Pseudochaetosphaeronema ginkgonis sp. nov., an endophyte isolated from Ginkgo biloba

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An endophytic strain (designated as SYPF 7195T) was isolated from a branch of a ginkgo tree in Liaoning province of China. Strain SYPF 7195T was characterized by its grey to greyish-green aerial mycelium, velvety to floccose surface and swelling near the septa. Phylogenetic analyses, which were inferred from the internal transcribed spacer (ITS) and partial sequences of the LSU and SSU of the rDNA and translation elongation factor 1-alpha (TEF1), showed that strain SYPF 7195T belonged to the genus Pseudochaetosphaeronema, and was distinct from all other species with high bootstrap-supported values (92 %). Strain SYPF 7195T constitutes a separate evolutionary clade with Pseudochaetosphaeronema lairense and Pseudochaetosphaeronema martinelli, with P. martinelli as its closest phylogenetic neighbour. The nucleotide differences between strain SYPF 7195T and P. martinelli were 71 substitutions in the ITS region. Strain SYPF 7195T could also be distinguished from P. martinelli by a number of physiological characteristics. Combined with morphology and molecular analyses, strain SYPF 7195T merits recognition as a representative of a novel species of the genus Pseudochaetosphaeronema, for which the name Pseudochaetosphaeronema ginkgonis sp. nov. is proposed. The type strain is CBS 140953T (=CGMCC 3.17865T=SYPF 7195T). The Mycobank number is MB 816567.

The genus Pseudochaetosphaeronema was described based on anamorph morphology. Morphological characteristics of the species of the genus Pseudochaetosphaeronema mainly include production of black obpyriform pycnidia with a long neck, hyaline and phialidic conidiophores, and unicellular subspherical to ellipsoidal conidia. No sexual morphology was found (Ahmed et al., 2014). Recent studies have demonstrated that translation elongation factor 1-alpha (TEF1) and the rDNA LSU, SSU and internal transcribed spacer (ITS) loci play important roles in classification of the family Macrodiplodiopsidaceae (Ahmed et al., 2015; Crous et al., 2015; Wijayawardene et al., 2014).

In this study, an endophyte attributed morphologically to the genus Pseudochaetosphaeronema was isolated from a branch of Ginkgo biloba. The ITS sequence of this strain did not match significantly to any other known sequences in the GenBank database. Based on the morphology and DNA sequence analyses, a novel species of the genus Pseudochaetosphaeronema is proposed.

Samples of healthy branches of G. biloba were collected from Dandong city, Liaoning Province, northeast of China. Samples were surface sterilized (Khieu et al., 2015; Qin et al., 2011), sliced into small pieces and transferred to PDA medium (PDA: 20 % potato extract, 1 % glucose and 2 % agar) supplemented with 100 mg streptomycin L−1. All

Abbreviations: CI, consistency index; HI, homoplasy index; ITS, internal transcribed spacer; RC, rescaled consistency index; RI, retention index; TEF1, translation elongation factor 1-alpha; TL, tree length.

The GenBank/EMBL/DDBJ accession numbers for the ITS, 28S rDNA, 18S rDNA and translation elongation factor 1-alpha gene sequences of the type strain SYPF 7195T are KU365986, KU365985, KU365983 and KU365984, respectively.

One supplementary table is available with the online Supplementary Material.
plates were incubated at 28 °C for 12/12 h with and without fluorescent light equally, and examined daily. Small colonies were transferred to another PDA plate. Mycelia were stored in 20 % glycerol at −80 °C for further study.

Two microlitres mycelia suspension was added to PDA, corn meal agar (CMA: 0.7 % cornmeal and 1.5 % agar), malt extract agar (MEA: 3 % malt extract, 0.3 % soy peptone and 1.5 % agar), oatmeal agar (OA: 3 % oatmeal and 2 % agar) and pine-needle agar (PNA: 20 % pine needle extract and 2 % agar). All plates were incubated at 26 °C for 12/12 h with and without the fluorescent light for 20 days. Growth rates of the fungus was calculated by measuring the colony diameters. Mycelium structure was observed through a Nikon Eclipse 80i light microscope.

