

Cylindrocladiella hahajimaensis, a new species of *Cylindrocladiella* transferred from *Verticillium*

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Abstract

We used phylogenetic analyses based on ribosomal internal transcribed spacer (ITS) and beta-tubulin (*TUB*) sequences and determined that the correct name of '*Verticillium hahajimaense*' was *Cylindrocladiella hahajimaensis*. A closest relative could not be determined, since *C. hahajimaensis* clustered within the *C. infestans* species complex, a poorly resolved group of taxa. *Cylindrocladiella hahajimaensis* differed from all other members of the *C. infestans* species complex by at least 18 substitutions at the two loci. Morphological characters supported the placement within *Cylindrocladiella*. In addition to the verticillate conidiophores mentioned in the type description, we found evidence for the presence of penicillate conidiophores. Other differences to the type description included the presence of yellow to brown-pigmented hyphae, and the hyaline instead of pigmented chlamydo-spores.

Keywords

Ascomycetes, Nectriaceae, taxonomy, phylogenetics

Introduction

The genus *Cylindrocladiella* comprises a small, monophyletic group of fungi in the Nectriaceae related to *Cylindrocladium* (Schoch et al. 2000). *Cylindrocladium* and *Cylindrocladiella* are morphologically similar, they form two different synanamorphs that resemble *Penicillium* and *Verticillium* in terms of branching pattern and phialide arrangement, but may bear long hyphal projections with swollen tips that are referred to

as filaments with terminal vesicles. Chlamydospores may also be present (Boesewinkel 1982). Differences between *Cylindrocladiella* and *Cylindrocladium* include aspects of conidiophore branching, filament morphology, appearance in culture, and conidia morphology and size (Boesewinkel 1982). The teleomorph of *Cylindrocladiella* where known, belongs to *Nectricladiella* (Schoch et al. 2000). *Cylindrocladiella* comprises ten species that have been isolated from soil as well as plant tissues where they may act as pathogens (van Coller et al. 2005; Zhang and Chi 1996).

Verticillate conidiophores that consist of narrow, elongate phialides arranged in whorls along a main axis, have evolved multiple times as determined by molecular data and phylogenetic analyses. Taxonomic conclusions from these studies have resulted in the traditional genus *Verticillium* being restricted to a small, monophyletic group of plant pathogens in the Plectosphaerellaceae (Gams et al. 2005; Zare et al. 2007) with the majority of former *Verticillium* species transferred to other genera, including *Lecanicillium* (Gams and Zare 2001; Zare and Gams 2001a; Zare and Gams 2008), *Pochonia* and *Haptocillium* (Zare and Gams 2001b; Zare et al. 2001), as well as *Gibellulopsis* and *Musicillium* (Zare et al. 2007).

During recent studies of *Verticillium* evolution and taxonomy (Inderbitzin et al. 2011a; Inderbitzin et al. 2011b), we received the ex-holotype culture of ‘*V. habajimaense*’ isolated from soil in Japan using cucumber seeds as bait (Watanabe et al. 2001). Phylogenetic analyses showed that ‘*V. habajimaense*’ belonged to *Cylindrocladiella* instead as documented and discussed below.

Methods

Taxon sampling, origins of fungal strains and growth conditions

An ex-holotype culture of ‘*V. habajimaense*’ (strain MAFF 238172, PD684) was obtained free of charge from the National Institute of Agrobiological Sciences Genebank (NIAS), Japan, single spore purified and maintained in a glycerol solution at -80°C (Inderbitzin et al. 2011a). For all 42 ingroup taxa used by van Coller et al. (2005), ribosomal internal transcribed spacer sequences (ITS) and beta-tubulin sequences (*TUB*) were downloaded from GenBank.

DNA extraction, PCR amplification, and DNA sequencing

For complete details see Inderbitzin et al. (2011b). The ITS region was PCR amplified using primers ITS1-F (Gardes and Bruns 1993) and ITS4, and sequenced with ITS5 and ITS4 (White et al. 1990), part of *TUB* was PCR amplified and sequenced with VTubf2/VTubR (Inderbitzin et al. 2011b).

