Cryptogamie, Mycologie, 2013, 34 (3): 233-241

A novel species of *Gliocladiopsis* from freshwater habitat in China

*Fang LIU & Lei CAI**

State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, P.R. China

Abstract – *Gliocladiopsis guangdongensis* sp. nov. is introduced on the basis of both morphological and on multilocus phylogenetic analysis (β-tubulin, histone H3, internal transcribed spacer region and translation elongation factor 1-α). The species is compared to other morphologically similar and phylogenetically closely related taxa. Species of *Gliocladiopsis* are mostly known from soil and terrestrial plants whereas this new species is the first isolation from decaying wood from freshwater habitat.

Phylogeny / Submerged wood / Taxonomy

INTRODUCTION

The genus *Gliocladiopsis* S.B. Saksena (Saksena 1954) was introduced to accommodate *G. sagariensis*, a soil-born species. *Gliocladiopsis* was characterized by penicillate conidiophores resembling *Gliocladium*, and straight cylindrical conidia similar to that of *Calonectria* and *Cylindrocladiella*.

Glionectria tenuis was described as the teleomorph state of *Glicladiopsis tenuis,* the first report of telemorph for *Gliocladiopsis* (Schoch *et al.*, 2000). However, *Ga. tenuis* was shown to be distinct from *G. tenuis* by further sequence data and morphological comparisons, and is provided with a new name *G. pseudotenuis* (Lombard and Crous 2012).

Recently, the taxonomy of *Gliocladiopsis* was investigated on the basis of multilocus phylogenetic inferences and morphological comparisons (Lombard and Crous 2012). Currently, nine species are recognized, i.e. *G. irregularis* (Crous and Peerally 1996), *G. sumatrensis* (Crous *et al.*, 1997), *G. tenuis* (Crous and Wingfield 1993), *G. sagariensis* (Saksena 1954), *G. elghollii*, *G. mexicana*, *G. indonesiensis*, *G. pseudotenuis*, and *G. curvata* (Lombard and Crous 2012). In addition, two unique sterile strains remain unnamed. Lombard and Crous (2012) named all the species in the anamorph genus *Gliocladiopsis* (Saksena 1954) instead of *Glionectria* (Schoch *et al.*, 2000) following the "strict priority" option as applied by Gräfenhan *et al.* (2011) and Lombard *et al.* (2010, 2012). Phylogenetic studies revealed that genus *Gliocladiopsis* is closely related to *Gliocephalotrichum*/*Leuconectria* (Nectriales, Ascomycota) (Schoch *et al.*, 2000).

During a survey of freshwater fungi in Guangdong Province, southern China, a species of *Gliocladiopsis* was isolated but could not be identified as any

* Correspondence: Lei CAI, email: mrcailei@gmail.com

doi/10.7872/crym.v34.iss2.2013.233

of the known species. In a multi-locus phylogenetic inferences (β-tubulin, histone H3, internal transcribed spacer region and translation elongation factor 1-α), it also formed a terminal clade distinct from all other species clades. Consequently, the species is described as *Gliocladiopsis guangdongensis* sp. nov.

MATERIALS AND METHODS

Isolation. — Submerged wood samples were collected from streams at Dinghu Mountain Nature Reserve in Zhao Qing, Guangdong Province, China in Dec 29, 2010. Samples were returned to the laboratory and treated as described by Liu *et al.* (2012). Single-spore isolations were made on water agar (WA) with antibiotics following the method described by Choi *et al.* (1999). Morphological characterization of *Gliocladiopsis* isolate was done on submerged wood and synthetic nutrient-poor agar (SNA; Nirenberg 1976). Inoculated plates were incubated at room temperature under normal light condition and examined after 7 d. Measurements and photographs of characteristic structures were made according to Liu *et al.* (2012). The specimens are deposited in the herbarium of Microbiology, Academia Sinica (HMAS), while living cultures deposited in China General Microbiological Culture Collection Center (CGMCC). Descriptions, nomenclature and illustrations were deposited in MycoBank.

