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Humicola phialophoroides sp. nov. from soil with potential for biological control of plant diseases

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ABSTRACT. A fungus isolated from soil amended with organic matter and with evidence for biological control of plant diseases, produced both *Phialophora*- and *Humicola*-like synanamorphs. Based on ITS1-5.8S-ITS2 sequence analyses, this previously undescribed fungus had a closer relationship with the genus *Humicola* than with *Phialophora* and was thus named *Humicola phialophoroides*.

Keywords: Biological control; *Humicola phialophoroides*; Soil fungus; Synanamorphs; Taxonomy.

INTRODUCTION

Recently, microorganisms capable of utilizing organic vegetable matter for multiplication in soil were selectively isolated and cultured in a broth prepared from this same matter. The liquid cultures that resulted were each tested for their ability to control plant diseases (Ko et al., 2010a). Liquid cultures of six morphologically similar isolates of a fungus obtained from a farm soil collected from southern Taiwan reduced disease incidence of Phytophthora blight of pepper, caused by *Phytophthora capsici,* and black leaf spot of spoon cabbage (*Brassica campestris* subsp. *chinensis*), caused by *Alternaria brassicicola,* from 100% in the control to less than 10%. During the early stage of growth, the fungus formed branched *Phialophora*-like conidiophores bearing phialidic cells, later producing *Humicola*-type aleuriospores. The fungal characteristics differed from those of recognized species of *Phialophora* (Schol-Schwarz, 1970; Cole and Kendrick, 1973; Gams and Holubova-Jechova, 1976; de Hoog et al., 1999) or *Humicola* (Fassatiova, 1964; Fergus, 1964; Ellis, 1971; Nicoli and Russo, 1974; de Bertoldi, 1976). Molecular phylogenetic study showed that the fungus was phylogenetically closer to the genus *Humicola* than to *Phialophora*. The organism was confirmed as a new species of *Humicola* by Dr. G. J. M. Verkley of CBS Fungal Diversity Center, the Netherlands.

MATERIALS AND METHODS

Isolation and culture of organisms from soil

Soil collected from Tienliau, Kaohsiung, in southern Taiwan, was sifted and moistened to about 65% water-holding capacity. For soil amendment, tomato fruit (*Solanum lycopersicum*), sweet potato tubers (*Ipomoea batatas*) and greens of spinach (*Spinacia oleracea*), ong choy (*Ipomoea aquatica*) and common purslane (*Potulaca oleracea*) were purchased at a local market and chopped into small pieces. About 500 g soil was mixed with 20 g of each chopped vegetable in a 1000-ml bottle, and incubated at 24°C for two weeks. To isolate fungi from soil, 1.3 g soil was mixed with 100 ml sterile distilled water in an Omni mixer chamber at 5,000 rpm for 30 s, and the suspension was diluted to 10^{-4}, 10^{-5} and 10^{-6}. One ml of the diluted soil suspension in a 9-cm Petri plate was mixed with 20 ml of 45°C molten selective medium consisting of 10% soil extract, 0.02% urea, 0.1% Tergitol NP-7 (Sigma-Aldrich, St. Louis, MO) and 1.5% agar (Ko et al., 2010b). Tergitol NP-7 was added to retard spreading of fast-growing fungi. After autoclaving, the medium was supplemented with 50 ppm chloramphenicol and 50 ppm streptomycin sulfate to inhibit bacteria and actinomycetes. Soil extract was prepared by autoclaving 100 g sieved soil, 0.2 g $CaCO_3$ and 1000 ml water for 20 min, filtering the suspension through three layers of cheesecloth and centrifuging the filtrate at 1500 *g* for 5 min to clarify the extract. Five plates were used for each dilution.

Single spore isolate, KVF-2, was obtained by spreading conidial suspension on 2% water agar and transferring the colony originating from a single conidium to a potato dextrose agar (PDA) plate as previously described (Ko, 1981). For the study of colony morphology and the effect of temperature on growth, an 8-mm culture disc obtained from the 6-day-old colony margin was placed on the center of each PDA plate. The temperatures tested were 4, 8, 12, 16, 20, 24, 28, 32, 36, 40 and 48°C. Three plates were used for each temperature, and the experiment was repeated once.