Phylogenetic analyses

Three different datasets were analyzed, the single locus datasets ITS and *TUB* using parsimony as implemented in PAUP v.4.0b 10 (Swofford 2002), as well as a the combined ITS plus *TUB* dataset, using parsimony and likelihood implementations in PAUP, and a Bayesian approach using MRBAYES v3.0b4 (Ronquist and Huelsenbeck 2003). The likelihood and Bayesian analyses used an optimal model of DNA evolution determined using MODELTEST 3.7 (Posada and Crandall 1998). DNA sequences were assembled and aligned in GENEIOUS v4.8.5 (Drummond et al. 2010). For complete details of phylogenetic analyses, see Inderbitzin et al. (2011b).

Microscopy

Morphological observations were from cultures grown on PDA. Microscopy was performed using a Leica DM5000 B microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany), with differential interference contrast (DIC) illumination of specimens mounted in water. Photographs were taken with a Leica DFC310 FX camera, using LEICA APPLICATION SUITE VERSION 3.6.0 software.

Results

DNA sequence data obtained and phylogenetic analyses

The ITS and *TUB* sequences of *Cylindrocladiella habajimaensis* strain PD684 measured 462 and 480 bp in length, respectively (GenBank JN687561, JN687562), and were aligned with the *Cylindrocladiella* ingroup sequences by van Coller et al. (2005) resulting in 43 taxa datasets submitted to TreeBase (<http://purl.org/phylo/treebase/phylovs/study/TB2:S11935>). ITS and *TUB*-derived most parsimonious trees were identical on a 70% bootstrap support level (data not shown), and were combined into one, 950 character dataset that was analyzed using parsimony, likelihood and Bayesian inference of phylogeny, the latter two methods implementing a HKY+I+G model of nucleotide substitution. The 22 most parsimonious trees were 227 steps in length and differed by branches that were supported by less than 70% of the bootstrap replicates (data not shown). One most parsimonious tree was midpoint rooted and is shown in Fig. 1. The most likely tree (-ln likelihood = 2580.47), and the Bayesian tree were congruent with the most parsimonious tree on 70% bootstrap and posterior probability levels, respectively (data not shown, but see Fig. 1 for support values). The tree topology obtained was as in van Coller (2005) for the taxa shared between the two studies, but with lower branch supports probably because our analyses included only two and not three loci. *Cylindrocladiella habajimaensis* grouped in a well supported, but poorly resolved clade together with *C. lageniformis*, *C. infestans*, *Nectricladiella infestans*, *C. viticola* and a

Cylindrocladiella sp. (Fig. 1). This group, without *C. lageniformis*, was referred to as the ‘*C. infestans* species complex’ by van Coller et al (2005). Among its closest relatives, *C. habajimaensis* was most similar to *N. infestans* from which it differed by 18 substitutions in the 950 bp, combined ITS and *TUB* dataset.

