were run in four chains with sampling every 100 generations. Burn-in was determined using Tracer 1.4 (http://beast.bio.ed.ac.uk/Tracer).

Species diversity of Chinese isolates

Species diversity of fungal isolates from galleries and *D. valens*. were compared. The number of species (S), Simpson's index (D = Sum [P_i^2]), P_i is the number of a given species divided by the total number of isolates observed, and Simpson's index of diversity (1-D) were used to evaluate species diversity (Atlas and Bartha, 1998).

Results

Isolation of fungi

In total, 133 ophiostomatoid fungal isolates were obtained from *D. valens* from three localities in China. Of these, 114 were isolated from *D. valens* beetles from all three

sites, and an additional 19 came from 30 galleries of *D. valens* at the Tunlanchuan Forest Station. Small numbers of other fungi including yeasts were encountered but these were not considered.

Morphological studies

Based on culture and anamorph morphology, isolates could be divided into 10 groups (Table 4). Three groups (A, B, C) presented *Leptographium*-like anamorphs in culture. The remaining groups formed anamorphs typically associated with *Ophiostoma* species: two groups (D, E) formed *Hyalorhinocladiella* anamorphs, four groups (F, G, H, I) produced *Pesotum* anamorphs, and the remaining group (J) had a *Sporothrix* anamorph.

Sequence analyses

Initial comparisons of ITS2-LSU (for *Leptographium*-like), and ITS 1 and 2 (for *Ophiostoma*-like) sequences confirmed that the 10 morphological groups could be separated into two distinct phylogenetic groups within the Ophiostomatales. Groups A-D were related to *Grosmannia* and/or *Leptographium* species, while the remaining groups (E-J) showed affinity to species of *Ophiostoma*.

Analyses of the ITS2-LSU, β-tubulin and EF-1α sequences of the *Leptographium*-like isolates revealed that the three morphological groups (A, B, C), represented three distinct species. ITS sequences of Group D, with a *Hyalorhinocladiella*-like anamorph in culture, showed that this group was also related to *Leptographium*, rather than to *Ophiostoma* or *Ceratocystiopsis* species. The appropriate gene regions were thus sequenced for this group so it could be analyzed together with the other *Leptographium* species. Statistical data obtained from the different analyses are presented in Table 5.

Although the ITS data (not shown) confirmed that groups A to D represented four species, they did not distinguish clearly between closely related species such as *Grosmannia aurea*, *G. robusta*, *L. wingfieldii*, *L. terebrantis*, *L. pyrinum* and *G. clavigera*. However, both β-tubulin and EF-1α sequences (Fig. 1) did distinguish between these closely related taxa and confirmed that groups A to D represented four known species (dark grey blocks, Fig. 1). Group B isolates were monophyletic with *L. pini-densiflorae*, and the group C isolate with *L. truncatum*. The two isolates with *Hyalorhinocladiella* anamorphs (Group D) clustered with *H. pinicola*.

Group A, representing the majority of the isolates, grouped with an authentic isolate of *L. procerum* from the original collection in the USA and used for the description of the species (Kendrick 1962). Two isolates (underlined in group A, Fig. 2) from *D. valens* in Vermont, USA, previously misidentified as *L. terebrantis* (Jacobs *et al.*, 2004), were also included in this group. None of the Chinese isolates grouped with *L. terebrantis* isolates from North America (light grey blocks, Fig. 1), which also included isolates from *D. valens* (underlined in Fig. 1). The *L. terebrantis* isolates from North America did not form a monophyletic lineage in either the β-tubulin or EF-1α trees (Fig. 1).

ITS data for the *Ophiostoma* spp. (Fig. 2) confirmed that the six remaining morphological groups (E-J) from *D. valens* in China represented six discrete taxa. In the ITS tree groups G and H grouped unequivocally with reference sequences of *O. floccosum* and *O. ips* respectively. β-tubulin sequences for these two groups (data not shown) also corresponded with those of reference strains of the two species.

ITS data could not clearly assign three other morphological groups (E, F, J) to known species due to a lack of resolution. For example, group F from China could not be distinguished from *O. piceae*, *O. setosum*, *O. canum* or *O. breviusculum* (ITS tree, Fig. 2). Separate analyses of the β-tubulin sequences for these three groups (Table 5) were necessary because they differed in the presence and absence of introns (Fig. 3). When analyzed with closely related species, the two Chinese isolates in group F clearly grouped with *O. piceae* isolates. The group E isolate clustered with the European isolates of *O. minus*, and group J among several isolates of *O. abietinum*.

Group I represented the only Chinese isolates distinct from all known species. They grouped most closely with *O. rectangulosporium* and *Pesotum fragrans* based on ITS. However, no β-tubulin sequences were

Table 5. Statistics resulting from various phylogenetic analyses.