For spore production, the fungus was grown on PDA at 24°C in darkness or under cool white fluorescent light (2,000 lx). To compare the amount of spores produced, the PDA plate culture was triturated with 100 ml water in

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a blender at high speed for 1 min and the spore concentration was determined using a Pipeman microliter pipette (West Coast Scientific Inc., Oakland, California) (Ann et al., 2010). Three plates were used for each treatment and the experiments were repeated at least once.

DNA extraction, sequencing and phylogenetic analysis

DNA of KVF-2 was extracted from 0.1 g of 3-day-old mycelium, grown on cellophane and placed on V-8 agar (Wang et al., 2010) using the plant DNA extraction kit (GenMark Technology Co., Taichung, Taiwan). Nucleic acid sequences of the ITS1-5.8S-ITS2 were amplified with primer pairs of ITS1 and ITS4 (White et al., 1990). PCR was performed in a 25 µl reaction solution containing 50 ng DNA, 0.5 pmol each of ITS1 and ITS4 primers and 1.5 units of SuperTaq polymerase (Protech Technology Entexprise Co., Ltd., Taiwan) using the buffer system recommended by the manufacturer. Cycling conditions of PCR: initial denaturation at 94°C for 2 min, 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and the final elongation at 72°C for 6 min.

The amplified PCR product was analyzed by electrophoresis in 1.2% agarose gel and cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, California) according to manufacturer's instructions. Plasmid clones with expected size DNA inserts were screened and used for sequencing. Sequencing of the target DNA insert was done using an automatic DNA sequencer (ABI PRISM 377, Perkin-Elmer, California) with the BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystem, California).

To examine phylogenetic relationships, the new sequence and those of species in the genera *Humicola* and *Phialophora*, available in GenBank (Table 1), were aligned using the ClustalX Version 1.81 (Thompson, 1997). The multiple alignment was adjusted after visual examination with Sequence Alignment Editor (Se-A1) version 1.0 alpha 1 (Rambaut, 1996). Phylogenetic tree building based on neighbor-joining (NJ) (Saitou and Nei, 1987) and distance matrix for the aligned sequences were performed with Kimura's two-parameter method (Kimura, 1980) of the program PAUP* 4.0 (Swofford, 1998). Bootstrap values were generated with 1000 replicate heuristic searches to estimate support for clade stability of the consensus tree (Felsenstein, 1985).

RESULTS AND DISCUSSION

Humicola phialophoroides sp. nov. formed *Phialophora*-like conidiospores (Figure 1a, b, c) and *Humicola*-like aleuriospores (Figure 1d, e). The morphological characteristics of this fungus were different from those of recognized species of *Phialophora* (Schol-Schwarz, 1970; Cole and Kendrick, 1975; Gams and Holubova-Jechova, 1976; de Hoog et al., 1999) or *Humicola* (Fasatiova, 1964; Fergus, 1964; Ellis, 1971; Nicoli and Russo, 1974; de Bertoldi, 1976) in both shape and size of conidiophores and aleuiospores. *H. phialophoroides* shared only 52.2 to 76.0 % ITS sequence identity with the *Phialophora* species available in GenBank, but 86.1 to 87.0% with the *Humicola* species (Table 1). The fungus was therefore placed in *Humicola*. *H. phialophoroides* was distinct from other species of *Humicola* in its production of bottle-like phialides

Table 1. Sequence identity of ITS regions between *Humicola phialophoroides* and species in the genera of *Humicola* and *Phialophora* retrieved from GenBank for phylogenetic analysis.