DNA sequencing and phylogenetic analyses. — Total genomic DNA was extracted from single-spore isolates using a Biospin Fungus Genomic DNA Extraction Kit (Bio-Flux®) according to the instructions of the manufacturer. Four loci were amplified using primers ITS1 and ITS4 for ITS (White *et al.*, 1990), EF1-728F and EF1-986R for TEF-1α (Carbone and Kohn 1999), CYLH3F and CYLH3R for HIS3 (Crous *et al.*, 2004), and T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) for TUB2. PCR protocols are same as that outlined by Liu & Cai (2012). The PCR products were sequenced in both directions at SinoGenoMax Company ltd.

Sequences of our isolates, along with reference sequences (Lombard and Crous 2012) obtained from GenBank, were aligned using MAFFT v.6 (Katoh and Toh 2010), and manually corrected where necessary with MEGA5 (Tamura *et al.*, 2011). Phylogenetic analysis was performed using PAUP* 4.0b10 (Swofford 2002). Ambiguously aligned regions were excluded from all analyses. Maximum parsimony (MP) analysis was performed. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved. Clade stability was assessed in a bootstrap analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa. Trees were visualized in Treeview (Page 1996).

Model of evolution was estimated by using MrModeltest 2.3 (Nylander 2004) for each gene region and included in the analyses. Posterior probabilities (PP) (Rannala and Yang 1996; Zhaxybayeva and Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v.3.2.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), using the estimated model of evolution. Two analyses of four MCMC chains were run from random trees for ten million generations and sampled every 1000 generations. All runs converged on the same likelihood score and tree topology, and therefore the

first 25% trees were discarded as the burn-in phase of each analysis and posterior probabilities determined from the remaining trees. Sequences derived in this study were deposited in GenBank.

RESULTS

Phylogenetic analyses

Eight sequences of the new species were deposited in GenBank (KC776118–KC776125). The alignment was deposited in TreeBASE under accession number S14051. Phylogenetic relationships were inferred by analyzing a combined ITS, HIS3, TUB2, and TEF 1-α regions data set. *Calonectria brachiatica* (CBS 123700) was used as outgroup taxon. The resulting dataset comprises 1835 characters with gaps, including 1404 constant and 229 parsimony-uninformative characters. Analysis of the 202 parsimony-informative characters yielded three equally parsimonious trees, one of the trees (Length = 622; CI = 0.814; RI = 0.896; RC = 0.729; HI = 0.186) is presented in Fig. 1. The Bayesian tree confirmed both the tree topology and support of the branches obtained with maximum parsimony. Bayesian posterior probability ≥ 0.95 are shown as thickened branches on the phylogenetic tree. The two isolates of *Gliocladiopsis guangdongensis* clustered in a distinct clade (Bootstrap support value = 100; PP = 1.00, shown as thick branch) and appeared basal to *G. curvata* (Fig. 1).

Gliocladiopsis guangdongensis F. Liu & L. Cai, **sp. nov.** **Figs 2-3**

MycoBank: MB 803846

Etymology: "*guangdongensis*", referring to the Province where the type was collected.

Colonies on natural substratum effuse, white. Mycelium partly immersed, partly superficial. Conidiophores hyaline, penicillate. Conidiogenous apparatus with two levels of hyaline branches: primary branches aseptate or 1-septate, 14.5-15.5 × 3-3.5 μm; secondary branches aseptate, 10-10.5 × 2.5-3 μm; phialides cymbiform to cylindrical, 11-16 × 2-3 μm, arranged in terminal whorls of 3-6 per branch, collarettes present. Conidia cylindrical, hyaline, smooth, guttulate, straight with obtuse ends, 0- to 1-septate, 13.5-16 (–22.5) × 2-3 μm (› = 15.0 × 2.5 μm, n = 50). Teleomorphic stage not observed.