Data set	No. of taxa	No. of [a]bp	Outgroup	Maximum Parsimony						ML	MrBayes	
				[b]PIC	No. of trees	Tree length	[c]CI	[d]RI	[e]HI	[f]Subst. model	[f]Subst. model	Burn-in
Leptographium												
ITS2 and LSU (not shown)	63	579	*L. americanum*	88	1000	113	0.929	0.982	0.071	TrN+G	GTR+G	100000
β-tubulin	64	432	*L. americanum*	244	1000	411	0.888	0.981	0.112	HKY+I	HKY+I	20000
EF-1α	64	870	*L. americanum*	500	1000	894	0.872	0.984	0.128	TrN+G	GTR+G	100000
Ophiostoma												
ITS	57	728	*G. penicillata*	439	72	1312	0.654	0.908	0.346	GTR+I+G	GTR+I+G	100000
β-tubulin (*O.piceae*)	15	331	*O. setosum*	35	2	40	0.950	0.975	0.050	TIM+I	GTR+G	500000
β-tubulin (*O.minus*)	15	363	*O. piliferum*	114	1	171	0.953	0.980	0.047	K81uf+G	HKY+G	50000
β-tubulin (*O.abietinum*)	23	243	*O. stenoceras*	62	2	72	1.000	1.000	0.000	K81uf+I	GTR+I	50000

[a]bp = base pairs; [b]PIC = number of parsimony informative characters; [c]CI = consistency index; [d]RI = retention index; [e]HI = homoplasy index; [f]Subst. model = best fit substitution model.

Table 6. Numbers of isolates of the different fungal species obtained from *D. valens* and their galleries on *P. tabuliformis* in China during the course of this study.

Fungal Species	Total no. of Isolates	Shaanxi Province	Shanxi Province		
		Yaopin Forest Station	Qinyuan County	Tunlanchuan Forest Station	
		From beetles	From beetles	From beetles	From galleries
Leptographium procerum	94	23	40	27	4
Ophiostoma flocossum	16	0	12	2	2
O. ips	7	0	1	4	2
L. pini-densiflorae	3	2	0	0	1
O. abietinum	3	0	0	0	3
O. rectangulosporium-like	3	0	0	0	3
H. pinicola	2	0	0	2	0
L. truncatum	2	0	0	0	2
O. piceae	2	0	0	0	2
O. minus (European variety)	1	0	0	1	0
Total	133	25	53	36	19

available for these two species and they could not be identified with certainty.

Species diversity of Chinese isolates

More species were isolated from galleries than from *D. valens* (Galleries in Tunlanchuan Forest Station: S (the number of species) = 8; *D. valens* in Tunlanchuan Forest Station: S = 5; *D. valens* in Qinyuan County: S = 3; and *D. valens* in Yaopin Forest Station: S = 2). The isolates from galleries were also more diverse as indicated by the Simpson's index of diversity (1-D) (Galleries in Tunlanchuan Forest Station: (1-D) = 0.86; *D. valens* in Tunlanchuan Forest Station: (1-D) = 0.42; *D. valens* in Qinyuan County: (1-D) = 0.38; and *D. valens* in Yaopin Forest Station: (1-D) = 0.15).

Four species, *L. truncatum*, *O. abietinum*, *O. piceae* and the *O. rectangulosporium*-like species, were found only in the galleries and not on the beetles. Six species, *L. truncatum*, *O. abietinum*, *O. piceae*, *H. pinicola*, *O. minus*, and the *O. rectangulosporium*-like species were obtained only from Tunlanchuan Forest Station (Table 6). *Leptographium procerum* was the only species found with *D. valens* at all collection sites, and it accounted for 70.7 % of the total isolations (56.3 % of isolations from the Tunlanchuan Forest Station; 75.5 % of the isolations from Qinyuan County; and 92 % of isolations from the Yaopin Forest Station). This fungus was isolated from 90 of the 515 beetles sampled, representing about 17.5 % of all the beetles.