The mycelia grown in PDB (PDB: 20 % potato extract and 1 % glucose) at 26 °C for 5 days were prepared for DNA isolation. Total genomic DNA was extracted from fresh mycelium by using the NuClean PlantGen DNA Kit (CWBI0) according to the manufacturer’s instructions. Target regions of the ITS, SSU, LSU and TEF1 were amplified by using fungal-specific primers: ITS4 and ITS5 for ITS (White et al., 1990); NS1 and NS8 (Noda & Kodama, 1996; White et al., 1990) for partial SSU; LROR (Rehner & Samuels, 1995) and LR7 (Vilgalys & Hester, 1990) for partial LSU, EF1728F (Chaverri et al., 2003) and TEF1LErev (Jaklitsch et al., 2005) for partial TEF1. A G1000 Thermal Cycler (BIOER) was used to amplify the above DNA sequences. The amplifications were verified with 1 % agarose gel electrophoresis, and the fluorescent bands were collected and purified with an AxyPrepgel purification kit (Axygen). The fragment was cloned into the pMD18-T vector (TaKaRa Biotech) followed by sequencing (Sangon Biotech, Shanghai, China).

The phylogenetic tree was reconstructed based on the combined sequences of ITS, SSU, LSU and TEF1. Sequences from this study, along with reference sequences obtained from GenBank, were edited with BioXM 2.6 (Huang & Zhang, 2004) and aligned by CLUSTAL X (Larkin et al., 2007). Phylogenetic analyses were accomplished by MEGA 5.0 (Tamura et al., 2011) by using the neighbour-joining method and adopting Kimura 2 as the nucleotide substitution model, and by PAUP* 4.0b10 (Swofford, 2003) with the criterion of maximum-parsimony. The tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were recorded. A heuristic search option was chosen with random addition of sequences as 1000 replications, and gaps were treated as missing data.

Species delineation, classification and ecology

Based on the combination of multilocus (ITS, SSU, LSU and TEF1) analysis and phenotypic features, a novel species named Pseudochaetosphaeronema ginkgonis sp. nov. is proposed and described. It grew slowly on PDA, CMA, MEA, OA and PNA plates and increased 1–2 mm per day. P. ginkgonis sp. nov. showed variable morphology on different media. The colonies showed a velvety surface with round grooves on PNA (Fig. 1c) while they were round with radial grooves on MEA (Fig. 1g). When growing on PDA, CMA and OA, the colonies showed floccose surface (Fig. 1a, e, i). Radial cracks were observed in the center of the reverse colonies on PDA and MEA (Fig. 1b, h). P. ginkgonis sp. nov. was characterized by the production of smooth-walled hyphae and swelling near the septa, which was similar to that observed in P. martinelli (Ahmed et al., 2015). Key morphological features that differentiated P. ginkgonis sp. nov. from P. martinelli were that P. martinelli produces wart-like incrustation and brown, swollen and coiled hyphae (Ahmed et al., 2015). The pycnidia, conidiophores and conidia were only found in P. larense (Ahmed et al., 2014).

With the primers described above, four loci (ITS, SSU, LSU and TEF1) were amplified from strain SYPF 7195T along with type strains from previous studies for the phylogenetic analysis (Table S1, available in the online Supplementary.

