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Full paper

Grosmannia tibetensis, a new ophiostomatoid fungus associated with *Orthotomicus* sp. (Coleoptera) in Tibetan subalpine forests

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ABSTRACT

Few ophiostomatoid fungi have been reported from the margin of the Tibetan Plateau and none have been found in the central portion of the region. In a survey of ophiostomatoid fungi associated with spruce bark beetles in Tibetan subalpine forests, numerous strains of *Leptographium* s. l. (Ophiostomataceae) were isolated from *Orthotomicus* sp. (Coleoptera: Scolytinae) and its galleries infesting *Picea likiangensis* var. *balfouriana*. Morphological characters and phylogenetic analysis based on multiple DNA sequence data (ITS2-partial LSU rDNA region, beta-tubulin and transcription elongation factor-1α genes) revealed a new species in the "*Grosmannia penicillata* complex", which is proposed as *G. tibetensis*. The species is characterized by both *Leptographium* and *Pesotum* asexual states, which is unique in the "*G. penicillata* complex". Additionally, sequences of the *tubC* paralogue gene were found combining with *tub2* sequences in many species of the "*G. penicillata* complex", resulting in incongruent trees. This is the first report of tubulin paralogue genes in ophiostomatoid fungi. Gene duplication and losses make beta-tubulin a potentially challenging locus for use as a molecular marker for tracing speciation.

1. Introduction

Ophiostomatoid fungi have symbiotic relationships with bark beetles. The fungi provide a direct benefit to their beetle partners through producing bark beetle aggregation pheromones and semiochemicals (Zhao et al., 2019a). In many cases, the fungi are virulent to host plants (Brasier, 1979; Harrington, Fraedrich, & Aghayeva, 2008; Wingfield, Seifert, & Webber, 1993), and this may indirectly help the beetles overcome the defensive system of their host plants (Zhao et al., 2019b). This fungal group is polyphyletic and includes numerous genera in two orders, Ophiostomatales (Sordariomycetidae) and Microascales (Hypocreomycetidae) (de Beer & Wingfield, 2013). *Ophiostoma* and *Leptographium* sensu lato are the two genera with the greatest species diversity.

Many species of *Leptographium* s. l. were previously classified in different genera such as *Ceratocystis*, *Grosmannia*, or *Ophiostoma*. Phylogenetic analysis has been based on DNA sequence data such as the nuclear ribosomal large subunit region (LSU), internal transcribed spacer regions 1 and 2 of the nuclear ribosomal DNA operon, including the 5.8S region (ITS), the beta-tubulin gene region (BT), and the transcription elongation factor-1α gene region (EF). These analyses can reveal the relationships and provide accurate classification of the fungi. However, since a single name is now used for the fungi in this group, two generic names, including the older and broader name *Leptographium* and a teleomorphic name, *Grosmannia*, are used by taxonomists and require reevaluation (de Beer & Wingfield, 2013; Jacobs & Wingfield, 2013).

In mainland China, the taxonomy of *Leptographium* is now reasonably well known. Numerous studies using both morphological and DNA-based phylogenetic approaches have identified at least 37 *Leptographium* species, of which 23 were new (Zhou et al., 2000; Lu, Decock, Zhang, & Maraite, 2008; Lu, Decock, et al., 2009; Lu, Zhou, et al., 2009; Paciura et al., 2010; Yin, Duong, Wingfield,

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Zhou, & de Beer, 2014; Chang et al., 2019, 2017; Liu et al., 2017; Wang et al., 2019; Wang et al., 2020; Yin, Wingfield, Zhou, & de Beer, 2020). However, only 8 of the 34 Chinese provinces have been surveyed and large areas harboring diverse forest ecosystems remain unexplored. Thus, there is great potential for discovering new species in China. The Tibetan Plateau is an area that is unexplored for ophiostomatoid fungi.

The Tibetan Plateau is the highest and largest plateau in the world. The unique geographical conditions there have produced diverse ecosystems and created favorable environments for species maintenance and formation of new species (Gansser, 1964). As such, the Tibetan Plateau has rich biological diversity (Liu, Wang, Wang, Hideaki, & Abbott, 2006). Many native coniferous trees occur on the Tibetan Plateau (China flora editorial committee of Chinese academy of sciences, 1978). Spruces (*Picea* spp.) are the dominant trees in the forests. More than half of the 34 world species of *Picea* occur on the plateau and adjacent regions (Sun et al., 2014). Tibetan spruce forests are often infested by bark beetles (Yin, Huang, & Li, 1984). Nine ophiostomatoid fungus species have been reported in association with four bark beetle species that infest spruces on the plateau margin in Qinghai province (Yin, Wingfield, Zhou, & de Beer, 2016, 2020).

In a survey of the ophiostomatoid fungi on bark beetles infesting *Picea likiangensis* var. *balfouriana* (Rehder & E.H. Wilson) Hillier. in forests of the Tibetan Plateau, an undescribed species of *Leptographium* s. l., was isolated from *Orthotomicus* sp. adults. Their galleries were characterized by morphological observations and multilocus DNA sequence data. This species is described as *Grosmannia tibetensis* sp. nov. This is the first report of the presence of tubulin paralogue genes in ophiostomatoid fungi.

2. Materials and methods

2.1. Collection of samples and fungus isolations

Fungi were isolated from adults and breeding galleries of *Orthotomicus* sp., infesting the bark of *P. likiangensis* var. *balfouriana* in Zuogong county (29°14′2″N; 98°2′52″E, altitude 3780 m), Tibet autonomous region. Adult beetles and their galleries were individually placed in sterile Eppendorf tubes and envelope bags, respectively. They were stored at 4 °C until fungal isolations were made. Each adult beetle was dismembered into about 15 pieces on the surface of 2% water agar without previous surface disinfection. Breeding galleries were disinfected for 1 min with 1.5% sodium hypochlorite, rinsed with sterile water 3 times, then cut into ca. 3×3 mm^2 tissue pieces in a sterile environment and transferred onto 2% water agar. After incubation at 25 °C in continuous darkness, all strains were purified by single-spore isolation and/or mycelium apex and grown on 2% malt extract agar (MEA). After an initial analysis of macro- and micro-characteristics, representative strains of the morphotype (Table 1) were selected for detailed morphological, physiological, and molecular studies. For the preparation of dried specimens, the ex-holotype strain was inactivated by complete drying using a drying oven at 70 °C for 3 d. All strains were deposited in the culture collection of the forest pathology laboratory at the Chinese Academy of Forestry (CXY). Representative samples were also deposited at the China Forestry Culture Collection Center (CFCC).