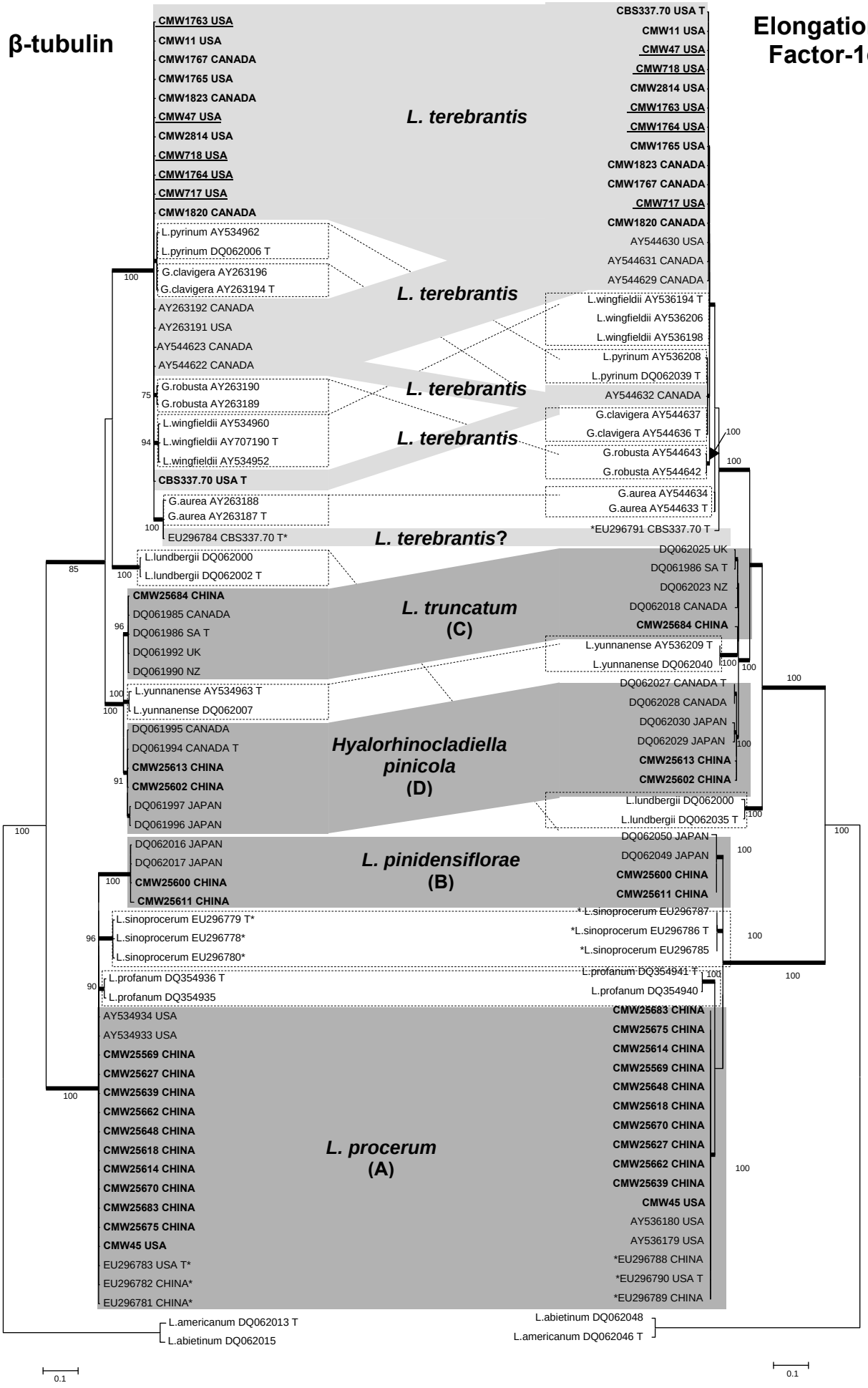
β-tubulin
Elongation Factor-1α
CMW1763 USA
CMW11 USA
CMW1767 CANADA
CMW1765 USA
CMW1823 CANADA
CMW47 USA
CMW2814 USA
CMW718 USA
CMW1764 USA
CMW717 USA
CMW1820 CANADA
L.pyrinum AY534962
L.pyrinum DQ062006 T
G.clavigera AY263196
G.clavigera AY263194 T
AY263192 CANADA
AY263191 USA
AY544623 CANADA
AY544622 CANADA
G.robusta AY263190
G.robusta AY263189
L.wingfieldii AY534960
L.wingfieldii AY707190 T
L.wingfieldii AY534952
CBS337.70 USA T
G.aurea AY263188
G.aurea AY263187 T
EU296784 CBS337.70 T*
L. terebrantis
L. terebrantis
L. terebrantis
L. terebrantis?
CBS337.70 USA T
CMW11 USA
CMW47 USA
CMW718 USA
CMW2814 USA
CMW1763 USA
CMW1764 USA
CMW1765 USA
CMW1823 CANADA
CMW1767 CANADA
CMW717 USA
CMW1820 CANADA
AY544630 USA
AY544631 CANADA
AY544629 CANADA
L.wingfieldii AY536194 T
L.wingfieldii AY536206
L.wingfieldii AY536198
L.pyrinum AY536208
L.pyrinum DQ062039 T
AY544632 CANADA
G.clavigera AY544637
G.clavigera AY544636 T
G.robusta AY544643
G.robusta AY544642
G.aurea AY544634
G.aurea AY544633 T
*EU296791 CBS337.70 T
L.lundbergii DQ062000
L.lundbergii DQ062002 T
CMW25684 CHINA
DQ061985 CANADA
DQ061986 SA T
DQ061992 UK
DQ061990 NZ
L. truncatum (C)
L.yunnanense AY534963 T
L.yunnanense DQ062007
DQ061995 CANADA
DQ061994 CANADA T
CMW25613 CHINA
CMW25602 CHINA
DQ061997 JAPAN
DQ061996 JAPAN
Hyalorhinocladiella pinicola (D)
DQ062016 JAPAN
DQ062017 JAPAN
CMW25600 CHINA
CMW25611 CHINA
L. pinidensiflorae (B)
L.sinoprocerum EU296779 T*
L.sinoprocerum EU296778*
L.sinoprocerum EU296780*
L.profanum DQ354936 T
L.profanum DQ354935
AY534934 USA
AY534933 USA
CMW25569 CHINA
CMW25627 CHINA
CMW25639 CHINA
CMW25662 CHINA
CMW25648 CHINA
CMW25618 CHINA
CMW25614 CHINA
CMW25670 CHINA
CMW25683 CHINA
CMW25675 CHINA
CMW45 USA
EU296783 USA T*
EU296782 CHINA*
EU296781 CHINA*
L. procerum (A)
L.americanum DQ062013 T
L.abietinum DQ062015
DQ062025 UK
DQ061986 SA T
DQ062023 NZ
DQ062018 CANADA
CMW25684 CHINA
L.yunnanense AY536209 T
L.yunnanense DQ062040
DQ062027 CANADA T
DQ062028 CANADA
DQ062030 JAPAN
DQ062029 JAPAN
CMW25613 CHINA
CMW25602 CHINA
L.lundbergii DQ062000
L.lundbergii DQ062035 T
DQ062050 JAPAN
DQ062049 JAPAN
CMW25600 CHINA
CMW25611 CHINA
*L.sinoprocerum EU296787
*L.sinoprocerum EU296786 T
*L.sinoprocerum EU296785
L.profanum DQ354941 T
L.profanum DQ354940
CMW25683 CHINA
CMW25675 CHINA
CMW25614 CHINA
CMW25569 CHINA
CMW25648 CHINA
CMW25618 CHINA
CMW25670 CHINA
CMW25627 CHINA
CMW25662 CHINA
CMW25639 CHINA
CMW45 USA
AY536180 USA
AY536179 USA
*EU296788 CHINA
*EU296790 USA T
*EU296789 CHINA
L.abietinum DQ062048
L.americanum DQ062046 T
0.1
0.1