Species (isolate)	Location	GenBank accession no.	Identity
Humicola fuscoatra (MTCC 6329)	India	EF550969	87.0%
Humicola grisea (DAOM 232586)	Canada	AY706334	86.1%
Humicola grisea (IMI 126329)	Ireland	AJ131856	86.9%
Humicola insolens (IMI 126330)	Ireland	AJ131857	87.0%
Humicola insolens (MTCC 4617)	India	EF550968	87.0%
Phialophora botulispora (DAOM 75261)	USA	AF083198	67.5%
Phialophora brunnescens (A178)	USA	AY249079	72.3%
Phialophora calyciformis (A177)	USA	AY249077	75.4%
Phialophora europaea (CBS 129.96)	Netherlands	EF551553	52.2%
Phialophora lagerbergii (CBS 266.33)	USA	AF083197	67.8%
Phialophora lignicola (WRCF-AW4)	Canada	AY618677	75.5%
Phialophora mustea (DUKE 2314)	USA	AF083194	76.0%
Phialophora sessilis (CBS 243.85)	Netherlands	AY857542	53.1%
Phialophora verrucosa (IFM 5089)	Japan	AB369920	55.8%

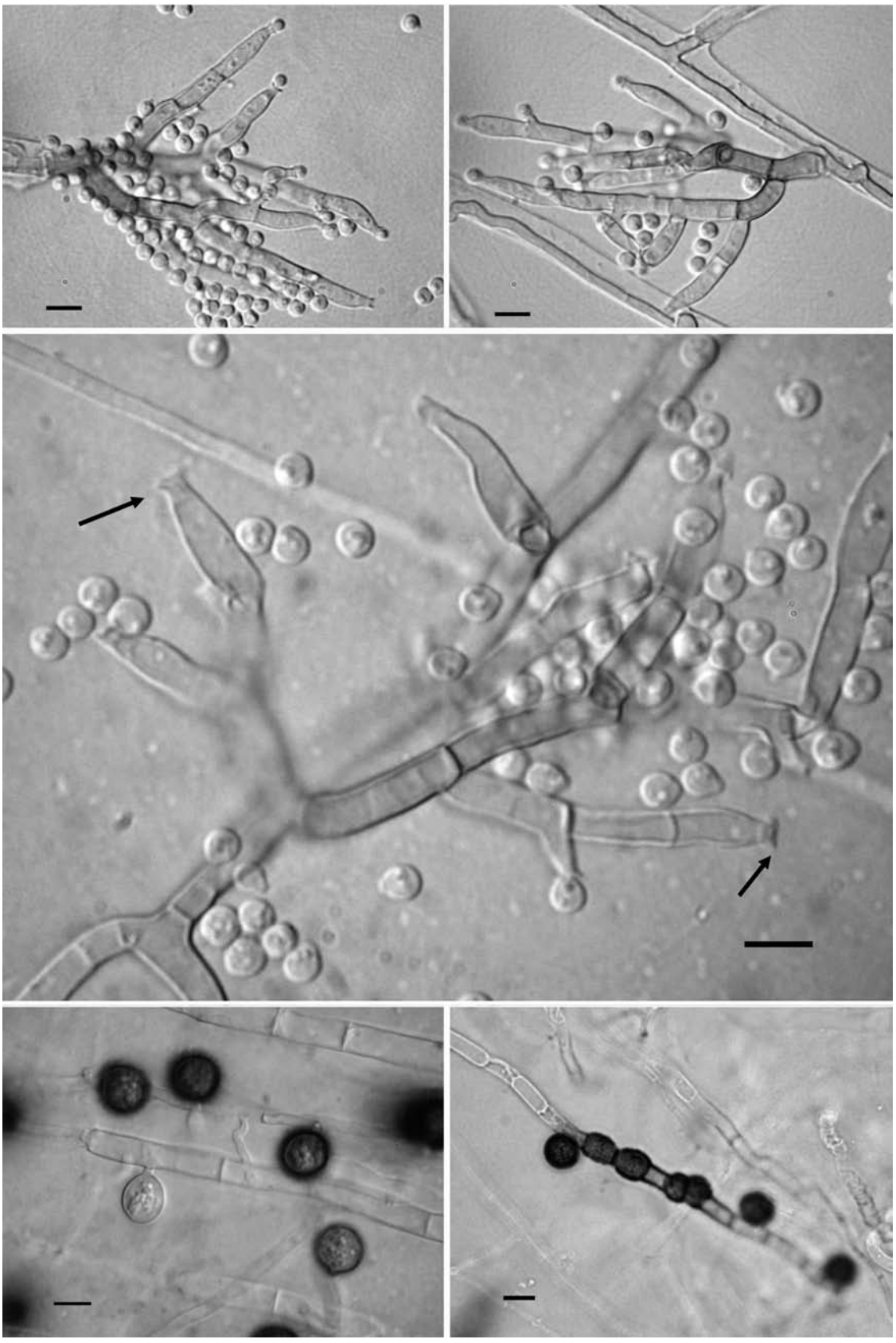

Figure 1. *Humicola phialophoroides* sp. nov. HAST 123948. (a-b) Branched conidiophores; (c) phialides with saucer-shaped top (arrows); (d) lateral or terminal aleuriospores; (e) intercalary chlamydospores. Bars, 5 μm (a-c), 10 μm (d-e).

with saucer-shaped tops, that formed on branched conidiophores (Figure 1). Phylogenetic relationships inferred from the ITS1-5.8S-ITS2 region sequences of *Humicola* species and *Phialophora* species are shown in Figure 2. The phylogenetic tree showed that all *Humicola* species including *H. phialophoroides* formed a single clade, while *Phialophora* species were divided into three clades.

TAXONOMY

Humicola phialophoroides Ko, Yang, Lin, Chen et Tsou sp. nov. Figure 1