2.2. Morphological and cultural studies

Morphological structures of each morphotype were observed and recorded using an OLYMPUS BX51 microscope, OLYMPUS SZX16 stereomicroscope and OLYMPUS DP70 digital camera (Olympus, Centre Valley, PA, USA). For the strain selected as the holotype, the lengths and widths of 30 reproductive structures per strain were measured. Mean, standard deviation (SD), minimum (min) and maximum (max) measurements were presented as format of (min−) (mean−SD)–(mean + SD) (−max). All relevant data pertaining to type specimens were deposited in MycoBank (www.MycoBank.org).

For the growth study, a round mycelium plug (5 mm diam), taken from an actively growing fungal colony, was placed in the center of a 90-mm-diam. Petri plate containing 2% MEA. The cultures were incubated in continuous darkness at 5–40 °C with different treatments at 5 °C intervals. Five replicate plates of each strain at each temperature were studied. Two diameter measurements, orthogonally, were recorded daily until the fastest growing mycelium reached the edge of the MEA plates. Color descriptions were based on the charts of Rayner (1970).

2.3. DNA extraction, amplification, and nucleotide sequencing

Prior to DNA extraction, the strains were grown in 2% MEA at 25 °C for 5 d. The actively growing mycelium from one MEA plate per strain was scraped from the surface of the media using a sterile scalpel and transferred into 1.5 mL Eppendorf tubes. DNA extractions and purification were carried out using an Invisorb Spin Plant Mini Kit (Tiangen, Beijing, China), following manufacturer's instructions. The primer pair ITS3/LR3 (Vilgalys & Hester, 1990; White, Bruns, Lee, & Taylor, 1990) was used for amplification of the internal transcribed spacer 2 and part of the 28S of the rDNA operon (ITS2-LSU). BT was amplified with two primer pairs of Bt2a/Bt2b (Glass & Donaldson, 1995) and T10/Bt2b (O'Donnell & Cigelnik, 1997); EF was amplified with primers EF1F/EF2R (Jacobs et al., 2004).

PCR assays were performed using the 2 × Taq PCR MasterMix (Tiangen, Beijing, China), following manufacturer instructions. The PCR conditions for the three regions were as follows: an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 45 s at 58 °C, and 1 min at 72 °C, and a final chain elongation at 72 °C for 8 min. The PCR products were separated by gel excision. PCR products were cleaned using a MSB Spin PCRapace Kit (250) (Invitek, Berlin, Germany), following manufacturer's instructions.

Sequencing reactions were performed using the CEQ DTCS Quick Start Kit® (Beckman Coulter, Brea, CA, USA), following manufacturer instructions and using the same PCR primers as above. Nucleotide sequences were determined using a CEQ 2000 XL capillary automated sequencer (Beckman Coulter, Brea, CA, USA).

2.4. Phylogenetic analysis

Preliminary identifications of the strains were made using standard BLAST searches. Referenced sequences in the analyses were downloaded from GenBank (Table 1). Datasets were compiled in Molecular Evolutionary Genetic Analyses (MEGA) 7.0 (Kumar, Stecher, & Tamura, 2016). Alignments were constructed with the online tool MAFFT v.7 (Katoh & Standley, 2013). The ITS2-

Table 1
Strains of *Leptographium* spp. included in this study.