Fig. 1. Two 50% majority rule trees obtained from Bayesian analyses of the partial β-tubulin and EF-1α gene regions respectively, revealing the identity of *Leptographium* species isolated from *Dendroctonus valens* infesting pine forests in China (dark grey blocks). Numbers of isolates sequenced in this study are presented in bold type. These include additional isolates of *L. terebrantis* (light grey blocks) and *L. procerum* from North America, some of which were isolated from *D. valens* (numbers underlined). Sequences from the study of Lu et al. (2008) are marked with *. Ex-type isolates of species are indicated with T. Bootstrap values >75% are presented at nodes for Maximum Parsimony (MP). Posterior probabilities >90% obtained from Bayesian analyses are shown as bold branches.

Discussion

In this study, ten fungal species were found in association with the exotic bark beetle, *D. valens*, infesting pine forests in China. These were *Leptographium procerum*, *L. pini-densiflorae*, *L. truncatum*, *Hyalorhinocladiella pinicola*, *Ophiostoma floccosum*, *O. ips*, *O. abietinum*, *O. piceae*, *O. minus*, and an undescribed taxon close to *O. rectangulosporium. Leptographium truncatum*, *L. pini-densiflorae*, *H. pinicola*, *O. floccosum*, and the European variety of *O. minus*, are reported for the first time from China. This list increases the number of ophiostomatoid fungi known from China from nine (Table 1) to fifteen (including the undescribed taxon).

Of all the species collected in this study, *L. procerum* was the fungus most consistently isolated from *D. valens*. This fungus is known to be associated with the beetle in the eastern part of its native range in North America (Wingfield, 1983). Seven fungi (Table 2) have been reported in association with *D. valens* in North America and only two of these, *L. procerum* and *O. ips*, correspond with the suite of fungi that have been found associated with this insect in China thus far. Other fungi associated with *D. valens* in North America but not found in China include *L. terebrantis*, *L. wingfieldii*, *L. wageneri*, *Grosmannia clavigera* and an undescribed *Graphium*, possibly *Pesotum*, species (Harrington, 1982; Goheen, 1976). It seems likely that *L. procerum* was introduced into China with *D. valens* and thismight provide interesting clues as to the source of the founder population of the insects.

