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FULL PAPER

The anamorphic genus *Calcarisporiella* is a new member of the Mucoromycotina

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Abstract The phylogenetic position of the anamorphic genus *Calcarisporiella* was investigated. Three isolates of *Calcarisporiella*, including an authentic strain and a newly obtained isolate, were analyzed phylogenetically using rDNA sequences. The result indicated that *Calcarisporiella*, which was classified as an ascomycetous anamorph, is a member of Mucoromycotina. It formed an independent clade separated from the other known orders of this subphylum.

Keywords *Calcarisporiella* · Mucoromycotina · Phylogeny · rDNA

Introduction

Calcarisporiella is a monotypic anamorphic genus containing *C. thermophila* (H.C. Evans) de Hoog. It was initially described as a species of *Calcarisporium* (*C. thermophile* H.C. Evans) by Evans (1971a,b) based on an isolate obtained from coal spoil tip soil in Staffordshire, England. Evans (1971a) classified it in the group of thermotolerants based on the cardinal temperature range for growth of the species. de Hoog (1974) erected the genus *Calcarisporiella* to accommodate *Calcarisporium thermophila* because it differs from other *Calcarisporium* species in several characteristics; these include the production of wide, undulating, and fragile hyphae, absence of differentiated conidiophores, shape of the conidiogenous cells, wide conidium-bearing denticles, and the shape and size of conidia.

During a survey of soil microfungi, an isolate of *Calcarisporiella* was obtained from a soil sample aseptically collected at a depth of 100 cm in a *Miscanthus sinensis* grassland in the Sugadaira Montane Research Center, University of Tsukuba, located in the central part of Japan. It is similar to *C. thermophila* in both colony and morphological characters, except that it was mesophilic.

The genus *Calcarisporiella* was previously thought to be an ascomycetous anamorphic genus of Pezizomycotina judging from morphology of the anamorph, the process of spore formation, and the presence of septate hyphae (Kirk et al. 2008). Because there has been no published molecular analysis of this fungus, we investigated its phylogenetic position using nuclear small subunit (18S), nuclear large subunit (LSU), and internal transcribed spacer (ITS) rDNA sequence data, including isolates of *C. thermophila* and our new mesophilic isolate.

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Materials and methods

Fungal strains

Strain data are shown in Table 1. In addition to our new mesophilic isolate, an authentic strain and three Japanese isolates of *C. thermophila* were included in the analyses. These strains represent all the known cultures available from culture collections.

Table 1 Strains used in this study

Taxon	Strain[a]	Date isolated	Strain data	DDBJ accession no.	
				18S rDNA	ITS + 28S rDNA
Calcarisporiella thermophila	CBS 279.70 (=ATCC 22718; IMI 144750; MUCL 19326)	March 1968	Type of *Calcarisporiella thermophila* Coal spoil tip soil, Staffordshire, Keele, England, UK Isolated by H.C. Evans	AB597205	AB617741
	NBRC 33169 (=IFO 33169)		Soil, Tatsugo-cho, Amamioshima Is., Kagoshima Pref., Japan	–	–
	NBRC 33269 (=IFO 33269)		Pineapple field soil, Iriomote Is., Okinawa Pref., Japan	–	–
	NBRC 33279 (=IFO 33279)		Soil, Haemita, Iriomote Is., Okinawa Pref., Japan	AB597204	AB617739
Calcarisporiella sp.	NBRC 105922 (=NUH 037)	May 2008	Subsoil, Sugadaira, Ueda-shi, Nagano Pref., Japan	AB597203	AB617740

[a] ATCC, American Type Culture Collection, Manasas, VA; CBS, Centraalbureau voor Schimmelcultures, Baarn and Delft, Netherlands; IMI, International Mycological Institute, Egham, UK; IFO, Institute for Fermentation, Osaka, Japan; MUCL, Mycotheque del'Universite Catholique de Louvain, Louvain-la-Neuve, Belgium; NBRC, NITE Biological Resource Center, Chiba, Japan; NUH, Dai Hirose's private fungal collection at Nihon University, Chiba, Japan

Culture studies

To examine temperature ranges for growth, four Japanese isolates were incubated on potato dextrose agar (PDA) at 10°, 15°, 20°, 25°, 30°, 35°, and 40°C for 10 days in the dark. The isolates were also incubated on cornmeal agar (CMA; Nissui Pharmaceutical, Tokyo, Japan) and Miura agar plates at 25° and 30°C to observe colony appearance and morphological characteristics of the hyphae and reproductive structures. Additional cultures were incubated on a laboratory bench for longer periods to investigate possible sexual reproduction.