Species[a]	Isolate no[b,c,d]	Host[e]	Insect	Location	GenBank accession no[f]		
					ITS2-LSU	BT (tub2/ tubC)	EF
Grosmannia abieticola (Yamaoka & Masuya) Masuya & Yamaoka	CMW17199 T	*Abies mariesii* Mast.	*Dryocoetes hectographus* Reitter	Japan	–	MN647790/-	MN647879
	CMW17200	*A. mariesii*	*D. hectographus*	Japan	–	MN647791/-	MN647880
G. abietina	CMW276 T	*Picea engelmannii* Parry ex Engelm.	-	Canada	–	MN647798/-	MN647887
	CMW2817	*P. engelmannii*	-	USA	–	MN647799/ DQ062014	MN647888
	CMW3083	*Picea* sp.	-	Canada	–	MN647800/ DQ062015	MN647889
G. abiocarpa R.W. Davidson	CMW65 T	*P. engelmannii*	*Ips* sp.	USA	AJ538339	MN647792/-	MN647881
	CMW24905	*P. engelmannii* × *glauca*	*I. tridens* Wood	Canada	–	MN647793/-	MN647882
	CMW24906	*P. engelmannii* × *glauca*	*I. tridens*	Canada	–	MN647794/-	MN647883
L. altius	CMW12471 T	*P. koraiensis* Nakai	-	China	HQ406851	-/HQ406899	HQ406875
	CMW12501	*Larix olgensis* Henry	*I. cembrae* Heer	China	–	-/HQ406901	HQ406877
G. americana	CMW123 T	*L. laricina* (Du Roi) K.Koch	*Dendroctonus simplex* LeConte	USA		MN647801/-	MN647890
	CMW495	*L. decidua* Mill.	-	USA	DQ062079	-/DQ062013	DQ062046
	CMW2929	*L. laricina*	*De. simplex*	USA		MN647802/ DQ062012	MN647891
	CMW2932	*L. laricina*	*De. simplex*	USA		MN647803/-	MN647892
G. bistate (J.J. Kim & G.H. Kim) M.L. Yin, Z.W. de Beer & M.j. Wingf.	CMW3802 T	*Pinus radiata* D. Don	-	Korea	AY348305	AY348306/-	MN647917
	CMW3814	*Pi. radiata*	-	Korea	–	AY348307/-	MN647918
G. chlamydata	CMW11592 T	*P. abies* (L.) Karst.	*D. autographus* Ratzeburg	Norway	EU979333	EU979341/-	EU979349
	CMW11597	*P. abies*	*Hylastes cunicularius* Erichson	Norway	–	EU979342/-	EU979350
	CMW11623	*P. abies*	*H. cunicularius*	Norway	–	EU979343/-	EU979351
G. crassifolia	CMW38885 T	*P. crassifolia* Kom.	*Polygraphus polygraphus* Linnaeus	China	–	MN647808/-	MN647897
	CMW39236	*P. purpurea*	*I. shangrila* Cognato & Sun	China	–	MN647809/-	MN647898
	CMW39243	*P. crassifolia* Mast.	*P. polygraphus*	China	–	MN647810/-	MN647899
L. curviconidium	CMW12425 T	*P. koraiensis*	*I. typographus* Wood & Bright	China	HQ406850	-/HQ406898	HQ406874
	CMW12441	*P. koraiensis*	*I. typographus*	China	–	-/HQ406896	HQ406872
G. curvispora (K. Jacobs, M.J. Wingf. & H. Solheim) M.L. Yin, Z.W. de Beer & M.j. Wingf.	CMW17260 T	*P. abies*	*D. autographus*	Norway	EU979328	MN647806/-	MN647895
	CMW17262	*P. abies*	*D. autographus*	Norway	–	MN647807/-	MN647895
G. eucalyptophila (K. Jacobs, M.J. Wingf. & Jol. Roux) M.L. Yin, Z.W. de Beer & M.j. Wingf.	CMW5193 T	*Eucalyptus urophylla* × *E. pellita*	–	Congo	–	MN647828/-	MN647919
	CMW5196	*E. urophylla* × *E. pellita*	–	Congo	–	MN647829/-	MN647920
L. fenglinhense	CMW44579 T	*P. koraiensis*	*I. typographus*	China	–	MH124323/-	MH124403
G. fruticetum (Alamouti, J.J. Kim & C. Breuil) M.L. Yin, Z.W. de Beer & M.j. Wingf.	CMW20604	*P. engelmannii* × *glauca*	*I. perturbatus* Wood & Bright	Canada	–	MN647795/-	MN647884
	CMW20605 T	*P. engelmannii* × *glauca*	*I. perturbatus*	Canada	DQ097847	MN647796/-	MN647885
	CMW20606	*P. engelmannii* × *glauca*	*I. perturbatus*	Canada	–	MN647797/-	MN647886
L. gestamen de Errasti & Z.W. de Beer	CIEFAP453 T	*Nothofagus dombeyi* (Mirb.) Oerst.	Ambrosia beetle gallery dying tree	Argentina	–	KT381297/-	KT381300
	CIEFAP457	*N. pumilio* (Poepp. & Endl.) Reiche	Ambrosia beetle gallery dead tree	Argentina	–	KT381298/-	KT381301
G. grandifoliae (R.W. Davidson) T.C. Harr	CMW703	*Fagus grandifolia* Ehrh.	–	USA	–	DQ296119/-	–
G. hughesii (K. Jacobs, M.J. Wingf. & T.C. Harr) M.L. Yin, Z.W. de Beer & M.j. Wingf.	CMW4053 T	*Aquilaria* sp.	–	Viet Nam	–	MN647831/-	MN647922
G. maixiuense	CMW38884 T	*P. crassifolia*	*P. polygraphus*	China	–	MN647811/-	MN647900
	CMW39235	*P. crassifolia*	*P. polygraphus*	China	–	MN647812/-	MN647901
	CMW39929	*P. purpurea*	*I. shangrila*	China	–	MN647813/-	MN647902
G. penicillata	CMW2642 T	*P. abies*	*I. typographus*	Sweden	–	MN647820/-	MN647909
	CMW2644	*P. abies*	–	Norway	–	MN647821/-	MN647910
	CMW39748	*P. abies*	–	Germany	DQ097851	MN647814/-	MN647903
G. purpurea	CMW38886 T	*P. purpurea*	*I. shangrila*	China	–	MN647825/-	MN647914
	CMW39927	*P. purpurea*	*I. shangrila*	China	–	MN647826/-	MN647915
	CMW39928	*P. purpurea*	*I. shangrila*	China	–	MN647827/-	MN647916
G. xeno-abietina M.L. Yin, Z.W. de Beer & M.j. Wingf.	CMW2407	*Pi. ponderosa* Dougl. ex Laws.	–	USA	–	MN647804/-	MN647893
	CMW2410 T	*Pi. ponderosa*	–	USA	–	MN647805/-	MN647894
G. xianmiense	CMW38892 T	*P. crassifolia*	*P. polygraphus*	China	–	MN647822/-	MN647911
	CMW38893	*P. purpurea*	*I. shangrila*	China	–	MN647823/-	MN647912
	CMW39926	*P. crassifolia*	*I. nitidus* Eggers	China	–	MN647824/-	MN647913
L. taigense Linnakoski, Z.W. de Beer & M.J. Wingf.	CMW36629	*P. abies*	*I. typographus*	Russia	–	JF280016/-	JF280061
	CMW36630 T	*Pi. sylvestris*	*Hylurgops palliates* Wood & Bright	Russia	JF279980	JF280017/-	JF280062

Table 1 (*continued*)

Species[a]	Isolate no[b,c,d]	Host[e]	Insect	Location	GenBank accession no[f]		
					ITS2-LSU	BT (tub2/ tubC)	EF
G. tibetensis	CXY2029 (CFCC53458)	*P. likiangensis* var. *balfouriana*	*Orthotomicus* sp.	China	–	MT268735/ MT268745	MT268755
	CXY2030 (CFCC53415) T	*P. likiangensis* var. *balfouriana*	*Orthotomicus* sp.	China	MT269759	MT268736/ MT268746	MT268756
	CXY2031 (CFCC53409)	*P. likiangensis* var. *balfouriana*	*Orthotomicus* sp.	China	–	MT268737/ MT268747	MT268757
	CXY2032 (CFCC53408)	*P. likiangensis* var. *balfouriana*	*Orthotomicus* sp.	China	–	MT268738/ MT268748	–
	CXY2033 (CFCC53410)	*P. likiangensis* var. *balfouriana*	*Orthotomicus* sp.	China	–	MT268739/ MT268749	–
	CXY2034 (CFCC53459)	*P. likiangensis* var. *balfouriana*	Gallery of *Orthotomicus* sp.	China	–	MT268740/ MT268750	MT268758
	CXY2035 (CFCC53460)	*P. likiangensis* var. *balfouriana*	Gallery of *Orthotomicus* sp.	China	–	MT268741/ MT268751	MT268759
	CXY2036 (CFCC53417)	*P. likiangensis* var. *balfouriana*	Gallery of *Orthotomicus* sp.	China	–	MT268742/ MT268752	MT268760
	CXY2037 (CFCC53402)	*P. likiangensis* var. *balfouriana*	Gallery of *Orthotomicus* sp.	China	–	MT268743/ MT268753	MT268761
	CXY2038 (CFCC53414)	*P. likiangensis* var. *balfouriana*	Gallery of *Orthotomicus* sp.	China	–	MT268744/ MT268754	MT268762

[a] Species named in black bold are novel species in this study.
[b] CFCC: China Forestry Culture Collection Center, Beijing, China.
[c] CXY (Culture Xingyao): Culture collection of the forest pathology laboratory at Chinese Academy of Forestry.
[d] T = ex-holotype isolate.
[e] *D.* = *Dryocoetes*; *De.* = *Dendroctonus*; *P.* = *Picea*; *Pi.* = *Pinus*.
[f] ITS2-LSU = the internal transcribed spacer 2 region and partial large subunit of the nrDNA operon; BT = beta-tubulin; EF = translation elongation factor 1-alpha.

