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RESEARCH ARTICLE

Morphological and phylogeny of *Plenodomus sinensis* and *P. collinsoniae*, two unreported species isolated from soil in Korea

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ABSTRACT

Two unreported fungal isolates, KNU-GW1901 and KNU-AP100C, were collected from soil sample in Gyeongsangbuk-do, Korea. Their cultural and morphological characteristics were examined after 4 weeks of incubation at 25°C on potato dextrose agar (PDA), malt extract agar (MEA), and oatmeal agar (OA). The conidial shape of KNU-GW1901 was aseptate, hyaline, globose to ellipsoidal, oblong, and reniform to pyriform and 2.61-4.97×1.93-3.61 µm in size, whereas no conidial structures were observed in KNU-AP100C. The internal transcribed spacer (ITS) regions, large subunit (LSU), and small subunit (SSU) sequences were used to determine the taxonomic positions of the strains using the maximum likelihood phylogenetic tree. The isolate KNU-GW1901 was closely clustered with *Plenodomus sinensis* MFLUCC 17-0767, and KNU-AP100C was closely matched with *P. collinsoniae* CBS 120227. Based on the findings of morphological, cultural, and phylogenetic analysis, the isolates KNU-GW1901 and KNU-AP100C were identical to the previously described *P. sinensis* and *P. collinsoniae* isolates, respectively, which are first reported in Korea.

Keywords: Genus *Plenodomus*, Morphological characteristics, Phylogenetic analysis

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INTRODUCTION

Dothideomycetes is the largest class of Ascomycota, comprising more than 100 families and 19,000 species, and Pleosporales represents the largest order in Dothideomycetes, consisting of a quarter of all Dothideomycetous species [1,2]. The family Leptosphaeriaceae was introduced by Barr in 1987, which is an economically important family of the order Pleosporales for plant pathogens, and the genus *Plenodomus* was included in the family [3,4]. Species of this family are generally saprophytes but also parasitic on herbaceous or woody plants on the land and in water bodies [5]. Members of this family are well known for their immersed perithecial ascomata possessing single papillate ostioles, hyaline to brown cylindrical asci, and their transverse septate ascospores [3,5]. They can also exist as asexual morphs, which are either Coelomycetes or Hyphomycetes [6,7]. The genus *Plenodomus* was first introduced in 1851 by Preuss with

P. rabenhorstii as a type species [3,8]. *P. ravenhorstii* was replaced by Borerema & Kesteren (1964) with *P. lingam* (Tode) Hohn, which is a sexual morph because the former material was lost during World War II [3,9]. Currently, there are 100 epithets of *Plenodomus* listed in Index Fungorum in 2020 [10]. The members of the genus *Plenodomus* have also been reported in some Asian and European countries while *P. biglobosus*, *P. lindquistii*, *P. tracheiphilus*, and *P. wasabiae* have been known as important pathogens [11]. In the present study, we aimed to investigate the fungal species in Korea as well as to establish the stable taxonomy and phylogeny for the fungal isolates collected from a field soil sample and to identify the species based on morphological characteristics and phylogenetic analysis. We provide detailed information about the cultural and morphological characteristics of two unreported species for the first time in Korea.

MATERIALS AND METHODS

Collection of soil samples

The fungal isolates KNU-GW1901 and KNU-AP100C were isolated from soil, collected from a disused apple orchard in Gyeongsangbuk-do (36°16′32.7″N, 128°27′59.1″E and 36°11′45.9″N, 128°34′10.8″E), Korea. These soil samples were collected from the ground at a depth of approximately 15-30 cm, air-dried, and then placed in a plastic bag at 4℃ till analysis. Each of the soil samples weighed approximately 1 g and were suspended in 10 mL of sterile distilled water. The soil solution was vortexed and diluted until suspended, and then 1 mL was spread on potato dextrose agar (PDA; Difco, Detroit, MI, USA) and incubated for 2-3 days at 25℃ [12]. Then, the individual colonies were transferred to new fresh PDA plates and incubated at 25℃ until the development of mycelium. Based on different cultural characteristics of these two strains, they were selected for further molecular analyses. These isolates, KNU-GW1901 and KNU-AP100C, were maintained in 20% glycerol at -80℃ for further studies.