**Fig. 1.** Cultural characters of Pseudochaetosphaeronema ginkgonis CBS 140953T (a to j). Colonies on PDA at 26 °C after 16 d (a, obverse; b, reverse), on PNA at 26 °C after 12 d (c, obverse; d, reverse), on CMA at 26 °C after 20 d (e, obverse; f, reverse), on MEA at 26 °C after 20 d (g, obverse; h, reverse) and on OA at 26 °C after 12 d (i, obverse; j, reverse).
Material. This united dataset comprised 4438 characters after alignment and represented 43 taxa, including the outgroup. During the maximum parsimony analysis, 3060 characters were constant, 563 were parsimony-uninformative and 815 were parsimony-informative. The phylogenetic tree resulting from the combined analysis of sequence data is shown in Fig. 2 (TL=3133, CI=0.622, RI=0.700, RC=0.435, HI=0.378). A total of 40 taxa from the Pleosporales were separated into eight groups with varying support. Our isolate was placed in the clade Macrodiplodiopsidaceae, forming a well-supported sister clade together with *P. larense* and *P. martinelli*. The phylogenetic tree also indicated that *P. ginkgonis* sp. nov. had a close relationship with *P. larense* and *P. martinelli*. The ITS sequence of *P. ginkgonis* sp. nov. had 71% identity with *P. ginkgonis* including *P. larense* and *P. martinelli*. The molecular data strongly indicated that *P. ginkgonis* sp. nov. had a close relationship with *P. ginkgonis* sp. nov. was different from *P. ginkgonis* including *P. larense* and *P. martinelli*. The ITS sequence of *P. ginkgonis* sp. nov. had 86% identity with *P. ginkgonis*. The ITS sequence of *P. ginkgonis* sp. nov. proposed in this study. *P. martinelli* was first isolated from a patient as an agent of subcutaneous phaeohyphomycosis (Ahmed et al., 2015), and *P. larense* was isolated as an agent of human subcutaneous infection (Borelli et al., 1976). Here, *P. ginkgonis* sp. nov. isolated from a branch of a ginkgo tree is regarded as an endophyte. This finding expands the host range of species of the genus *Pseudochaetosphaeronema* and deepens the understanding of species in this genus.

**Description of Pseudochaetosphaeronema ginkgonis**

_Tianyuan Zhang, Xiaoyu Deng, Mengyue Zhang & Yixuan Zhang sp. nov._

_Pseudochaetosphaeronema ginkgonis_ (gink.go’nis. N.L. gen. n. ginkgonis of Ginkgo biloba).

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Figs. 2. Consensus tree derived from a maximum-parsimony (MP) analysis of the combined ITS, SSU, LSU and TEF1 sequences alignment. PAUP maximum-parsimony bootstrap support values (MPB; above 50%) and _MEGA_ neighbour-joining bootstrap support values (BS; above 50%) are given at the nodes (MPB/BS). Bar, expected number of changes per site. The tree is rooted to _Sordaria fimicola, Xyaria hypoxylon_ and _Dothidea insculpta_. _P. ginkgonis_ sp. nov. is denoted by bold letters.
Colonies on PDA are grey, velvety to floccose with irregular edges, raised in the centre, becoming darker with age and reaching 19.1 mm in diameter after 16 days at 26 °C while their reverse sides are grey to dark grey with 3–5 radial cracks in the centre (Fig. 1a, b). Colonies on PNA are greyish green, velvety with two deep round grooves, reaching 13.8 mm in diameter after 12 days at 26 °C and produce rust-coloured pigment while their reverse sides are grey (Fig. 1c, d). Colonies on CMA are grey, velvety in the centre, floccose and irregular at the edge with a round groove, reaching 25.4 mm in diameter after 20 days at 26 °C while their reverse sides are dark grey to black (Fig. 1e, f). Colonies on MEA are greyish green, velvety with deep round and radial grooves and reaching 24.4 mm in diameter after 20 days at 26 °C while their reverse sides are grey to brown with circular striation and 5–7 radial cracks in the centre (Fig. 1g, h). Colonies on OA are grey, floccose and reaching 20.1 mm in diameter after 12 days at 26 °C while their reverse sides are grey (Fig. 1i, j). Conidiation and sexual morphology have not been observed. Hyphae are hyaline, smooth-walled, 2.7–8.2 µm in diameter, branched, regularly septate and swollen near the septa (Fig. 3).

The type strain SYPF 7195T was isolated from healthy branches of *Ginkgo biloba*, which were collected from Dandong city, Liaoning Province, northeast of China, and is considered to be an endophyte. The living culture from the type strain is maintained in the lyophilized state as SYPF 7195T (Shenyang Pharmaceutical University, Shenyang, China), CGMCC 3.17865T (China General Microbiological Culture Collection Center, Beijing, China) and CBS 140953T (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands). The Mycobank number is MB 816567.

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**References**