Coloniae in PDA albidae, *hyphis aeriis abundis*, *flavidae primo*, *brunneolescens demum*. *Hyphae hyalinae*, *septatae*, *rimosae*, *4.0-5.0 µm latae*. *Conidiophora dichotome rimosa*, *brunneola basi*, *31-72 µm longa*. *Phialides lageniformes*, *9.5-15.3 µm longae*, *2.2-2.7 µm latae basi*, *attenuatae sursum*, *apicem patelliformem 0.8-1.0 µm latum facientes*. *Conidia hyalina, sphericus*, *1.8-2.6 µm diam*, *ad apicem phialidis accumulata catervatim*. *Aleurioconidia atrata*, *sphericus*, *unicellularia*, *8.8-12.5 µm diam*. *Chlamydosporae intercalares, 5.6 × 9.0-5.6 × 12.1 µm, atratae, oblongae vel obovatae*.

Etymology. Referring to its *Phialophora*-like morphology due to the presence of *Phialophora* synanamorph.

Holotype. HAST 123948 (dried culture), Herbarium, Biodiversity Research Center, Academia Sinica, Taipei, Taiwan.

A living culture from the holotype has been deposited at CBS Fungal Diversity Center, Utrecht, Netherlands (CBS 125784) and at the Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan (BCRC 34556).

The fungus is relatively slow-growing. It grew 7 mm/day at the optimum temperature of 32°C in the dark, on PDA. The minimum and maximum temperatures were 12 and 40°C, respectively. Colonies grown on PDA were white with abundant aerial hyphae. Reverse colony color was light yellow initially and later became light brown with irradiation. By the seventh day, conidia were produced on PDA at 24°C with irradiation. Conidiophores frequently branched dichotomously, and were pigmented on the basal portion, 31-72 µm long from the basal cell to top of the phialide. Phialides were terminal or lateral, 9.5-15.5 µm long, 2.2-2.7 µm broad at the base with an apical col-