LSU dataset was aligned using the L–INS–i strategy with a 200PAM/k = 2 scoring matrix, a gap opening penalty of 1.53 and an offset value of 0.00. The BT and EF datasets consisted of closely related DNA sequences and were thus aligned using the L–INS–i strategy with a 1 PAM/k = 2 scoring matrix, a gap opening penalty of 1.53 and an offset value of 0.00. Phylogenetic analyses of the aligned sequences were conducted using maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) methods.

ML phylogenetic analyses were conducted using RAxML-HPC v.8.2.3 (Stamatakis, 2014), available in the CIPRES science gateway (Miller et al., 2015, http://www.phylo.org/). The GTR model of site substitution including estimation of Gamma-distributed rate heterogeneity and a proportion of invariant sites (Stamatakis, 2006) were selected. ML bootstrap support values were estimated using 1000 bootstrap replicates.

PAUP* version 4.0b10 (Swofford, 2003) was used for MP analysis, with gaps treated as a fifth base. One thousand bootstrap replicates were generated to estimate the branch node confidence, with max trees set to 200. Clades compatible with the 50%-majority rule in the bootstrap consensus tree were retained. The analysis settings were: tree bisection reconnection branch swapping, starting tree obtained via stepwise addition, steepest descent not in effect and MulTrees effective.

For BI analyses, the best-fit substitution models for each dataset were established using the corrected Akaike Information Criterion (AICc) in jModelTest v.2.1.7 (Darriba, Taboada, Doallo, & Posada, 2012). With the GTR model, BI analyses using four Markov Chain Monte Carlo (MCMC) chains were run simultaneously in MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) from a random starting tree for 5,000,000 generations to calculate posterior probabilities. Trees were sampled every 100 generations. The burn-in value for each dataset was determined in Tracer v1.4.1 (http://beast.bio.ed.ac.uk/Tracer). The first 25% of trees sampled were discarded as burn-in and the remaining trees were utilized to generate a majority rule consensus tree for determining the posterior probability values. Phylogenetic trees were edited using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) and Adobe Illustrator CS6. The final alignments and the retrieved topologies were deposited in TreeBASE (No. 24415).

3. Results

3.1. Collection of samples, isolation of fungi, and sequence comparisons

Twenty adults and 50 breeding galleries of *Orthotomicus* sp. were collected from *P. likiangensis* var. *balfouriana* in Zuogong county, Tibet autonomous region. A total of 225 strains were obtained, of which 52 were identified as ophiostomatoid fungi based on morphological characteristics. Among them, 29 strains were derived from the adult beetles and 23 strains were derived from the galleries. The 52 strains were characterized by olivaceous colored colonies, typical *Leptographium* and *Pesotum* asexual morphs, and chlamydospores. Ten representative strains were selected for in depth morphological study and phylogenetic analysis (Table 1). Amplifications of ITS2-LSU, BT and EF sequences yielded 1215–1270 bp, 374–486 bp, and 786–870 bp nucleotides in length, respectively. All the representative strains had almost identical sequences for each DNA locus especially ITS2-LSU and EF sequences. After a preliminary blast search against NCBI GenBank, the strains were shown to have close affinities with members of the "*Grosmannia penicillata* complex", more specifically *G. purpurea* M.L. Yin, Z.W. de Beer & M.j. Wingf.

Primer pair Bt2a/Bt2b could amplify two different sequences in each of the representative strains (Supplementary Fig. S1), *tub2* 374 bp (one intron) and *tubC* 433–435 bp (two introns) in length. They differed in 192 positions (out of 451 bp, including gaps, in the alignment matrix) (Supplementary Fig. S2). Another pair of primers, T10/Bt2b yielded a sequence with high similarity to the *tub2* sequence amplified by Bt2a/Bt2b but differed by length (486 bp *vs*. 374 bp) (Supplementary Figs. S1 and S2).

3.2. Phylogenetic analysis

Alignments for the ITS2-LSU, BT, and EF datasets contained 591, 455, and 710 characters (including gaps), respectively. Tree topologies obtained from ITS2-LSU analyses were unable to distinguish closely related species, but every species complex could be divided explicitly. Phylograms obtained by ML are presented for all the datasets, with nodal supports obtained from ML, MP, and Bayesian analyses indicating on the trees.

Our strains nested in the "*G. penicillata* complex" in the *Leptographium* s. l. lineage (Fig. 1). Although the strains formed a separate branch, there were no significant nodal supports. Therefore, additional loci offering higher resolution are required to designate strains to a species. In phylogenetic analysis based on the BT subset, the two amplification results of our strains were represented by *tub2* group (amplified with primer pairs of Bt2a/Bt2b and T10/Bt2b) and *tubC* group (amplified with primer pair of Bt2a/Bt2b), respectively (Fig. 2). The *tub2* group of our strains represented a distinct lineage with good supports, while *tubC* group of our strains resided in a lineage comprised of *tubC* group of *G. abietina* (Peck) M.L. Yin, Z.W. de Beer & M.J. Wingf., *L. altius* Paciura, Z.W. de Beer & M.J. Wingf., *L. curviconidium* Paciura, Z.W. de Beer & M.J. Wingf. and *G. americana* K. Jacobs & M.J. Wingf., which separated from the "*G. penicillata* complex" (Fig. 2). In the phylogenetic analysis based on the EF subset, the stains clustered within a distinct lineage with good support, closely related to *G. purpurea* (Fig. 3). Based on the robust supports from multiple phylogenetic analyses and the phenotype observations, the Tibet strains appear to represent an undescribed species and are therefore described as a new species as follows.

3.3. Taxonomy

Grosmannia tibetensis Z. Wang & Q. Lu, sp. nov. Fig. 4.

MycoBank No: MB 831638.