Karyological observation (Hoechst staining)

Nuclei of conidia were stained with 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole, trihydrochloride (Hoechst 33258) solution (Dojindo Laboratories, Kumamoto, Japan), and the number of nuclei per cell was observed using a fluorescence microscope (IX70; Olympus, Tokyo, Japan) under UV light.

DNA extraction and PCR amplification

DNA of three *Calcarisporiella* strains (CBS 279.70, NBRC 33279, and NBRC 105922) was extracted from mycelia that had been cultured in 2.5% malt extract liquid medium following the modified CTAB method described by Matsuda and Hijii (1999). The 18S rDNA was amplified with polymerase chain reaction (PCR) primers NS1 (White et al. 1990) and SR6 (TGTTACGACTTTTACTT; Vilgalys, unpublished data). To PCR amplify the region including the rDNA ITS and 28S rDNA D1–D2 domain, the primer pair ITS1f (Gardes and Bruns 1993) and LR3 (Vilgalys and Hester 1990) was used. Polymerase chain reactions were performed using a HotStarTaq Plus Master Mix (Qiagen, Tokyo, Japan). Each PCR reaction contained a 50 µl mixture [16 µl distilled water, 25 µl master mix, 3 µl template DNA, 5 µl Coral Load PCR buffer, and 0.5 µl each primer (final, 0.25 µM)]. Each DNA fragment was amplified using a PCR thermal cycler (DNA engine; Bio-Rad Laboratories, Tokyo, Japan) using the following thermal cycling schedule: the first cycle consisted of 5 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 58°C for annealing, 1 min at 72°C, and a final cycle of 10 min at 72°C. The reaction mixture was then cooled at 4°C for 5 min. PCR products were purified with a QiAquick PCR Purification Kit (Qiagen).

DNA sequencing

Purified PCR products were sequenced by Macrogen Japan (Tokyo, Japan). Sequencing reactions were performed in a PTC-225 Peltier Thermal Cycler (MJ Research) using a ABI PRISMR BigDyeTM Terminator Cycle Sequencing Kit with AmpliTaqR DNA polymerase (FS enzyme) (Applied Biosystems, Tokyo, Japan), following the protocols supplied by the manufacturer. The fluorescent-labeled fragments were purified from the unincorporated terminators using an ethanol precipitation protocol. The samples were resuspended in formamide and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Sequences determined in this study were deposited in the DDBJ (Table 1). Sequences of the three strains were

compared with known species using a BLAST search, and the most closely related fungal sequences were determined. In addition to this result, 13 sequences used in White et al. (2006) were also included in the phylogenetic analyses.

Phylogenetic analysis

Phylogenetic analyses of the 18S rDNA sequences were conducted using neighbor-joining (NJ) and maximum-likelihood (ML) methods. MAFFT ver. 6 (Katoh and Toh 2008) was used for preliminary multiple alignments of nucleotide sequences. Final alignments were manually adjusted using BioEdit (Hall 1999). Alignment gaps were treated as missing data, and ambiguous positions were excluded from the analysis. For the NJ and ML analyses, the TIM + I + G model was selected as the best-fit evolutionary model using Modeltest ver. 3.7 (Posada and Crandall 1998) under the Akaike information criterion (AIC). The model was used to construct phylogenetic trees with the NJ and ML methods using PAUP ver. 4.0b (Swofford 2000). Node support was evaluated by bootstrap analysis (Felsenstein 1985) using 1,000 replications in the NJ analyses and 100 replications in the ML analysis.

Results

Temperature range for growth

Results of the growth test using different incubation temperatures are shown in Table 2. Three isolates of *C. thermophila* did not grow at 10° and 15°C and only grew slightly at 20°C. They grew faster as incubation temperature increased and showed maximum growth at 40°C. By contrast, NBRC 105922 grew at 15°–35°C with the optimum at 20°–25°C, but growth was extremely restricted at 35°C and absent at 40°C.