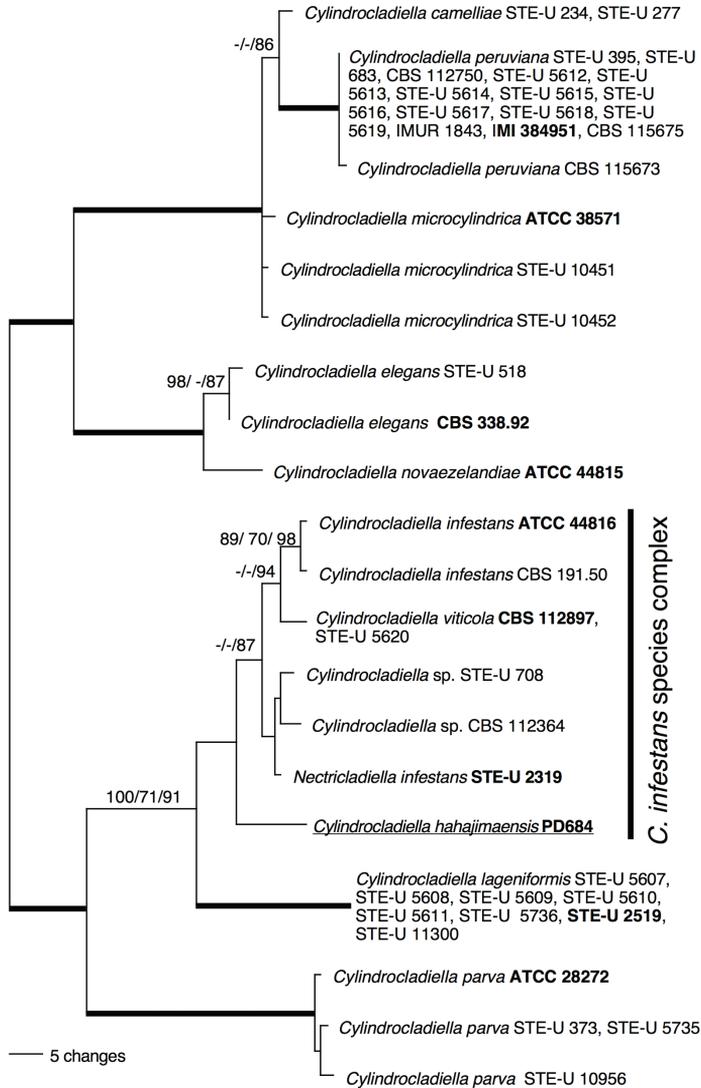


Figure 1. Phylogenetic position of *Cylindrocladiella habajimaensis* within *Cylindrocladiella* based on a combined ITS and *TUB* dataset of 950 characters and 43 taxa. The tree is midpoint rooted. One of the most parsimonious trees is shown. Species names are followed by strain identifiers for each strain included in this study, identifiers in bold face represent ex-type cultures. *Cylindrocladiella habajimaensis* is underlined. The *C. infestans* species complex is delimited by a vertical bar on the right. Numbers by the branches are parsimony, Bayesian and likelihood support values above 70 in that order, branches in bold had maximal support in all analyses.

Taxonomy

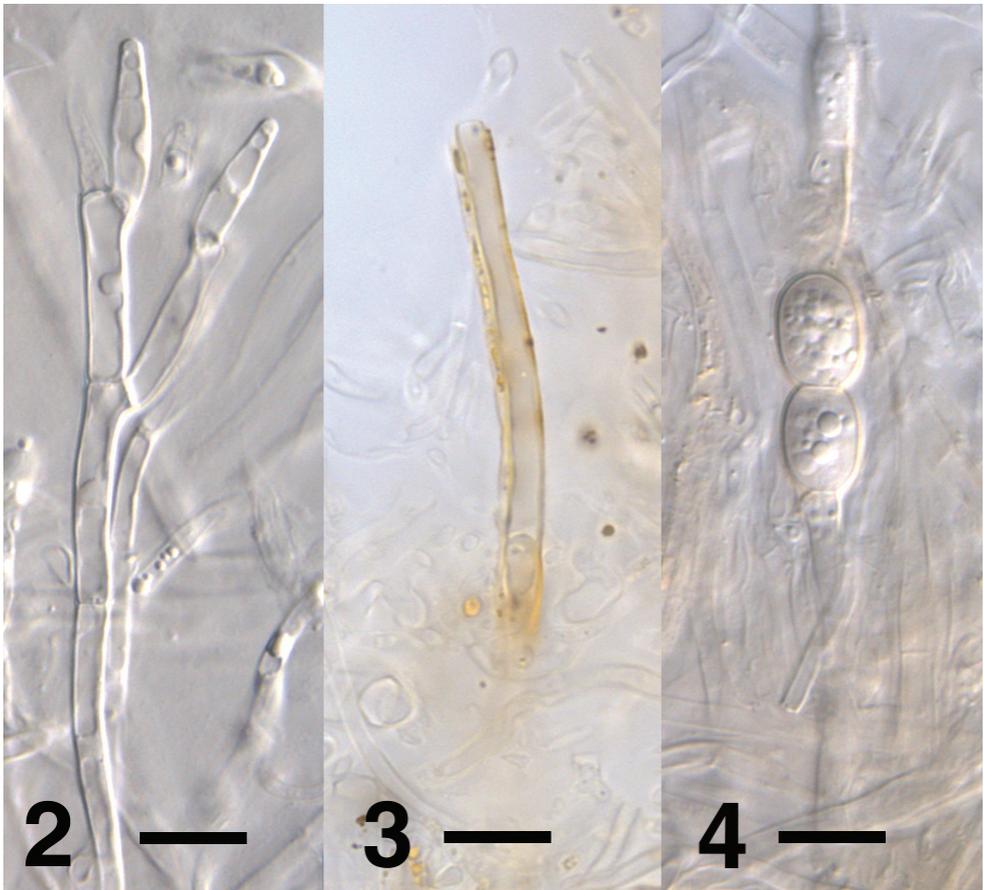
Cylindrocladiella habajimaensis (Ts. Watanabe) Inderb., R.M.Bostock and K.V.Subbarao, comb. nov.