Dendroctonus valens exists in three geographically isolated populations in its native range including the northeastern USA, the western USA, and Mexico (Smith, 1971). There has not been any detailed study of the fungi associated with this insect in these different environments but general observations (Six, personal communication), supported by the few existing reports of fungi associated with *D. valens*, indicate that these fungal associates differ in the different areas. Five fungi, *L. terebrantis*, *L. wageneri*, *L. wingfieldii*, *G. clavigera* and *O. ips* have been reported as associates of the beetle from the west coast of North America (California and Oregon) (Goheen and Cobb, 1978; Harrington and Cobb, 1983; Owen *et al.*, 1987; Six *et al.*, 2003), the presumed source of the Chinese infestation (Cognato *et al.*, 2005). In Minnesota and Wisconsin, part of the eastern range of *D. valens*, *L. procerum* was found together with *L. terebrantis* and *O. ips* (Wingfield, 1983; Klepzig *et al.*, 1995). Further east, in Vermont, only *L. procerum* and *L. wingfieldii* have been reported from *D. valens* (Jacobs *et al.*, 2004). Very little is known about the fungal associates of the insect in Mexico and only *Ceratocystiopsis collifera* has been recorded (Marmolejo and Butin, 1990). These studies suggest that *L. procerum* is not an associate of *D. valens* in the western United States, which is contrary to the consistent association of the beetle and fungus in China. Certainly, the results of this study suggest that the origin of the Chinese population of *D. valens* deserves further consideration and a more thorough sampling of the fungi associated with the insect in North America should be undertaken.

The role that *L. procerum* may play in the biology of *D. valens* in China deserves further study. In North America, this fungus has been associated with a disease known as white pine root decline, although it is generally considered a weak pathogen associated with insects that infest stressed trees (Wingfield *et al.*, 1988). Its consistent association with *D. valens* in China is intriguing. The fact that the insect has resulted in the death of large numbers of trees in China raises the question as to whether *L. procerum* might have contributed to the aggressiveness of the beetle in its introduced manifestation. Answers to these questions may lie in pathogenicity tests with the fungus on