Morphological characteristics

Good sporulation was observed on plates incubated at 25° or 30°C for all five isolates. Cultural characteristics and morphological characters were almost identical for the authentic strain of *C. thermophila* and the three Japanese isolates of this species, and they agreed well with the original description by Evans (1971a) and the one by de Hoog (1974). In addition, the mesophilic isolate NBRC 105922 was morphologically similar to the other isolates of *Calcarisporiella*. Spores of the mesophilic isolate (6–8 × 4.5–5.5 μm) were also similar to those of the thermotolerant isolates [6–9 × 4–6 μm (Evans 1971a); 7–9(–11) × 4–6 μm (de Hoog 1974)]. Colonies of the mesophilic and *C. thermophila* isolates lacked a diagnostic garlic-like odor typical of many mortierellaceous fungi.

Spores examined by Hoechst staining of NBRC 105922 and CBS 279.70 were uninucleate (Fig. 1).

Phylogenetic analysis

Comparison of rDNA ITS and 28S rDNA D1–D2 domain sequences of three *Calcarisporiella* strains showed that sequence identity between the mesophilic isolate NBRC 105922 and two *C. thermophila* strains (ITS 83%, 28S 92%) were lower than that between two *C. thermophila* strains (ITS 93%, 28S 97%). The result of a BLAST search using the 28S rDNA D1–D2 domain and 18S rDNA sequences of three *Calcarisporiella* strains as the query showed that the most closely related fungi were members of the Mortierellaceae (accession numbers EU688963 and EU688964) with >90% similarity. Based on this result, we conducted phylogenetic analyses to clarify the phylogenetic position of *Calcarisporiella* within the Mucoromycotina.

Trees obtained by NJ and ML analyses shared a similar topology. Mucoromycotina was paraphyletic as shown in White et al. (2006). In NJ and ML analyses of the 18S rDNA sequence data, the three strains of *Calcarisporiella* formed a well-supported monophyletic clade (100/100%

Table 2 Colony diameters (mm) of four *Calcarisporiella* strains incubated on potato dextrose agar (PDA) at different temperatures for 10 days

Strain	Temperature (°C)						
	10	15	20	25	30	35	40
Calcarisporiella thermophila							
NBRC 33169	0	0	<1	6–8	10–12	16–18	18–20
NBRC 33279	0	0	<1	5–6	10–14	20	22–24
NBRC 33269	0	0	<1	6–8	10–12	16–18	18–20
Calcarisporiella sp.							
NBRC 105922	0	3–4	10–12	10–12	6–8	1–2	0

ML/NJ bootstrap value; Fig. 2). The *Calcarisporiella* clade was resolved as a sister group of a clade that included the Endogonales, Mucorales, Glomales, and Dikaryomycota (82/70%).

Discussion

Phylogenetic analysis of the 18S rDNA sequence data revealed that *Calcarisporiella* species are members of the Mucoromycotina. *Calcarisporiella* formed an independent clade clearly separated from the other known orders of Mucoromycotina. *Calcarisporiella* isolates did not produce multispored sporangia or homothallic zygosporangia, regardless of the cultural condition, using various media in addition to PDA. In addition, no sexual reaction was observed in the mating experiments, although all available isolates were crossed in possible pairs and inoculated 10 mm apart on media used to induce zygospores in the Mortierellales [CMA (Gams and Williams 1963), modified PABA (diluted Pablum agar, using Gerber cereal mix instead of Pablum; Kuhlman 1972), hemp seed agar (HSA; Kuhlman 1972; Chien et al. 1974), and shrimp agar (ShA; Degawa and Tokumasu 1998) at 10° or 20°C for 2 weeks. In members of the Mucoromycotina, several taxa of the Mortierellales, e.g., sect. Stylospora, sect. Schmuckeri, etc., and Mucorales, e.g., *Umbelopsis nana*, are known to produce only unispored sporangioles. In the strict sense, except for aerially produced chlamydospores (so-called stylospores), all asexual spores of these fungi are regarded as endogenously produced sporangia or sporangioles. The sporophores of *Calcarisporiella* are also morphologically similar to those of the Mortierellales in that they are strongly or abruptly tapered toward the tips. To assess whether *Calcarisporiella* should be included in the Mortierellales, more comprehensive molecular phylogenetic and ultrastructural studies are required.