Morphological characterization

For morphological observations, the strains KNU-GW1901 and KNU-AP100C were transferred onto potato dextrose agar (PDA; Difco, Detroit, MI, USA), oatmeal agar (OA; Difco, MI, USA), and malt extract agar (MEA; Difco, MI, USA) and incubated at 25℃ for 4 weeks [13]. After incubation, the fungal growth of each strain was measured, and colony characteristics such as the shape, color, and size were recorded. Morphological characteristics of the isolates were observed under a light microscope (BX-50, Olympus, Tokyo, Japan).

Genomic DNA extraction, PCR amplification, and sequencing

Fungal mycelia were obtained by scraping from PDA plates for the extraction of genomic DNA. Then, the HiGene Genomic DNA Prep Kit (Biofact, Daejeon, Korea) was used to extract genomic DNA from the two isolates according to the manufacturer's instructions. For the isolate KNU-GW1901 and KNU-

AP100C, the large subunit (LSU), small subunit (SSU), and the internal transcribed spacer (ITS) regions were amplified using the primers LROR/LR5 or LROR/LR7, NS1/NS4, and ITS1F/ITS4 [14-16], respectively. The thermal conditions for PCR amplification were followed as described by a previous report [7]. Then, the amplified PCR products were purified using ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced by Macrogen Co. Ltd. (Daejeon, Korea). Finally, the obtained sequences from KNU-GW1901 and KNU-AP100C isolates were deposited in NCBI GenBank under the accession number of LC550566 and LC550567 for ITS, LC550568 and LC550569 for LSU, LC550570 for SSU.

Phylogenetic analysis

The partial sequences of large subunit (LSU), small subunit (SSU), and ITS regions of the allied species with *P. sinensis* and *P. collinsoniae* were retrieved from NCBI for phylogenetic analysis (Table 1). The consent sequences were compared with other allied sequences in the NCBI database using BLAST search results. The MEGA 7 software program was used to construct the phylogenetic tree with the bootstrap analysis of 1,000 replications [17]. Evolutionary distances were calculated using the maximum likelihood method based on the Tamura-Nei model [18].

Table 1. The isolates KNU-GW1901 and KNU-AP100C and culture collection strains of GenBank accession numbers used in this study for phylogenetic analyses.

Species	Strain number	GenBank Accession Numbers		
		ITS	LSU	SSU
Plenodomus chrysanthemi	CBS 539.63	NR111622	GU238151	GU238230
P. guttulatus	MFLU 15-1876	KT454721	KT454713	KT454729
P. sinensis	MFLU 17-0767	MF072721	MF072717	MF072719
P. sinensis	MFLU 17-0757	MF072722	MF072718	MF072720
P. sinensis	**KNU-GW1901**	**LC550567**	**LC550569**	**LC550570**
P. visci	CBS 122783	NR119957	EU754195	EU754096
Didymella exigua	CBS 183.55	GU237794	EU754155	EU754056
P. biglobosus	CBS 119951	JF740198	JF740274	JF740102
	CBS 127294	JF740199	JF740275	-
P. wasabiae	CBS 120119	JF740257	JF740323	-
	CBS 120120	JF740258	JF740324	-
P. collinsoniae	CBS 120227	JF740200	JF740276	-
P. collinsoniae	**KNU-AP100C**	**LC550566**	**LC550570**	-
P. pimpinellae	CBS 101637	JF740240	JF740309	-
P. hendersoniae	CBS 113702	JF740225	JF740295	-
	CBS 139.78	JF740226	JF740296	-
P. libanotidis	CBS 113795	JF740231	JF740300	-
P. enteroleucus	CBS 142.84	JF740214	JF740287	-
	CBS 831.84	JF740215	JF740288	-
P. inflluorescens	CBS 143.84	JF740228	JF740297	-
	PD 73/1382	JF740229	JF740298	-
P. lindquistii	CBS 386.80	JF740232	JF740301	-
	CBS 381.67	JF740223	JF740302	-
Alternaariaster helianthi	CBS 327.69	KC609335	KC584369	-

The species isolated in this study is indicated in bold.

CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; KNU: Kyunpook National University, Daegu, South Korea.