Colonies on SNA effuse, white (growth 26-30 mm/week). Aerial mycelium hyaline. Conidiophores hyaline, penicillate. Conidiogenous apparatus with 4 series of hyaline branches: primary branches aseptate or 1-septate, 12.5-21.5 × 1.5-4 μm; secondary branches aseptate, 7.5-21.5 × 1.5-3.5 μm; tertiary branches non-septate, 12-16.5 × 2.5-3.5 μm; quaternary branches rare to absent, aseptate, 14.5-17 × 2.5-3 μm; phialides cymbiform to cylindrical, 7-19.5 × 1.5-2.5 μm, arranged in terminal whorls of 3-6 per branch, collarettes present. Conidia cylindrical, hyaline, smooth, guttulate, straight with obtuse ends, aseptate to 1-septate, 8-17.5 × 2-3.5 μm ($\bar{x}$ = 12.5 × 2.5 μm, n = 30), lacking a visible abscission scar.

Teleomorph: not observed.

Habitat: Saprobic on submerged wood.

Known distribution: China.

Holotype: China, Guangdong Province, Zhaoqing Dinghu Mountain, on submerged wood, in a stream, 29 Dec. 2010, F. Liu, HMAS 244829, ex-type living culture CGMCC 3.15260 = LC 1340; Zhaoqing Dinghu Mountain, in a stream, on submerged wood, 29 Dec 2010, *F. Liu*, HMAS 244830, living culture CGMCC 3.15261 = LC 1349.