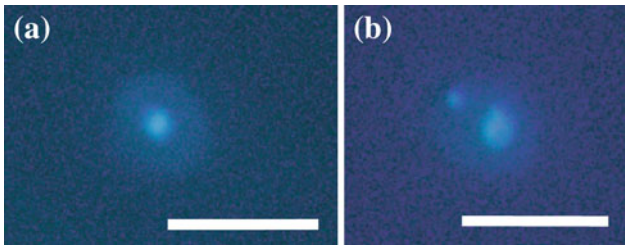

Fig. 1 Hoechst-stained nuclei in conidia of *Calcarisporiella thermophila* CBS 279.70 (**a**) and *Calcarisporiella* sp. NBRC 105922 (**b**). *Bars* 10 μm

The culture studies indicated that NBRC 105922 is mesophilic, whereas other Japanese isolates of *C. thermophila* are thermotolerant, as is the authentic strain of *C. thermophila* (CBS 279.70). Japanese isolates of *C. thermophila* were obtained from surface soil samples collected in the subtropical islands of Japan, Iriomote Island, and Amamioshima Island (Table 1). The type strain CBS 279.70 was isolated from soil of a coal spoil tip located at a latitude of approximately 53° N, but the soil temperatures are higher than those of the surrounding area because of spontaneous combustion of the coal spoil

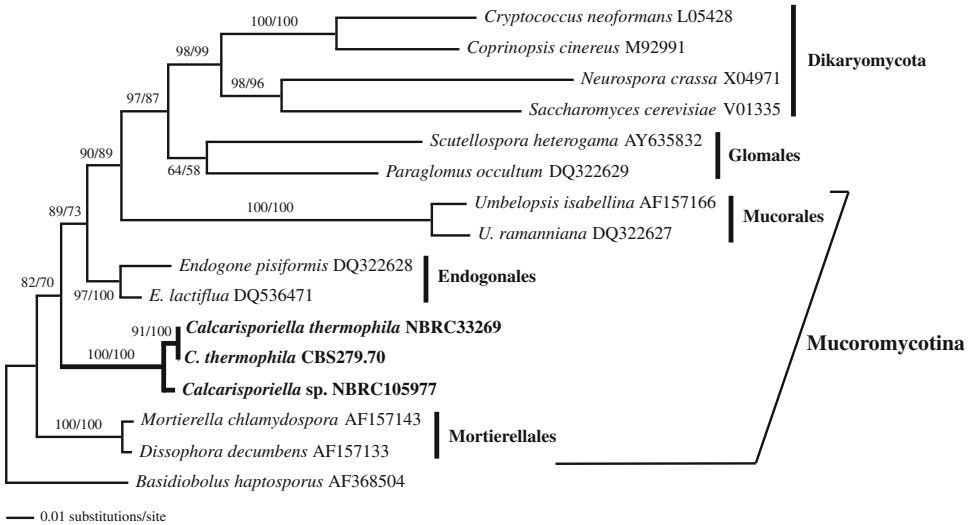

Fig. 2 Maximum-likelihood (ML) phylogeny inferred from 18S rDNA sequences including three strains of *Calcarisporiella*, 12 taxa including Mucoromycotina, Glomales, and Dikaryomycota, and an outgroup (*Basidiobolus haptosporus*). The evolutionary model used was TIM + I + G with unequal base frequencies (freqA = 0.2650, freqC = 0.1953, freqG = 0.2648, freqT = 0.2749), a substitution rate matrix ([A–C] = 1.0000, [A–G] = 2.7628, [A–T] = 1.2504, [C–G] = 1.2504, [C–T] = 6.1086, [G–T] = 1.0000), a proportion of invariable sites = 0.4497, and a gamma shape parameter = 0.5548. Bootstrap values for the ML/neighbor-joining (NJ) analyses are indicated for corresponding branches

(Evans 1971a). The available data suggested that *C. thermophila* has a worldwide distribution, especially in warm and hot climatic regions. The species may be isolated commonly when the isolation plates are incubated at 35°C or a higher incubation temperature. The mesophilic NBRC 105922 strain was isolated from a soil sample aseptically collected from a depth of 100 cm under grassland. One of the authors (Tokumasu, unpublished data) isolated the same fungus three times at a depth of 100 cm in the same grassland, but it is unknown whether the fungus is restricted to deep soil. It might be profitable to determine whether this mesophilic fungus is also distributed in surface soils. However, this may be difficult to determine by common plate techniques because the fungus is slow growing and may become overgrown with other faster-growing fungi. More data are required to clarify the ecology and habitat of this fungus.

Our research highlights the possibility that additional members of the Mucoromycotina may be misplaced within the Ascomycota. Additional molecular phylogenetic analyses are needed to clarify the evolutionary relationships of problematic anamorphic genera so as to catalogue the phylogenetic diversity of the Mucoromycotina.

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