RESULTS

Taxonomy of KNU-GW1901

Plenodomus sinensis **Tennakoon, Phookamsak & K.D. Hyde, Phytotaxa 324(1): 76(2017) (Fig. 1).**

Colony characteristics: The isolate KNU-GW1901 had a slow growth rate, with the average fungal growth rates on PDA, MEA, and OA being 48.5, 52.6, and 68.2 mm, respectively, at 25℃ for 4 weeks. The upper surface of the colonies on PDA was white to light gray in color in the center and reverse olivaceous green to black in the center with regular to irregular white margin (Fig. 1A). The colonies on MEA were covered by a gray-white to deep gray, circular margin, fluffy, and reverse olivaceous green to pale black with a light yellowish margin (Fig. 1B). Regarding the colonies on OA surface, the mycelium appeared white to dark gray with iron-gray spots and irregular margins. The elevation of the colonies on all media demonstrated an umbonate type (Fig. 1C).

Micromorphology: The isolate KNU-GW1901 produced pycnidia on the surface of PDA media after 4 weeks. The pycnidia measured 123 μm high and 110 μm wide and were scattered, solitary to gregarious, black and globose to subglobose (Fig. 1D). The conidia were aseptate, hyaline, globose to ellipsoidal, oblong, and reniform to pyriform and measured 2.61-4.97×1.93-3.61 μm (Fig. 1E and 1F). The morphology of the strain KNU-GW1901 was comparable with the previous research finding of *P. sinensis* [13].

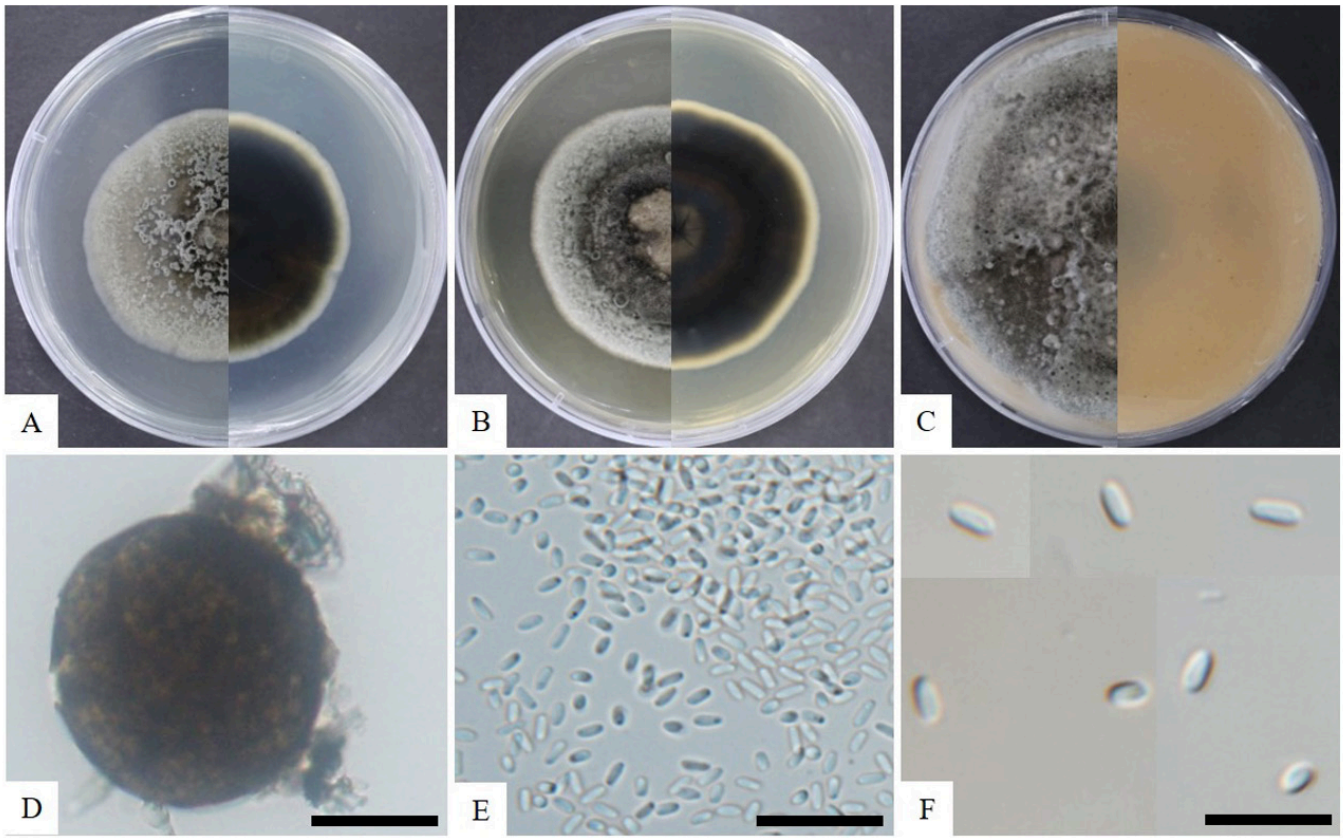

Fig. 1. Cultural and morphological characteristics of *Plenodomus sinensis* KNU-GW1901. A,colonies on potato dextrose agar; B, colonies on malt extract agar; C, colonies on oatmeal agar; D, pycnidium; E, F, conidia (Scale bars: D-F=10 μm).