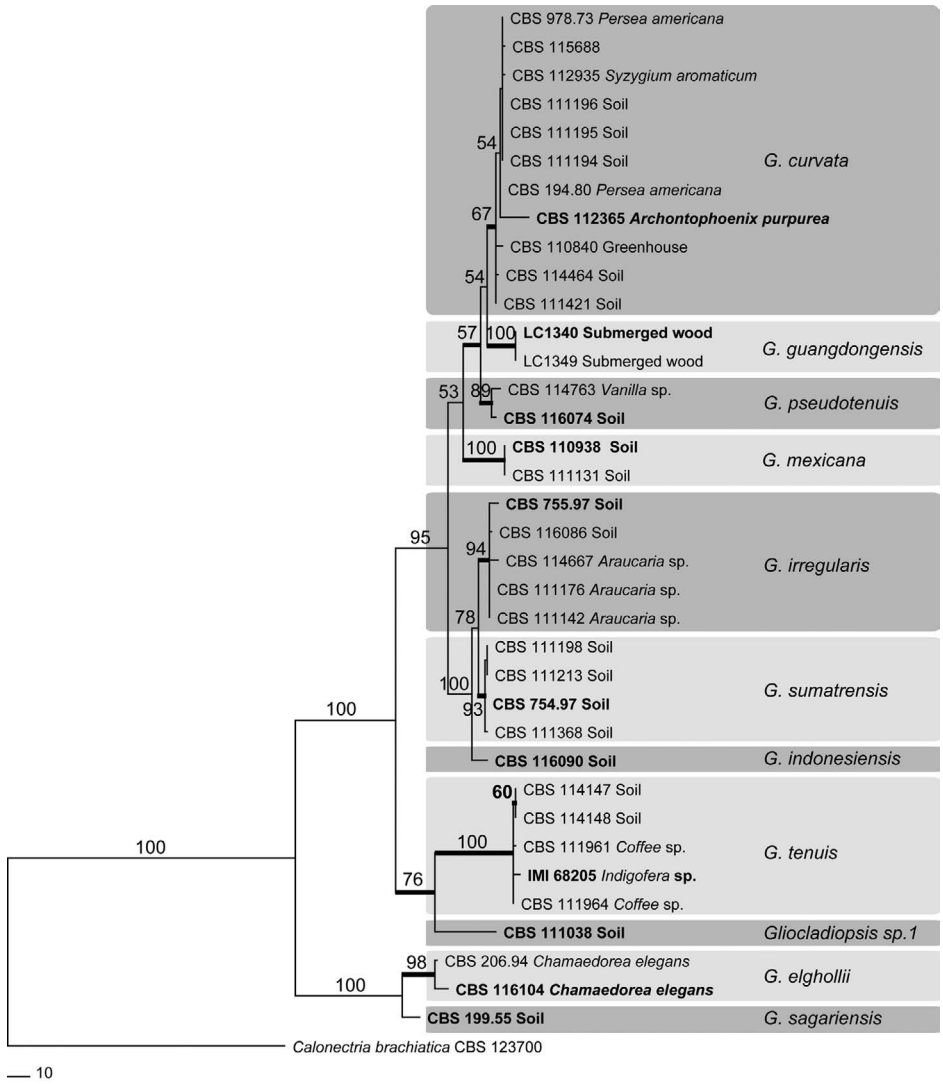

Fig. 1. Maximum parsimony phylogram showing phylogenetic relationships of *Gliocladiopsis guangdongensis* with closely related taxa based on combined sequences of internal transcribed spacer region, translation elongation factor 1-α, histon H3 and β-tubulin sequence alignments. Bootstrap support values above 50% are shown above the branches. Thickened branches represent significant Bayesian posterior probability (≥ 0.95). The tree is rooted with *Calonectria brachiatica*. Ex-type isolates are indicated in Bold.

DISCUSSION

Conidia of *G. guangdongensis* are hyaline, cylindrical with obtuse ends, 0-1-septate, with smooth wall. They are similar in shape to the conidia of *G. elghollii* and *G. tenuis*, but distinctly shorter (Table 1). The quaternary branches are rare or absent in *G. guangdongensis* but abundant in *G. elghollii*. Moreover, both species are well distinguished from each other by the multi-