Diagnosis: This species is clearly distinguished from other *Grosmannia* species by a combination of both *Leptographium* and *Pesotum* asexual morphs and production of chlamydospores.

Type: CHINA, Tibet autonomous region, Zuogong City, from *Orthotomicus* sp. infesting *P. likiangensis* var. *balfouriana*, Aug 2017, Z. Wang & Q. Lu, holotype CXY 2030, ex-holotype CFCC 53415.

Gene sequences ex-holotype: MT269759 (ITS2-LSU), MT268736 (BT), MT268756 (EF).

Etymology: The epithet *tibetensis* refers to the region where this species was isolated.

Description: Sexual state not observed.

Asexual state *Leptographium* and *Pesotum*.

Leptographium asexual state: Conidiophores macronematous,

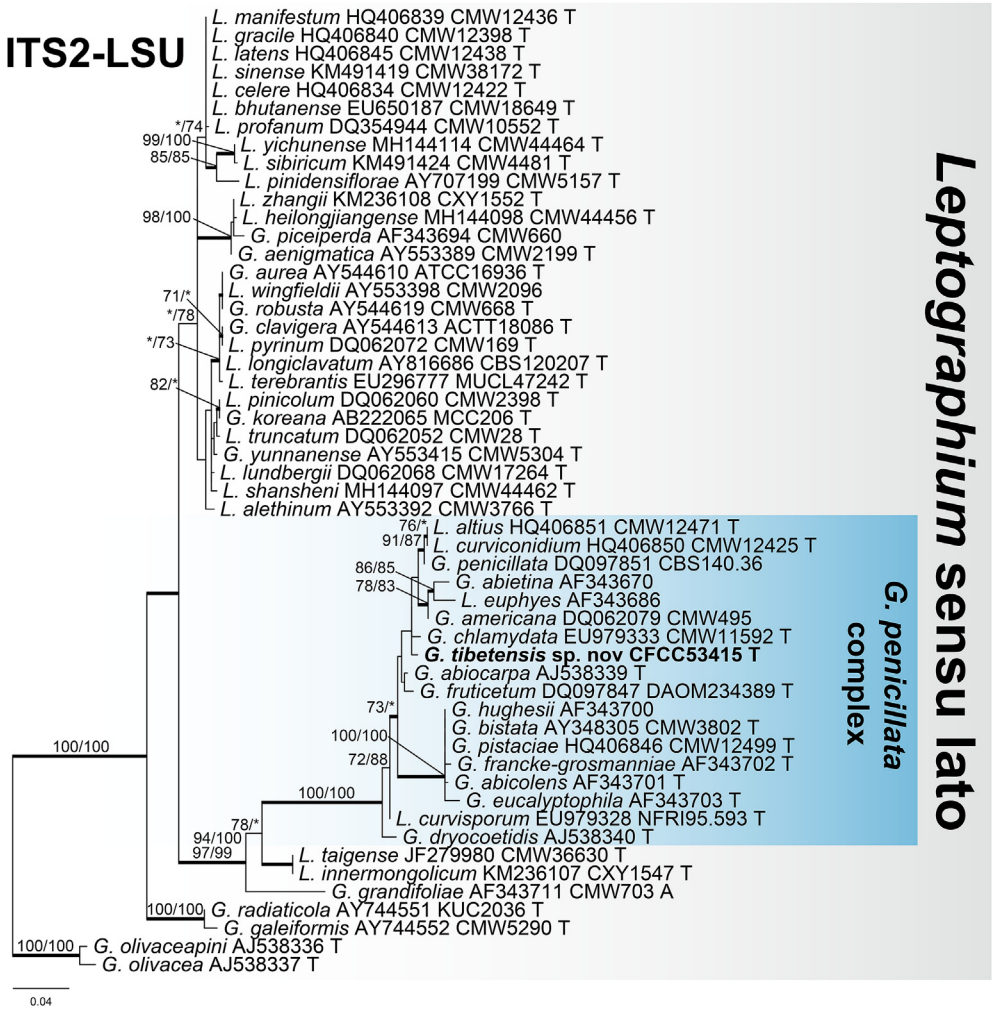

Fig. 1. ML tree of *Leptographium* generated from the ITS2-LSU sequence data. Sequences generated from this study are printed in bold type. Bold branches indicate posterior probability values ≥ 0.9. Bootstrap values of ML/MP ≥ 70% are recorded at nodes. Scale bars: branch length. T = ex-type isolates.