Phylogenetic analysis of KNU-GW1901

Sequences of the LSU, SSU, and ITS regions were used to construct the phylogenetic placement of the isolate KNU-GW1901. BLAST searches in the nucleotide database of GenBank (www http://blast.ncbi.nlm.nih.gov/) were used to obtain the closed taxa of the new isolate. After analyzing the nucleotide sequences, 595, 976, and 731 bp were obtained from the LSU, SSU, and ITS regions, respectively. Based on the BLAST search results, KUN-GW1901 exhibited high similarities in ITS (98.79%), SSU (100%), and LSU (99.59%) with the previously identified *P. sinensis* (MFLUCC 17-0767). The isolate KNU-GW1901 was closely clustered together with *P. sinensis* (MFLUCC 17-0767) in the maximum likelihood phylogenetic tree using the combined sequences of LSU, SSU and ITS regions (Fig. 2). Thus, the phylogenetic results support the morphological identification that the strain KNU-GW1901 was *P. sinensis*.

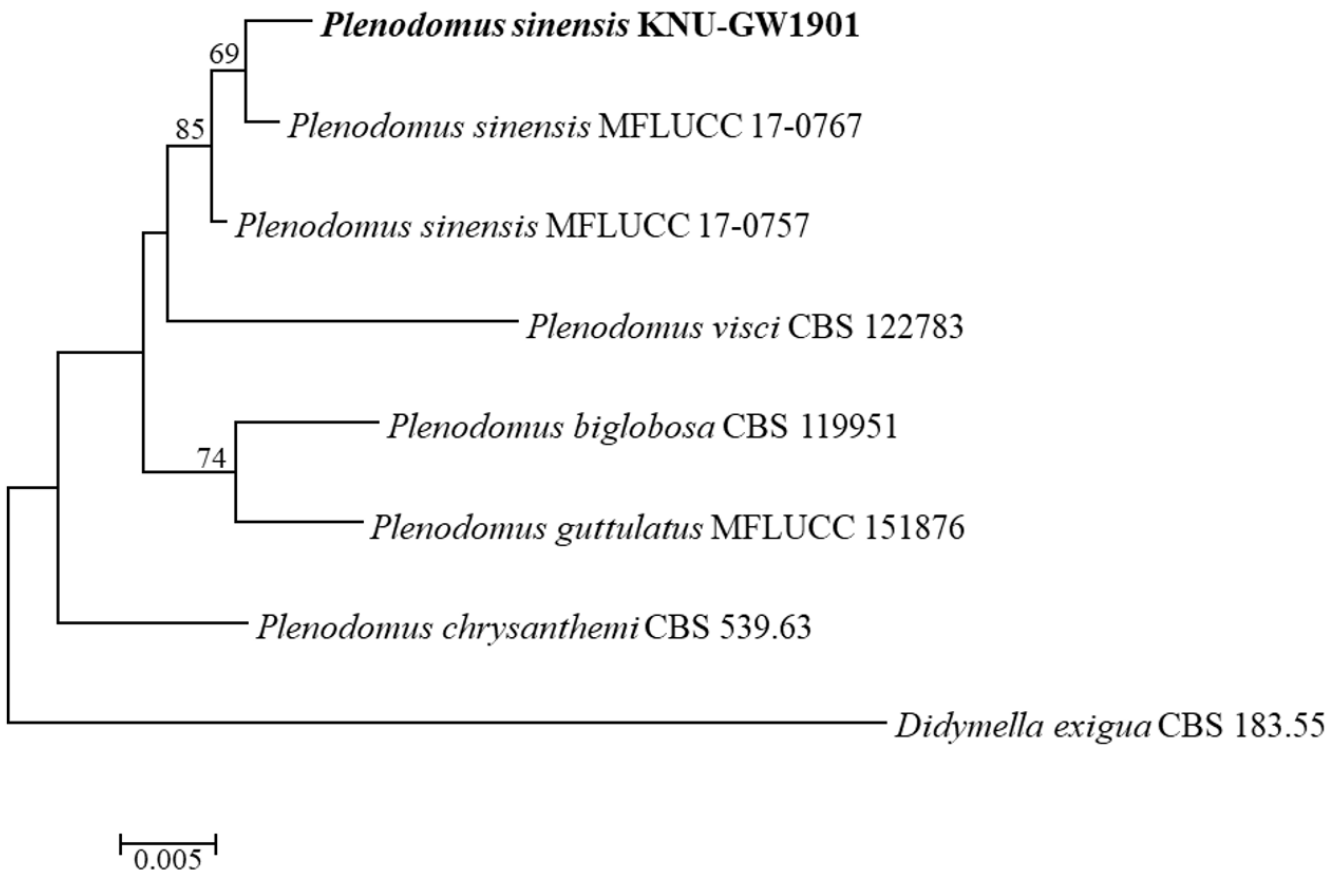

Fig. 2. Maximum likelihood phylogenetic tree based on the combined dataset of partial sequences of large subunit (LSU), small subunit (SSU), and internal transcribed spacer (ITS) regions. *Didymella exigua* CBS 183.55 was used as an outgroup. The isolated strain in this study is indicated in bold, and the bootstrap values (based on 1000 replications) higher than 70% is shown at the branch points. Bar, 0.005 substitutions per nucleotide position.