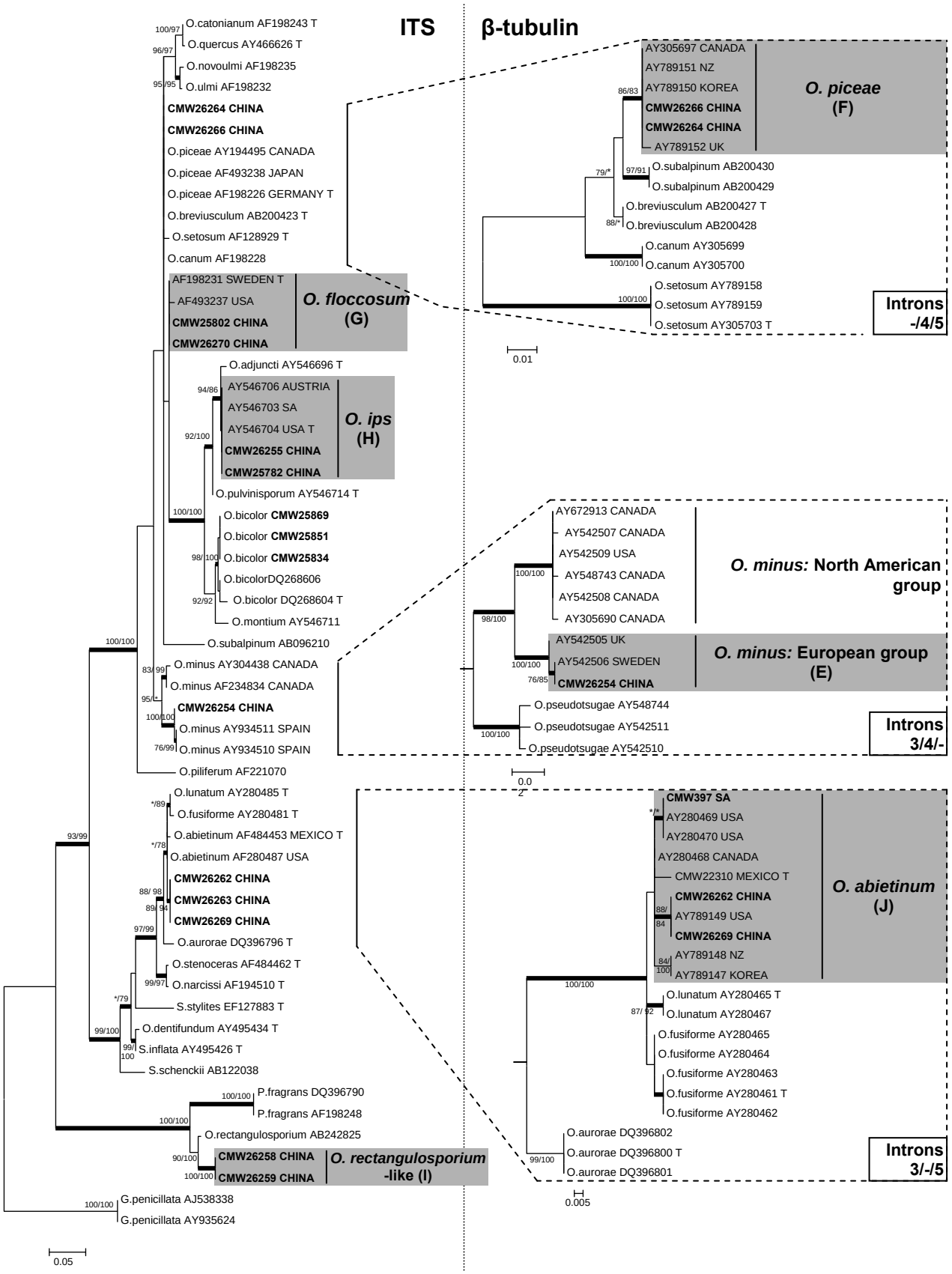

Fig. 2. Phylograms obtained from analyses of ITS sequence data, revealing the identity of *Ophiostoma* species isolated from *Dendroctonus valens* infesting pine forests in China (dark grey blocks). Phylograms of partial β-tubulin data are shown for species groups where ITS sequences did not resolve the identity of Chinese isolates. β-tubulin data sets were analysed separately from each other because of differences between the three groups in the presence and absence of introns 3, 4 and 5. Numbers of isolates sequenced in this study are presented in bold type. Ex-type isolates of species are indicated with T. All phylograms presented were obtained from Maximum Likelihood (ML) analyses. Bootstrap

values >75% are presented at nodes for ML and Maximum Parsimony (MP) as follows: ML/MP. Posterior probabilities >90% obtained from Bayesian analyses are shown as bold branches.

Chinese *Pinus* spp, as well as exploration of nutritional symbioses and chemicals-based interactions between insects and fungi.

Interestingly, one species we expected to find but did not recover was *L. sinoprocerum*, a species was recently described from the galleries of *D. valens* in China (Lu *et al.*, 2008). Our analyses did confirm that *L. sinoprocerum* and *L. procerum* are similar but distinct as revealed through DNA sequencing. It is difficult to explain why *L. sinoprocerum* was not isolated in our study, but several explanations are possible. Lu *et al*. (2008) only isolated *L. sinoprocerum* from galleries and it is possible that the species might be introduced to the *D. valens* galleries by secondary insects or mites. It is also possible that *L. sinoprocerum* is a native species with only a regional association with *D. valens* at present. To confirm this association, isolations of this fungus directly from beetles are needed.

The second most abundant species isolated in this study was *O. floccosum*. This fungus forms part of the *O. piceae*-complex with *O. piceae*, which was isolated only twice. Neither of these species is considered pathogens and both are commonly found on conifer timber (De Beer *et al.*, 2003). *Ophiostoma floccosum* was initially described causing sapstain of conifers in Sweden (Mathiesen, 1951), and has since been found in North America, New Zealand (Thwaites *et al.*, 2005), Korea (Kim *et al.*, 2005), and South Africa (De Beer *et al.*, 2003). *Ophiostoma piceae* is a ubiquitous sapstain fungus occurring on a wide range of coniferous and hardwood hosts in the northern hemisphere (Brasier and Kirk, 1993). Both species seem to be occasional associates of many different conifer-infesting beetles.

The results of this study have shown that a relatively large number of ophiostomatoid fungal species are associated with *D. valens* in China. Future isolations from *D. valens* and, especially, its galleries in China will most likely reveal additional ophiostomatoid fungi from this country. Some of these, particularly *L. procerum*, were probably introduced with the insect. Others are more likely native fungal species, historically associated with other bark beetles that infest the same trees as those attacked by *D. valens*. Fungi should be isolated from other bark beetles infesting the same trees as *D. valens* in China and their mycofloras compared. Novel insect-fungal associations appear to be emerging and it is possible that these could account in some way for the atypical behaviour of *D. valens* in China. Future studies will consider the functional aspects of these associations and will include pathogenicity tests to evaluate the relative importance of the fungi associated with *D. valens* in China. Studies to enhance our understanding of the fungi associated with *D. valens* in its native range will also add valuable insights into our understanding of the *D. valens* invasion in China.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (Project 30525009), the National Basic Research Program of China (973 Program 2009CB119204), and TPCP (Tree Protection Co-operation Program), South Africa. We thank the Forestry Bureaus of Shanxi and Shaanxi for their valuable assistance in field study. We are especially grateful to two anonymous reviewers whose comments helped improve the earlier version of the manuscript.

References

Aghayeva, D.N., Wingfield, M.J., de Beer, Z.W. and Kirisits, T. (2004). Two new *Ophiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. Mycologia 96: 866-878.

Atlas, R.M. and Bartha, R. (1998). Microbial Ecology: Fundametals and Applications. Benjamin/Cum mings Publishing Co, Menlo Park, CA, USA: 593.

Brasier, C.M. (1991). *Ophiostoma novo-ulmi* sp. nov. causative agent of the current Dutch elm disease pandemics. Mycopathologia 115: 151-161.

Brasier, C.M. and Kirk, S.A. (1993). Sibling species within *Ophiostoma piceae*. Mycological Research 97: 811-816.