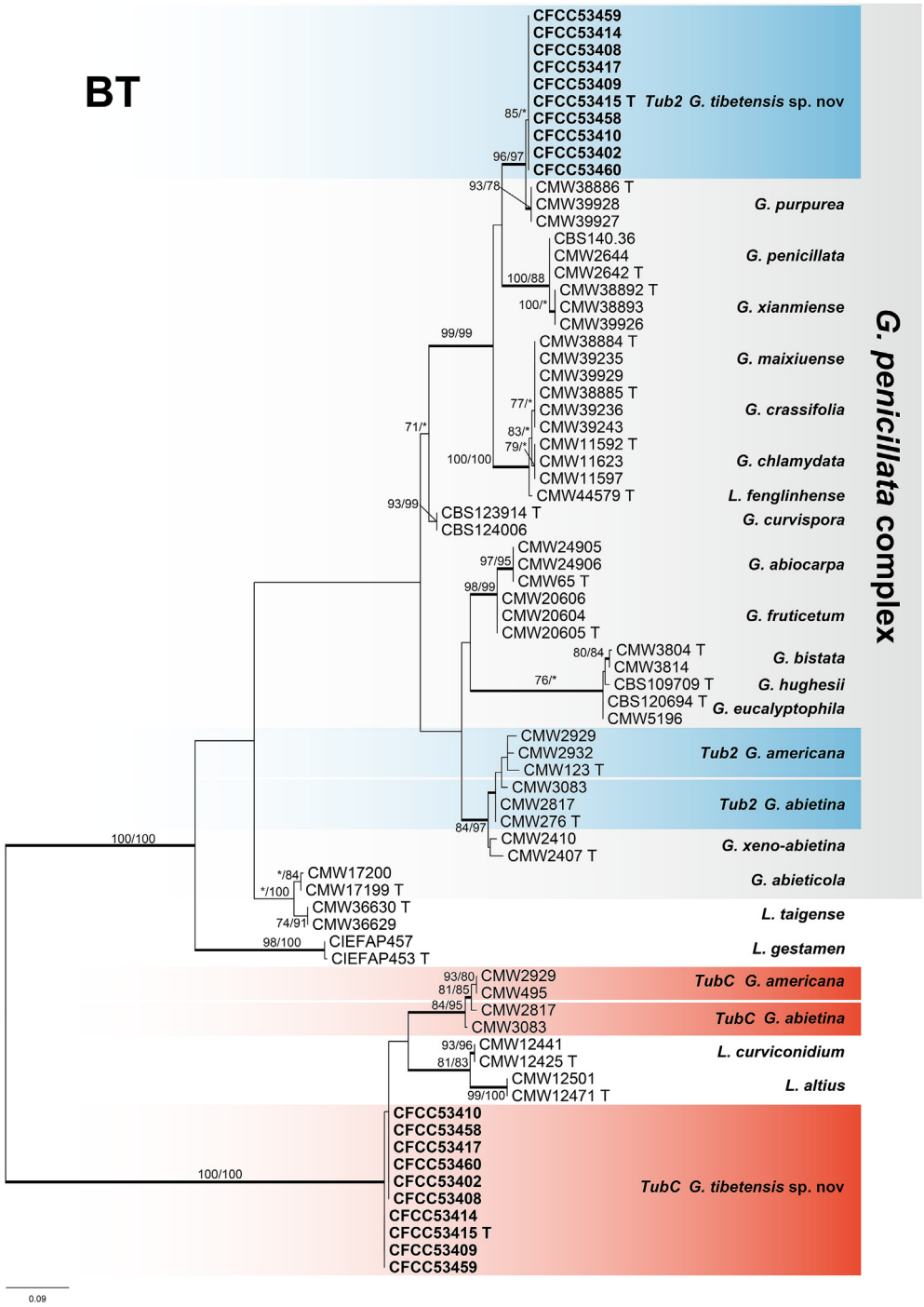

Fig. 2. ML tree of "*Grosmannia penicillata* complex" generated from the BT sequence data. Sequences generated are printed in bold type. Bold branches indicate posterior probability values ≥ 0.9. Bootstrap values of ML/MP ≥ 70% are recorded at nodes. Scale bars: branch length. T = ex-type isolates.

mononematous, erect, occurring singly or in groups, arising directly from the mycelium, (146.0–) 197.6–438.4 (–544.2) μm in length including the conidiogenous apparatus, rhizoid-like structures absent. Stipes light olivaceous, not constricted, cylindrical, simple, 3–10-septate, (75.5–) 96.5–235.3 (–310.3) long, (5.2–) 6.3–9 (–10.2) μm wide at base, the basal cell swollen or not, (6.1–) 6.8–10.0 (–12.1) μm wide below primary branches, apical cell not swollen. Conidiogenous apparatus (73.4–) 98.9–216.8 (–240.8) μm long, excluding the conidial mass, consisting of 2–4 series of branches, the primary branching type A. Primary branches light olivaceous, cylindrical, (11.6–) 13.6–21.9 (–26.6) × (3.2–) 4.2–7.2 (–9.2) μm, secondary branches light olivaceous, aseptate, (10.5–) 13.1–20.3 (–25.7) × (3.1–) 4.0–6.8 (–8.9) μm, tertiary branches light olivaceous, aseptate, (8.8–) 11.7–18.3 (–23.2) × (3.0–) 3.9–5.8 (–7.7) μm. Conidiogenous cells discrete, 2–3 per branch, smooth or rough, cylindrical, (11.3–) 12.6–18.3 (–21.5) × (1.7–) 1.8–2.4 (–3.0) μm. Conidia hyaline, smooth, aseptate, long oblong to obovoid, (5.2–) 5.4–9.2 (–11.2) × (1.9–) 2.1–3.2 (–4.3) μm.

Pesotum asexual state: synnemata solitary or in groups, (208.3–) 250.6–525.5 (–669.3) μm tall including the conidiogenous