Taxonomy of KNU-AP100C

***Plenodomus collinosinae* (Dearn. & House) Gruyter, Aveskamp & Verkley, Studies in Mycology 75: 21 (2012) (Figure 3).**

Colony characteristics: The isolate KNU-AP100C was cultured on PDA, MEA, and OA to observe the cultural and morphological characteristics. The growth of the colonies reached 57.2 mm on PDA, 58.2 mm on MEA, and 52.5 mm on OA, at 4 weeks of incubation at 25°C. The colonies on PDA were white to light gray color in the center with a circular margin and reverse orange to light black color in the center with a wide white margin (Fig. 3A). The colonies on MEA were white to light gray with black spots near the center and reverse pale yellow to orange with a circular margin (Fig. 3B). The surface of the colonies on OA were initially white to gray becoming brown in the center (Fig. 3C).

Micromorphology: The isolate produced numerous conidiomata on the surface of PDA after 4 weeks of incubation at 25°C. The conidiomata were round to irregular, solitary to aggregate, and dark brown to black on the surface of the medium (Fig. 3D). Wide hyphae, branched mycelium, septate, smooth, brown, and light brown chlamydospores were found on PDA media (Fig. 3E and 3F). No conidial structures were observed in KNU-AP100C despite being cultured on PDA, MEA, and OA media after 4 weeks of