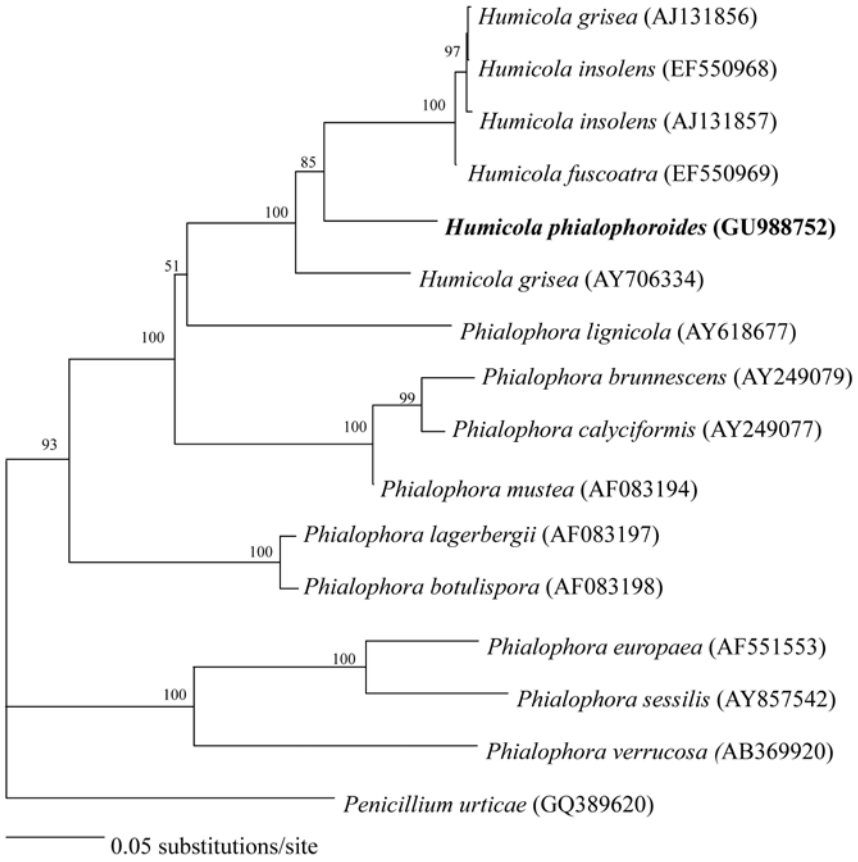

Figure 2. Phylogenetic relationship among species in the genera of *Humicola* and *Phialophora* available in GenBank based on rDNA sequence of the ITS1-5.8S-ITS2 region using neighbor-joining method (Saitou and Nei, 1987). The distance matrix for the aligned sequences was performed with Kimura's two-parameter method (Kimura, 1980) of the program PAUP* 4.0 (Swofferd, 1998). Bootstrap values were generated with 1000 replicate heuristic searches to estimate support for clade stability of the consensus tree (Felsenstein, 1985).

larette and 0.8-1.0 µm broad. Conidia were hyaline, spherical, 1.8-2.6 µm in diameter and accumulated in a ball-like mass at the apex of the phialide. After 30 days, the fungus produced a 4.0×10^8 conidia/plate under light but only a 3.2×10^6 conidia/plate in darkness. After incubation on PDA at 24°C for 15 days in darkness, *H. phialophoroides* produced terminal, lateral and intercalary dark chlamydospores. Aleuriospores were spherical, single-celled, 8.8-12.5 µm in diameter, while intercalary chlamydospores were oblong to obovate, 5.6 × 9.0-5.6 × 12.1 µm. After 30 days, the fungus produced 3.2×10^5 chlamydospores/plate in darkness, but none under light.

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由土壤中分離之新種真菌 *Humicola phialophoroides* 具植物病害生物防治的潛力

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由添加有機質土壤中分離到一株真菌，具有植物病害生物防治的潛力，且產生的孢子和 *Humicola* 屬及 *Phialophora* 屬的真菌相似。此尚未被發表敘述的真菌之 ITS1-5.8S-ITS2 序列，在演化親緣關係上，同 *Humicola* 屬的親緣比同 *Phialophora* 屬接近。所以將此微生物命名為 *Humicola phialophoroides*。

關鍵詞：生物防治；*Humicola phialophoroides*；土壤真菌；synanamorphs；分類學。