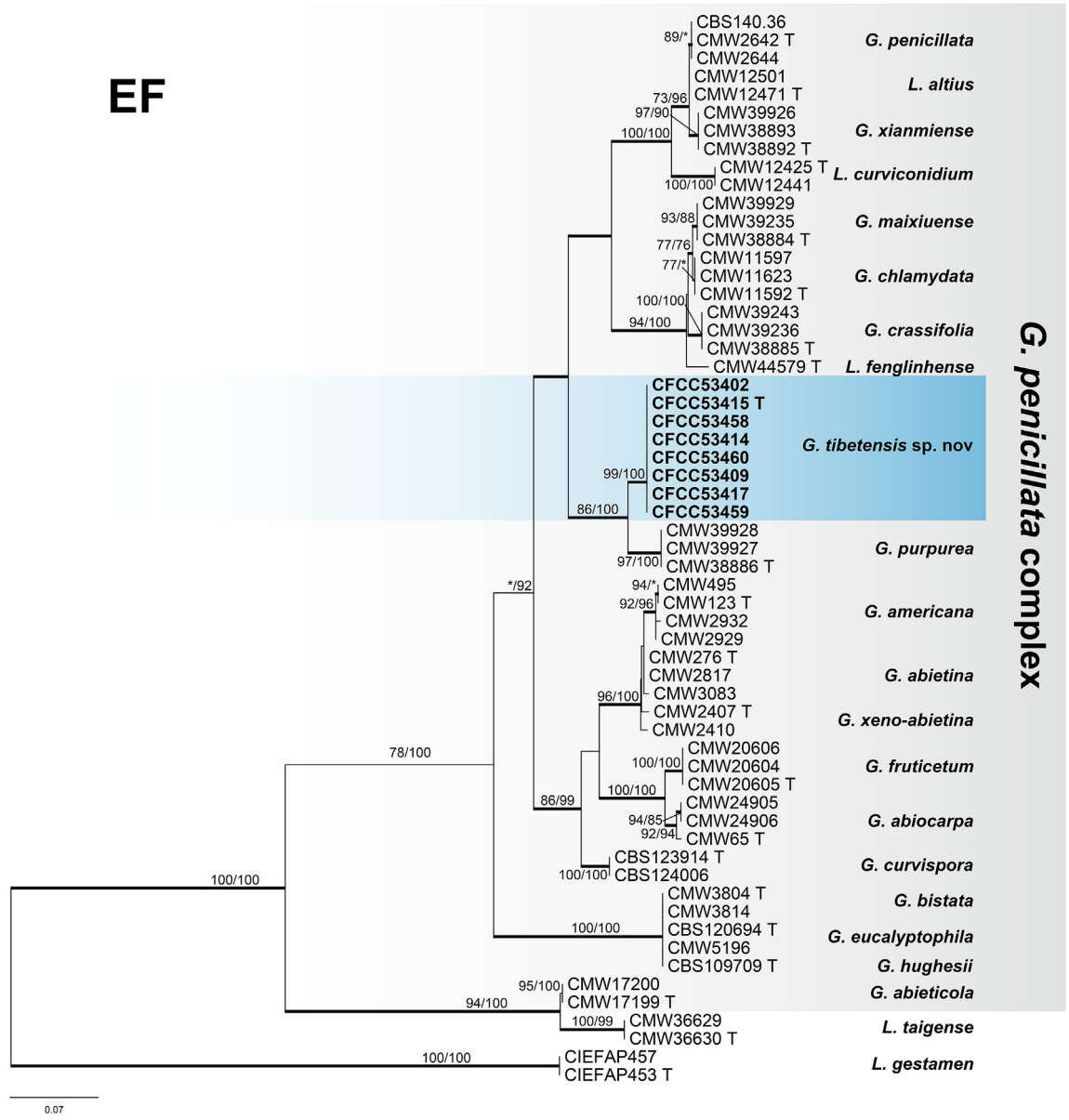

Fig. 3. ML tree of "*Grosmannia penicillata* complex" generated from the EF sequence data. Sequences generated from this study are printed in bold type. Bold branches indicate posterior probability values ≥ 0.9. Bootstrap values of ML/MP ≥ 70% are recorded at nodes. Scale bars: branch length. T = ex-type isolates.

apparatus, the base hyaline or pale yellow, (14.1−) 20.6−45.2 (−54.1) μm wide; conidiogenous cells (10.3−) 11.8−15.7 (−18.3) × (1.5−) 1.6−1.8 (−1.9) μm long; conidia hyaline, smooth, long oblong, aseptate, (3.9−) 4.3−5.4 (−6.2) × (1.6−) 1.7−2.1 (−2.2) μm.

Chlamydospore hyaline, smooth, aseptate, oval to elliptical, (8.2−) 9.0−11.8 (−13.2) × (6.4−) 7.8−10.0 (−10.5) μm.

Culture characteristics: Colonies on 2% MEA initially hyaline, colony edge thinning radially, later becoming olivaceous or pale olivaceous, aerial mycelium sparse. Reaching 73 mm diam in 5 d at 25 °C. No growth observed at 0 and 35 °C. Optimal temperature for growth at 25 °C.

Host: *Picea likiangensis* var. *balfouriana*.

Beetle vector: *Orthotomicus* sp.

Distribution: *Hitherto*, known only from Tibet autonomous region, China.

Additional specimen examined: CHINA, Tibet autonomous region, Zuogong City, from *Orthotomicus* sp. infesting *P. likiangensis* var. *balfouriana*. Aug 2017, Z. Wang & Q. Lu, CXY 2029 = CFCC 53458, CXY 2031 = CFCC 53409, CXY 2032 = CFCC 53408, CXY 2033 = CFCC 53410. Zuogong City, from gallery of *Orthotomicus* sp. infesting *P. likiangensis* var. *balfouriana*. Aug 2017, Z. Wang & Q. Lu, CXY 2034 = CFCC 53459, CXY 2035 = CFCC 53460, CXY 2036 = CFCC 53417, CXY 2037 = CFCC 53402, CXY 2038 = CFCC 53414.

Notes: Phylogenetic inferences show that *G. tibetensis* represents a distinct clade within the "*G. penicillata* complex", and is closely related to *L. altius*, *G. americana*, *G. chlamydata* (K. Jacobs, M.J. Wingf. & H. Solheim) M.L. Yin, Z.W. de Beer & M.j. Wingf., *G. crassifolia* M.L. Yin, Z.W. de Beer & M.j. Wingf., *L. curviconidium*, *L. fenglinhense* R. Chang, Z.W. de Beer & M.J. Wingf., *G. maixiuense* M.L. Yin, Z.W. de Beer & M.j. Wingf., *G. penicillata* (Grosmann) Goid., *G. purpurea*, and *G. xianmiense* M.L. Yin, Z.W. de Beer & M.j. Wingf. (Figs. 2 and 3). Morphologically, the species is characterized by a

Fig. 4. *Grosmannia tibetensis* (CFCC 53415, ex-holotype). **A:** *Leptographium*-like asexual morph; **B:** primary branch patterns: Type-A; **C, D:** conidiogenous cells of *Leptographium*-like asexual morph and conidia; **E, J:** chlamydospore; **F, G:** *Pesotum*-like asexual morph; **H, I:** conidiogenous cells of *Pesotum*-like asexual morph and conidia; **K, L:** Five-day-old cultures on 2% MEA. *Bars:* **A, B, J** 20 μm; **C**—**E, H, I** 10 μm; **F** 100 μm; **G** 50 μm.

combination of both *Leptographium* and *Pesotum* asexual morphs and production of chlamydospores, which is unique among species of the *G. penicillata* lineage. *Grosmannia chlamydata* and *L. curviconidium* develop a *Leptographium* and *Hyalorhinocladiella* asexual morphs (Jacobs, Krokene, Solheim, & Wingfield, 2010; Paciura et al., 2010); *L. altius*, *G. americana*, *G. crassifolia*, *G. maixiuense*, *G. penicillata*, *G. purpurea*, and *G. xianmiense* have a *Leptographium* morph (Jacobs & Wingfield, 2001; Paciura et al., 2010; Yin et al., 2020); and *L. fenglinhense* produces only a *Hyalorhinocladiella* asexual morph (Chang et al., 2019).

Additionally, *G. tibetensis* could be differentiated from *L. altius*, *L. curviconidium*, *G. maixiuense*, and *G. purpurea* by the absence of rhizoid-like structures subtending the *Leptographium* conidiophores, which are present in the two latter species (Paciura et al., 2010; Yin et al., 2020).