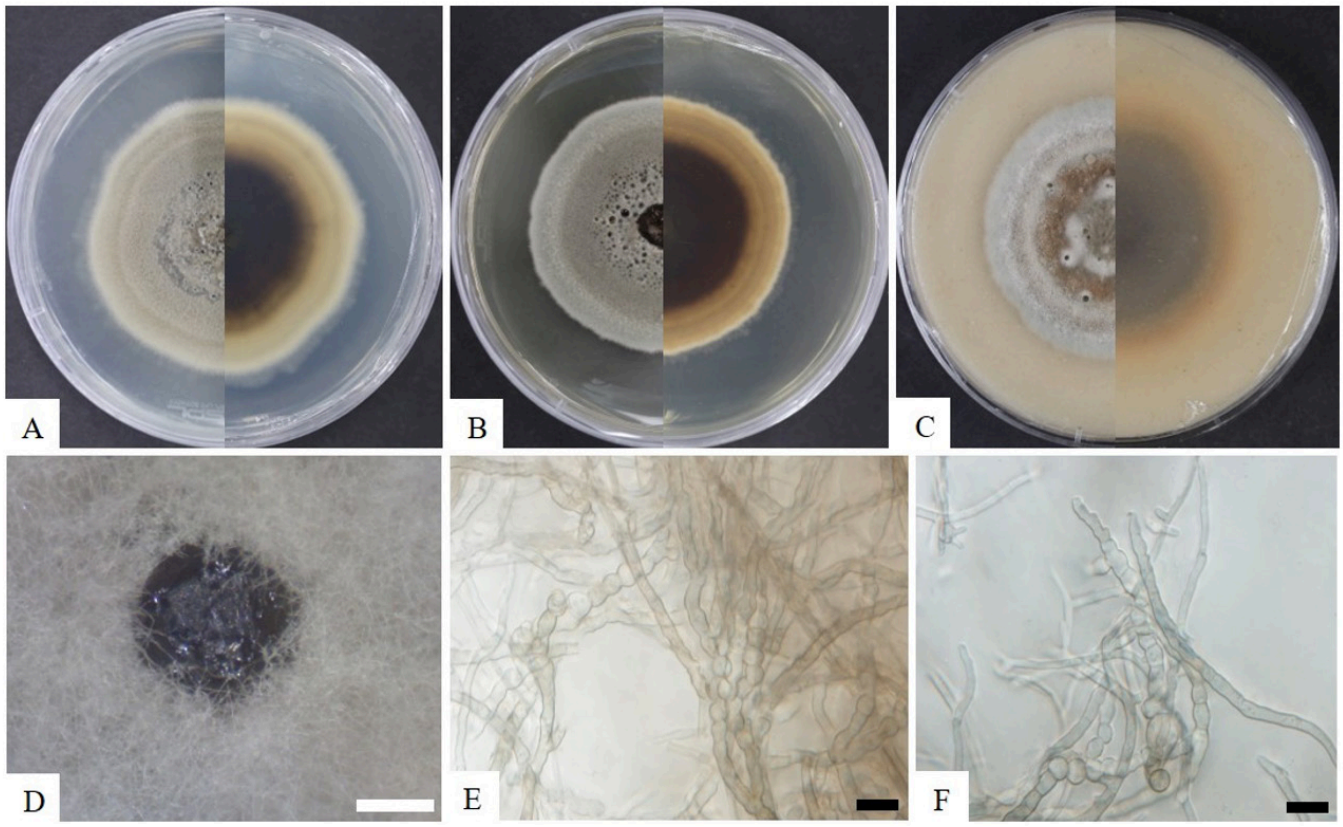

Fig. 3. Cultural and morphological characteristics of *Plenodomus collinsoniae* KNU-AP100C. A, colonies on potato dextrose agar; B, colonies on malt extract agar; C, colonies on oatmeal agar; D, conidiomata on potato dextrose agar (PDA); E, F, chlamydospore-like cells formed in culture. Scale bars: D=200 μm; E, F=10 μm.

incubation at 25°C. These media did not stimulate conidial production, and thus some special methods might be required to induce the production of conidia. Environmental factors such as temperature, humidity, and light should be taken into consideration to assess the production of conidia in KNU-AP100C.

Phylogenetic analysis of KNU-AP100C

Sequence analysis of KNU-AP100C resulted in 589 bp from the ITS regions and 1168 bp from the LSU. The isolate KNU-AP100C demonstrated 99% similarities with *P. collinsoniae* CBS 120227 in the ITS and LSU. The combined sequences of ITS and LSU were used to construct the maximum likelihood phylogenetic tree, and the isolate KNU-AP100C was matched with *P. collinsoniae* exhibiting 99% bootstrap values (Fig. 4). Thus, the molecular phylogenetic results supported the identification of the isolate KNU-AP100C as *P. collinsoniae*.