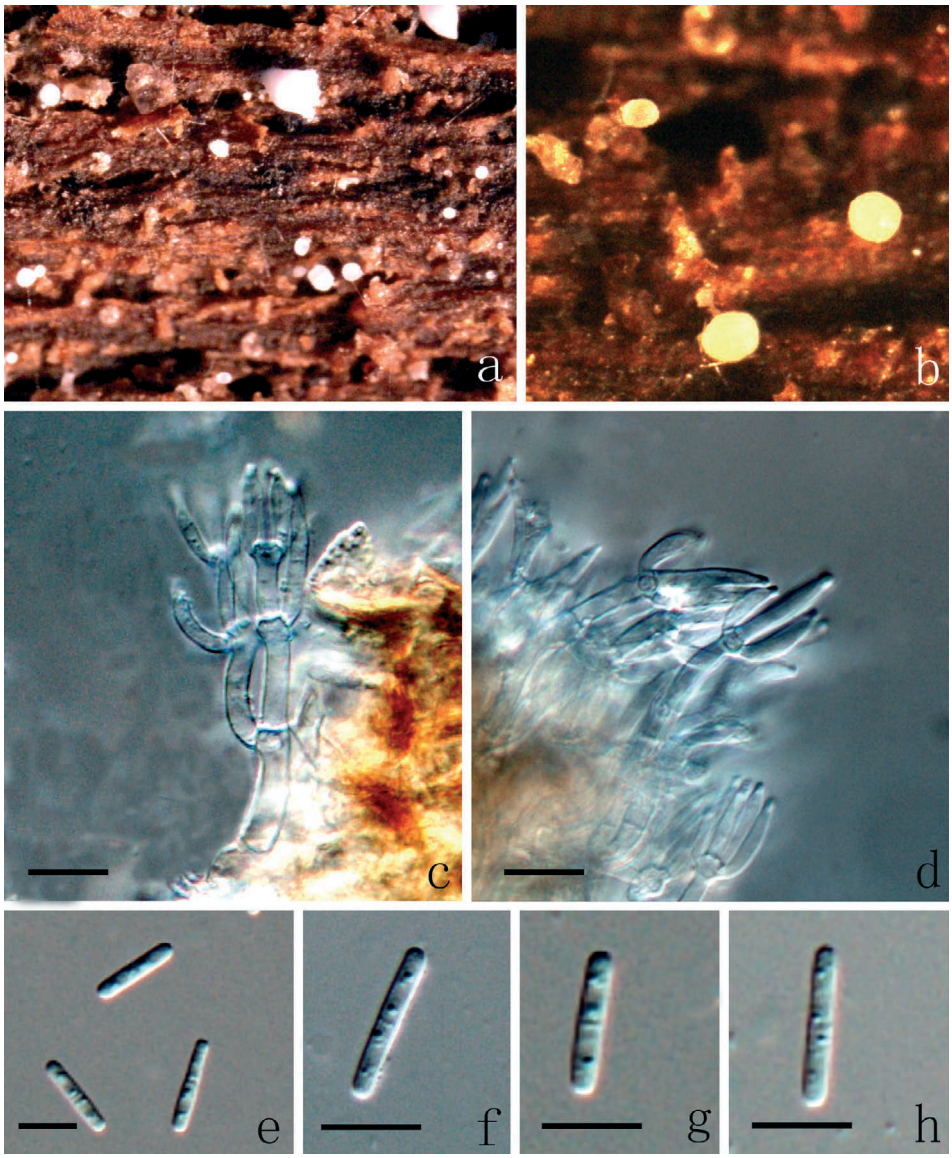

Fig. 2. *Gliocladiopsis guangdongensis* on submerged wood. **a-b.** Colonies on submerged wood; **c-d.** Penicillate conidiophores; **e-h.** Conidia (bar: c-h = 10 µm).

locus phylogenetic analysis (Fig. 1). *Gliocladiopsis guangdongensis* is phylogenetically related to *G. curvata*, but produced shorter and straight conidia (Table 1, Figs 2-3).