Grosmannia tibetensis shares with *G. chlamydata* the production of chlamydospores but these two species also differ in their conidial shape, long oblong to obovoid in *G. tibetensis* and oblong to obovoid with a truncate base and a rounded apex in *G. chlamydata* (Jacobs et al., 2010). The long oblong conidia of *G. tibetensis* also differ

from those of *L. altius*, which are obovoid and elongated with truncated bases (Paciura et al., 2010). The conidia of *G. americana* are obovoid to allantoid with subtruncated bases (Paciura et al., 2010). The conidia of *G. crassifolia*, *G. maixiuense*, *G. purpurea*, and *G. xianmiense* are obovoid (Yin et al., 2020). The conidia of *L. curviconidium* are curved and allantoid shape with truncate bases (Paciura et al., 2010). The conidia of *G. penicillata* are allantoid with truncate bases and rounded apices (Jacobs & Wingfield, 2001), as well as the elliptical conidium produced by *L. fenglinhense* (Chang et al., 2019).

Temperature, as a limiting growth factor, varies in the complex. *Grosmannia tibetensis,* contrary to the other species of the "*G. penicillata* complex", is able to grow at temperatures down to 5 °C. The optimal temperature for *G. tibetensis*, *L. altius*, *G. crassifolia*, *L. curviconidium G. purpurea*, and *G. xianmiense* are 25 °C, but it is 20 °C for *G. americana* and *G. maixiuense*, and 30 °C for *G. penicillata*. At the optimal growth temperature, the growth rate of *G. tibetensis* (73 mm diam in 5 d) is much faster than that of *L. altius* (44 mm diam in 8 d), *G. americana* (31 mm diam in 8 d), *G. crassifolia* (5.8 mm/d), *L. curviconidium* (52 mm diam in 8 d), *G. maixiuense* (5.7 mm/d), *G. purpurea* (4 mm/d), and *G. xianmiense* (5.5 mm/d). The growth rate of *G. tibetensis* was similar to *L. fenglinhense* (75 mm diam in 6 d), and *G. penicillata* (73 mm diam in 5 d) on 2% MEA at 25 °C (Chang et al., 2019; Jacobs et al., 2010; Jacobs & Wingfield, 2001; Yin et al., 2020).

4. Discussion

Fifty-two strains of ophiostomatoid fungi were isolated from adults and galleries of *Orthotomicus* sp. infesting *P. likiangensis* var. *balfouriana* in forest of the Tibetan Plateau. Using a combination of morphology and phylogeny, *G. tibetensis* sp. nov. was described and illustrated based on these strains. The species belongs to the "*G. penicillata* complex" and is the first record of ophiostomatoid fungi from Tibet.

Grosmannia penicillata is the type species of the genus. It forms a well-supported lineage together with 23 other species of *Leptographium* s. l. referred to as the "*G. penicillata* complex" (Chang et al., 2019; de Beer & Wingfield, 2013; Linnakoski et al., 2012; Six, de Beer, Duong, Carroll, & Wingfield, 2011; Yin et al., 2020). Jacobs and Wingfield (2001) summarized the morphological identification of *Leptographium*, and emphasis was placed on conidiophore length, presence and absence of rhizoids, primary branching pattern, and conidial morphology. Phylogenetic analysis based on the ITS2-LSU, BT, and EF genes supports the relatively stable relationships and accurate classification within the genus. Although the "one fungus one name" policy has been adopted over years (Barrie et al., 2012, p. 240; Hawksworth, 2011), the generic names of *Leptographium* and *Grosmannia* are still currently used for this group of fungi by most taxonomists. Following the suggestion by de Beer and Wingfield (2013) to "Describe new species in the "*G. penicillata* complex" in *Grosmannia*, irrespective of their morph", we used *Grosmannia* as the genus name for the new species.

Paralogous genes were well-known problems in phylogenetic analysis of taxonomy. Although the beta-tubulin gene was a common and valuable marker for fungal phylogeny, it underwent independent duplications and losses in different lineages and formed distinct paralogous and orthologous clades (Zhao et al., 2014; Tekpinar & Kalmer, 2019). The housekeeping gene for beta-tubulin protein is *tub2*, a third most utilized gene in fungal multilocus phylogenies (Feau, Decourcelle, Husson, Desprez-Loustau, & Dutech, 2011). Another gene, coding beta-tubulin (with identical or different function as *tub2* product) is *tubC*, which is paralogous to *tub2* (Weatherbee & Morris, 1984). Hubka and Kolarik (2012) reported that beta-tubulin paralogue *tubC* is frequently misidentified as the *tub2* gene in *Aspergillus*, and using primer pair Bt2a/Bt2b could amplify both of them. Our study provides similar results in ophiostomatoid fungi (Fig. 2). The lineage composed of *tubC G. americana* (Jacobs, Solheim, Wingfield, & Wingfield, 2005; Jacobs, Wingfield, & Wingfield, 2001), *tubC G. abietina* (Jacobs et al., 2005), *L. altius* (Paciura et al., 2010), *L. curviconidium* (Paciura et al., 2010) together with *tubC* group of new species were excluded from the "*G. penicillata* complex". In addition to *tubC* gene, *tub2* gene was also sequenced for *G. abietina* and *G. americana* (Massoumi Alamouti, Kim, Humble, Uzunovic, & Breuil, 2007; Yin et al., 2020), but not for *L. altius* and *L. curviconidium* (Paciura et al., 2010), which needs to be clarified as present in both species. Due to the importance of beta-tubulin gene as an ideal gene candidate in phylogenetics, the selection of the primer pair is a very important step (Tekpinar & Kalmer, 2019). Since the specificity of the Bt2a/Bt2b primer pair is apparently low in contrast to primer pair T10/Bt2b, which is highly *tub2*-specific (Supplementary Fig. S1), it is recommended that primer pair T10/Bt2b should be preferred in the phylogenic study of ophiostomatoid fungi.

Knowledge of fungal associates for *Orthotomicus* spp. is limited and mainly from European species. Only two species of *Orthotomicus*, *O. laricis* Fabricius. and *O. proximus* Gyllenhal., and two hosts, *Abies alba* Mill. and *Pinus sylvestris* Linn., have been studied so far (Jankowiak et al., 2017; Kirisits, 2004). To date, 27 species of ophiostomatoid fungi have been reported associated with the beetles worldwide, including 2 *Ceratocystiopsis* species, 2 *Ceratocystis* species, 2 *Graphilbum* species, 2 *Graphium* species, 8 *Leptographium* species, and 11 *Ophiostoma* species (Jankowiak et al., 2017; Kirisits, 2004). In China, at least five species of *Orthotomicus* spp. are widespread, infesting fir, spruce, and pine trees (Yin et al., 1984). There are likely to be more *Orthotomicus* spp. and their associated ophiostomatoid fungi awaiting discovery.

Declaration of competing interest

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.myc.2020.05.004.

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