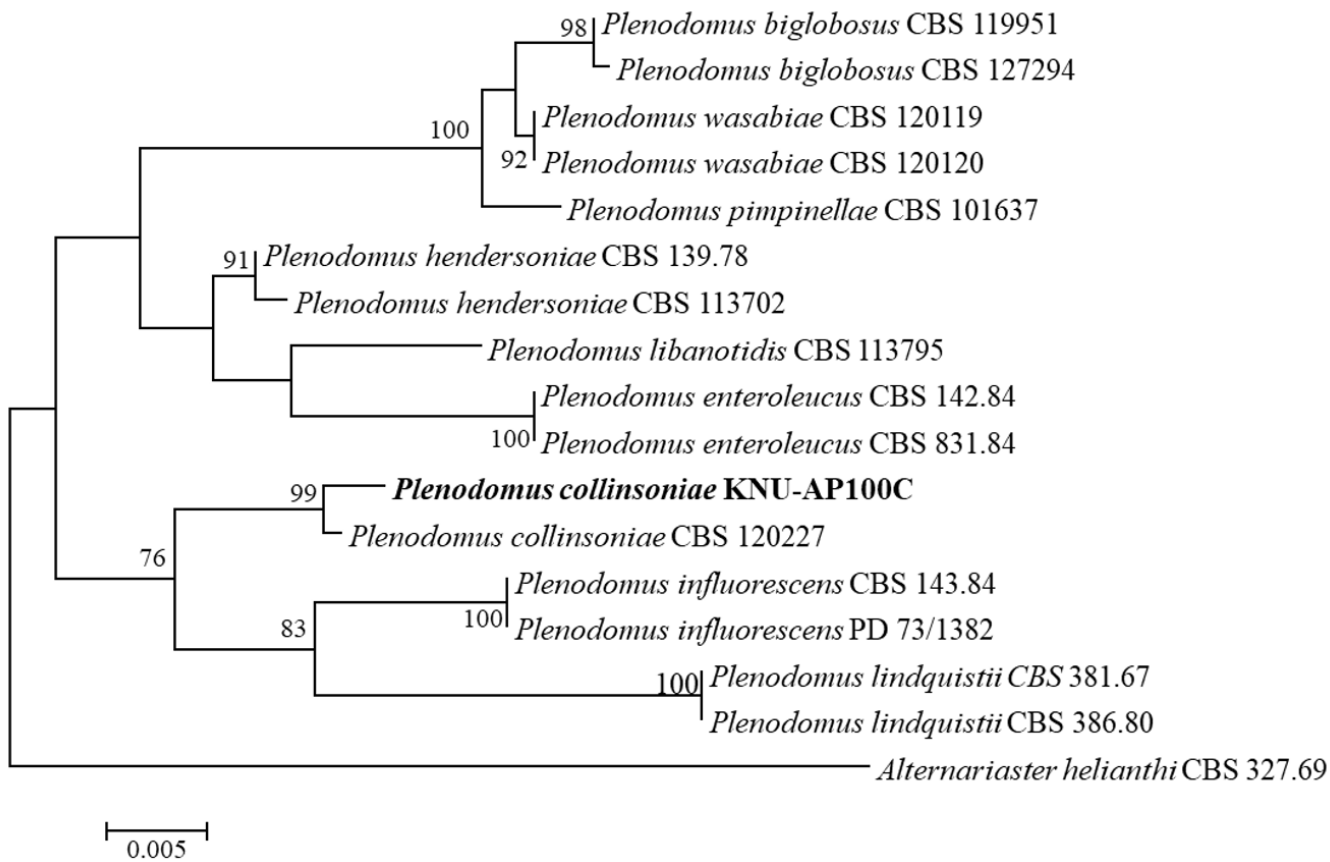

Fig. 4. Maximum likelihood phylogenetic tree based on the combined dataset of large subunit (LSU) and internal transcribed spacer (ITS) regions. *Alternariaster helianthi* CBS 327.69 was used as an outgroup. The fungal strain which examined in this study was indicated in bold, and the bootstrap value below 70% is not shown. Bar, 0.005 substitutions per nucleotide position.

DISCUSSION

In the present study, the two fungal isolates, KNU-GW1901 and KNU-AP100C, were collected and isolated from soil samples in Korea. These isolates were found to be morphologically similar to *P. sinensis* and *P. collinsoniae*, respectively, which have been described in previous studies [8,13]. The isolate KNU-AP100C, identified as *P. collinosinae*, was one of the combined novel species, and its previous name was *Leptosphaeria collinsoniae* [8]. *L. collinsoniae* was first reported by Dearn & House from USA who described its morphological characteristics as a sexual stage on the blackened stem of the stone root *Collinsonia canadensis* L. and asexual data were not determined [19]. It has been reported that *P. chrysanthemi* infected chrysanthemum plants in Greece, *P. biglobosus* caused upper stem lesions on *Brassica* species in France and Netherlands, and *P. collinsoniae* infected *Vitis coignetiae* plants in Japan [8]. In addition, *P. destruens* was isolated from the storage tuber rot on sweet potato in Korea [20]. Moreover, *Plenodomus* species caused cankers and leaf spots associated with a wide variety of substrates and were reported to be widely distributed from temperate to tropical countries throughout the world [21,22]. Although the specific host of the genus *Plenodomus* has not yet been confirmed, the species has been recorded from various plant families such as Asteraceae, Fabaceae, Lamiaceae, and Liliaceae [8]. To our knowledge, the isolates *P. sinensis* KNU-GW1901 and *P. collinsoniae* KNU-AP100C are the first reports of *Plenodomus* species in Korea.

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