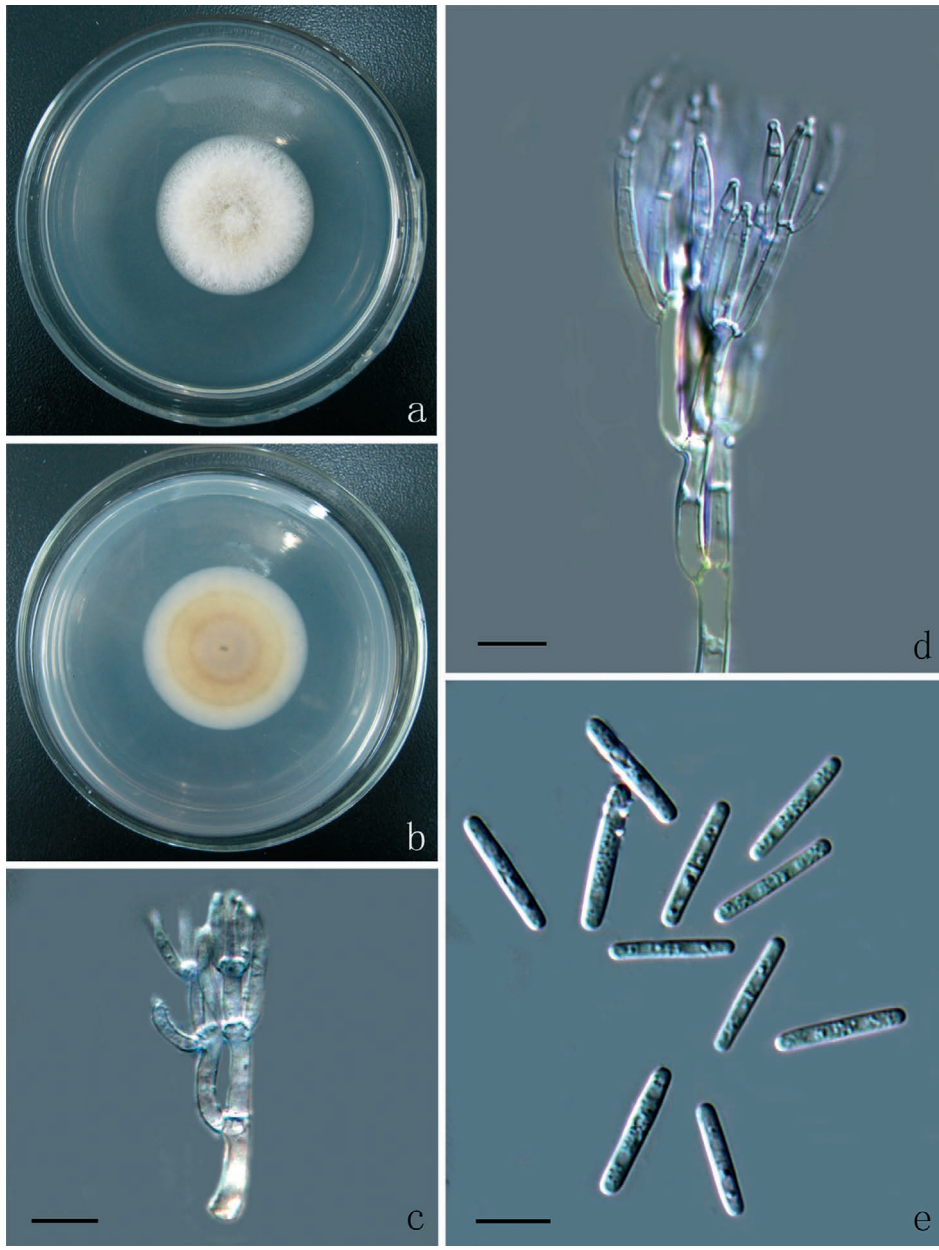

Fig. 3. *Gliocladiopsis guangdongensis* on medium after 7 days (ex-type culture). **a-b.** Colonies (a Surface, b Reverse); **c-d.** Penicillate conidiophores; **e.** Conidia (bar: c-e = 10 µm). **a-b.** on PDA; **c-e.** on SNA.