Cognato, A.I., Sun, J.-H., Anducho-Reyes, M.A. and Owen, D.R. (2005). Genetic variation and origin of red turpentine beetle (*Dendroctonus valens* LeConte) introduced to the People's Republic of China. Agricultural and Forest Entomology 7: 87-94.

De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. (2003). The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. Mycological Research 107: 469-476.

Eaton, B. and Lara, R.R. (1967). Red Turpentine beetle, *Dendroctonus valens* LeConte. In: *Important Forest Insects and Diseases of Mutual Concern to Canada, the United States and Mexico.* (eds. A.G. Davidson and R.M. Prentise). Canada Department Forestry and Rural Department, Ottawa, Canada: 21-24.

Gardes, M. and Bruns, T.D. (1993). ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118.

Glass, N.L. and Donaldson, G.C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous *Ascomycetes*. Applied and Environmental Microbiology 61: 1323-1330.

Goheen, D.J. (1976). *Verticicladiella wageneri* on *Pinus ponderosa*: Epidemiology and interrelationships with insects. Ph.D. thesis. University of California, Berkeley, California, U.S.A.

Goheen, D.J. and Cobb, F.W. (1978). Occurrence of *Verticicladiella wagenerii* and its perfect state, *Ceratocystis wageneri* sp. nov., in insect galleries. Phytopathology 68: 1192-1195.

Harrington, T.C. (1982). *Verticicladiella wageneri*: Taxonomy and vector relations. Ph.D. thesis. University of California, Berkeley, California, U.S.A.

Harrington, T.C. and Cobb, F.W. (1983). Pathogenicity of *Leptographium* and *Verticicladiella* spp. isolated from roots of western North American conifers. Phytopathology 73: 596-599.

Jacobs, K., Bergdahl, D.R., Wingfield, M.J., Halik, S., Seifert, K.A., Bright, D.E. and Wingfield, B.D. (2004). *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. Mycological Research 108: 411-418.

Katoh, K. and Toh, H. (2008). Recent developments in the MAFFT multiple sequence alignment program. Briefings in Bioinformatics 9: 286-298.

Kendrick, W.B. (1962). The *Leptographium* complex *Verticicladiella* Hughes. Canadian Journal of Botany 40: 772-797.

Kim, G.-H., Kim, J.-J., Lim, Y.W. and Breuil C. (2005). Ophiostomatoid fungi isolated from *Pinus radiata* logs imported from New Zealand to Korea. Canadian Journal of Botany 83: 272-278.

Kirisits, T. (2004). Fungal associates of European bark beetles with special emphasis on the Ophiostomatoid fungi. In: *Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis.* (eds. F. Lieutier, K.R. Day, A. Battisti, J.-C. Grégoire and H. Evans). Kluwer Academic Publishers, Dordrecht: 181-235.

Klepzig, K.D., Smalley, E.B. and Raffa, K.F. (1995). *Dendroctonus valens* and *Hylastes porculus* (Coleoptera: Scolytidae): vectors of pathogenic fungi (Ophiostomatales) associated with red pine decline disease. Great Lakes Entomologist 28: 81-88.

Lin, T.C., Huang, J.W. and Hsieh, W.H. (2003). Identification of ophiostomatoid fungi associated with Chinese fir wilt in Taiwan. Plant Pathology Bulletin 12: 33-42.

Linnakoski, R., De Beer, Z.W., Rousi, M., Niemelä, P., Pappinen, A. and Wingfeld, M.J. (2008). Fungi including *Ophiostoma karelicum* sp. nov., associated with *Scolytus ratzeburgi* infesting birch in Finland and Russia Mycological Research 112: 1475-1488.

Lu, M., Miller, D.R. and Sun, J.-H. (2007). Cross-Attraction between an exotic and a native pine bark beetle: a novel invasion mechanism? PLoS ONE 2: e1302.

Lu, Q., Decock, C., Zhang, X.Y. and Maraite, H. (2008). *Leptographium sinoprocerum* sp.nov., an undescribed species associated with *Pinus tabuliformis-Dendroctonus valens* in northern China. Mycologia 100: 275-290.

Marmolejo, J.G. and Butin, H. (1990). New conifer-inhabiting species of *Ophiostoma* and *Ceratocystiopsis* (Ascomycetes, Microascales) from Mexico. Sydowia 42:193-199.

Massoumi Alamouti, S., Kim, J.-J. and Breuil, C. (2006). A new *Leptographium* species associated with the northern spruce engraver, *Ips perturbatus*, in western Canada. Mycologia 98: 149-160.

Mathiesen, A. (1951). Einige neue *Ophiostoma*-Arten in Schweden. Svensk Botanisk Tidskrift 45: 203-232.

Owen, D.R., Lindahl, K.Q., Wood, D.L. and Parmeter, J.R. (1987). Pathogenicity of fungi isolated from *Dendroctonus valens*, *D. brevicomis*, and *D. ponderosae* to ponderosa pine seedlings. Phytopathology 77: 631-636.