Mycobank: MB 483222

Figures 2–4

◦ *Verticillium habajimaense* Ts. Watanabe, Mycoscience 42: 594 (2001).

Commentary: *Cylindrocladiella habajimaensis* was formerly placed in *Verticillium* because of the presence of verticillate conidiophores which were illustrated by Watanabe (2001), together with the chlamydo spores and the conidia. We confirmed the presence of these structures, and also found evidence for the presence of penicillate conidiophores (Fig. 2). However, *C. habajimaensis* strain PD684 conidiated sparsely, and we



Figures 2–4. Select morphological features of *Cylindrocladiella habajimaensis* strain PD684 (ex-holotype). **2** Potential penicillate conidiophore after eight days on PDA **3** Yellow-pigmented hypha after 15 days on PDA **4** A pair of thick-walled hyaline chlamydo spores with brown tinge after 27 days on PDA. Scale bar = 10 μ m; Imaging method: DIC.

were unable to conclusively assess the morphology of the penicillate conidiophores. In agreement with Watanabe (2001), no filaments with terminal vesicles were seen. Watanabe (2001) described the colony color as ‘Sudan brown or snuff brown’, and the chlamydospores were described as brown. However, we found that the brown colony color was caused primarily by yellow to brown-pigmented hyphae (Fig. 2) not mentioned by Watanabe (2001), the chlamydospores remained hyaline with a brown tinge after 27 days of incubation. We did not find any additional cultures of *C. habajimaensis* at CBS, NIAS or ATCC. More strains of *C. habajimaensis* will need to be examined to update the description of this fungus.

Discussion

Cylindrocladiella habajimaensis is the latest member of *Cylindrocladiella* which now consists of eleven species (van Coller et al. 2005; Zhang and Chi 1996). Our phylogenetic analyses showed that *C. habajimaensis* was nested within *Cylindrocladiella* with high support (Fig. 1), and was thus a member of this genus. Morphology did not contradict this placement. The original description of *C. habajimaensis* mentioned verticillate conidiophores and chlamydospores which are present in *Cylindrocladiella* (Boesewinkel 1982). However, the original description did not mention other characteristics of *Cylindrocladiella* including penicillate conidiophores and filaments with terminal vesicles. We found evidence for penicillate conidiophores (Fig. 1), but did not find any filaments with terminal vesicles. However, the ex-holotype culture we examined conidiated sparsely, preventing us from corroborating our observations and amending the *C. habajimaensis* type description.

Our analyses included DNA sequence data derived from ex-type cultures of all known *Cylindrocladiella* species, except for *C. tenuis* which has larger conidia than *C. habajimaensis* (Zhang and Chi 1996). Also, none of the species described by Lombard et al. (2012) were included in our analyses. This is because we were unable to replicate the topology of the phylogenetic tree in Lombard et al.’s (2012) figure 1, both based on DNA sequence data retrieved from GenBank, and based on a nexus file with MrBayes block provided by the authors. We do not know the cause of this divergence, but it was consistent and independent of the method of analysis and hardware used. Despite excluding the most recent data available, the analyses we presented here showed strong support that *C. habajimaensis* belonged to *Cylindrocladiella*, mainly to the *C. infestans* species complex (van Coller et al. 2005), a group with uncertain species boundaries. *Cylindrocladiella habajimaensis* differed from the most similar member of the *C. infestans* species complex included in van Coller et al. (2005) by a considerable 18 nucleotide substitutions at ITS and *TUB*. More analyses with a larger number of isolates, including the species in Lombard et al. (2012) are needed to resolve the branching order and species boundaries in the *C. infestans* species complex, and to determine whether any more recently described species of *Cylindrocladiella* may be synonyms of *C. habajimaensis*.

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