Table 1. Synopsis of characters of *Gliocladiopsis* species

Orgnism name	*Medium*[1]	*Quaternary branch*	*Conidial shape*	*Conidial size (µm)*	*Conidial av. size (µm)*	*Substrate*	*Country*	*References*
G. curvata	SNA	quaternary branches rare to absent	cylindrical, hyaline, smooth with rounded ends, straight to slightly curved, 0-1-septate	(16–)17–21(–23) × 3–5	19 × 3	*Archontophoenix purpurea*; *Persea americana*; Soil; *Syzygium aromaticum*	Belgium, Brazil, Ecuador,Indonesia, Japan, Mauritius, New Zealand	Lombard & Crous 2012
G. elghollii	SNA	quaternary branches present	cylindrical, hyaline, smooth with rounded ends, straight, 0-1-septate	(18–)19–23(–29) × 2–4	21 × 3	*Chamaedorea elegans*	USA	Lombard & Crous 2012
G. guangdongensis	SNA	quaternary branches rare to absent	cylindrical, hyaline, smooth with obtuse ends, straight, 0-1-septate	8–17.5 × 2–3.5	12.5 × 2.5	Submerged wood	China	This study
G. indonesiensis	SNA	quaternary branches present	cylindrical, hyaline, smooth with rounded ends, straight, 1-septate	(11–)13–15(–17) × 2–4	14 × 3	Soil	Indonesia	Lombard & Crous 2012
G. irregularis	CLA	quaternary branches absent	cylindrical, hyaline, smooth, apex obtuse, base subtruncate, straight to variously curved, 1-septate	(11–)13(–14) × 2.5(–3)	13 × 2.5	*Araucaria* sp.; Soil	Indonesia, Malaysia	Crous *et al.*, 1996; Lombard & Crous 2012
G. mexicana	SNA	quaternary branches absent	cylindrical, hyaline, smooth with rounded ends, straight, 1-septate	(15–)17–19(–21) × 2–4	18 × 3	Soil	Mexico	Lombard & Crous 2012
G. pseudotenuis	SNA	quaternary branches rare to absent	cylindrical, hyaline, smooth with rounded ends, 1-septate	(14–)15–19(–21) × 2–4	17 × 2	Soil; *Vanilla* sp.	China, Indonesia	Schoch *et al.*, 2000; Lombard & Crous 2012
G. sagariensis	Czapek agar	quaternary branches absent	cylindrical, slightly yellowish, smooth with rounded ends, 1-septate	18–24 × 1.5–2	–	Soil	India	Saksena 1954
G. sumatrensis	CLA	quaternary branches absent	cylindrical, hyaline, smooth with obtuse ends, straight, 1-septate	(10–)14–17(–18) × 2–2.5(–3)	–	Soil	Indonesia	Crous *et al.*, 1997
G. tenuis	CLA	quaternary branches rare to absent	cylindrical, hyaline, obtuse ends, 0-1-septate	(16–)16.5(–20) × 1.5(–2)	18 × 2	*Coffee* sp.; *Indigofera* sp.; Soil	India, Indonesia, Vietnam	Crous & Wingfield 1993; Lombard & Crous 2012

[1]SNA:Synthetic nutrient-poor agar; CLA: Carnation-leaf agar.

Gliocladiopsis was recently re-evaluated on the basis of multi-locus (ITS, TUB2, His3 and TEF 1-α) phylogenetic analysis (Lombard and Crous 2012). There were two distinct branches could not be assigned to any of the resolved species. These two clades were represented by the sterile strains CBS 116086 and CBS 111038 (Lombard and Crous 2012). In the current study, using same loci, we found out that the strain CBS 116086 nested confidently within the *G. irregularis* clade (Fig. 1) indicating conspecificity. The strain CBS 111038 still remains isolated and may correspond to an additional undescribed *Gliocladiopsis* species.

All the currently known *Gliocladiopsis* species were isolated from terrestrial habitats (roots of diseased plants, plant litter or soil) (Lombard and Crous 2012). Although *Gliocladiopsis* strains were isolated from symptomatic plant material hitherto, their relevance as plant pathogens has never been tested (Lombard and Crous 2012). Dann *et al.* (2012) conducted pathogenicity test of *Gliocladiopsis* isolates on avocado plant roots and revealed that they were non-pathogenic but strengthened the overall growth condition of the plants.

Beside those from diseased plant material and soil, *Gliocladiopsis* species have also been recovered as endophytes, which were isolated from *Paris polyphyla* var. *yunnanensis* (Li *et al.*, 2008, strain num. Ppf3, GenBank acc. num. EF495240) and *Rafflesia cantleyi* (Rojas-Jimenez K *et al.*, unpublished, strain num. INBio3709A, GenBank acc. num. GU827507). ITS phylogeny revealed that isolate INBio3709A from *R. cantleyi* nested within *G. curvata*, indicating possible conspecificity(unpubl.). The taxonomic status of isolate Ppf3 remains unclear using single ITS analysis.

Gliocladiopsis guangdongensis, isolated from submerged wood in a stream, is the first record of *Gliocladiopsis* species from freshwater. Freshwater fungi colonize submerged organic matter (e.g. leaves, twigs, branches) and play an important role in degrading woody debris and leaves in freshwater environments (Cai *et al.*, 2003, 2006, Ranghoo *et al.*, 2001, Simonis *et al.*, 2008).

Acknowledgement. This study was financially supported by NSFC 31322001 & 31093440.

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