Perry, T.J. (1991). A synopsis of the taxonomic revisions in the genus *Ceratocystis* including a review of blue-staining species associated with *Dendroctonus* bark beetles. General Technical Report, SO-86. New Orleans, LA: USDA Forest Service, Southern Forest Experiment Station: 1-16.

Posada, D. and Crandall, K.A. (1998). MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817-818.

Roets, F., de Beer, Z.W., Dreyer, L.L., Zipfel, R., Crous, P.W. and Wingfield, M.J. (2006). Multi-gene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences. Studies in Mycology 55: 199-212.

Ronquist, F. and Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

Rumbold, C.T. (1931). Two blue-staining fungi associated with bark-beetle infestation of pines. Journal of Agricultural Research 43: 847-873.

Schweigkofler, W., Otrosina, W.J., Smith, S.L., Cluck, D.R., Maeda, K., Peay, K.G. and Garbelotto, M. (2005). Detection and quantification of *Leptographium wageneri*, the cause of black-stain root disease, from bark beetles (Coleoptera: Scolytidae) in Northern California using regular and real-time PCR. Canadian Journal of Forest Research 35: 1798-1808.

Seifert, K.A. (1993). Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In:

Ceratocystis and Ophiostoma Taxonomy, *Ecology and Pathogenicity*. (eds. M.J. Wingfield, K.A. Seifert and J.F. Webber). American Phytopathological Society Press, St. Paul, Minnesota: 141-151.

Six, D.L. (2003). Bark beetle-fungus symbiosis. In: *Insect Symbiosis*. (eds. K. Bourtzis and T.A. Miller). CRC Press, Washington, D.C.: 97-114.

Six, D.L., Harrington, T.C., Steimel, J., McNew, D. and Paine, T.D. (2003). Genetic relationships among *Leptographium terebrantis* and the mycangial fungi of three Western *Dendroctonus* bark beetles. Mycologia 95: 781-792.

Smith, R.H. (1971). Red turpentine beetle. USDA Forest Service, Forest Pest Leaflet 55.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599.

Thwaites, J.M., Farrell, R.L., Duncan, S.M., Reay, S.D., Blanchette, R.A., Hadar, E., Hadar, Y., Harrington, T.C. and McNew, D. (2005). Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. New Zealand Journal of Botany 43: 653-663.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols*: *Genes for Phylogenetics* (eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White). Academic Press, San Diego, California: 315-322.

Wingfield, M.J. (1983). Association of *Verticicladiella procera* and *Leptographium terebrantis* with insects in the Lake States. Canadian Journal of Forest Research 13: 1238-1245.

Wingfield, M.J. and Swart, W.J. (1994). Integrated management of forest tree diseases in South Africa. Forest Ecology and Management 65: 11-16.

Wingfield, M.J., Capretti, P. and Mackenzie, M. (1988). *Leptographium* spp. as root pathogens on conifers. An international perspective. In: *Leptographium root diseases on conifers*. (eds. T.C. Harrington and F.W. Cobb). American Phytopathological Society, St. Paul, Minnesota: 113-128.

Wingfield, M.J., Crous, P.W. and Tzean, S.S. (1994). *Leptographium elegans*: a new species from Taiwan. Mycological Research 98:781-785.

Wood, S.L. and Bright, D.E. (1992). A catalog of Scolytidae and Platypodidae (Coleoptera), Part 2: Taxonomic Index. Great Basin Naturalist Memoirs A 13: 1-6.

Wright, E.F. and Cain, R.F. (1961). New species of the genus *Ceratocystis*. Canadian Journal of Botany 39: 1215-1230.

Yan, Z.L., Sun, J.H., Don, O. and Zhang, Z.N. (2005). The red turpentine beetle, *Dendroctonus valens* LeConte (Scolytidae): an exotic invasive pest of pine in China. Biodiversity and Conservation 14: 1735-1760.

Zhang, L.W. and Sun, J.H. (2006). Electrophysiological and behavioral response of *Dendroctonus valens* (Coleoptera: Scolytidae) to candidate pheromone components identified in hindgut extracts. Environmental Entomology 35: 1232-1237.

Zhou, X.D., De Beer, Z.W., Ye, H., Jacobs, K., Paciura, D., Yang, L.Y., Pang, Y.Z. and Wingfield, M.J. (2008). Ophiostomatoid fungi associated with conifer-infesting bark beetles in China. http://src.fabinet.up.ac.za/ophiostoma/pdf/Zhou%20et%20al%20Ophiostomatoid%20in%20China.pdf".

Zhou, X.D., De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. (2001). Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. Sydowia 53: 290-300.

Zhou, X.D., Jacobs, K., Morelet, M., Ye, H., Lieutier, F. and Wingfield, M.J. (2000). A new *Leptographium* species associated with *Tomicus piniperda* in Southwestern China. Mycoscience 41: 573-578.