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ORIGINAL ARTICLE

Curviclavula, a new genus of anamorphic Helotiales (Leotiomycetes) isolated from air

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Abstract A new genus *Curviclavula*, with *C. anemophila* as the type species, is established to accommodate a hyphomycetous anamorph isolated from a culturable air sample collected outdoors in New Mexico, USA. The fungus is morphologically distinct in having 1–5 times dichotomously branched conidiophores and integrated, terminal or intercalary, indeterminate, irregularly sympodial conidiogenous cells with 1–2 flattened, inconspicuous, non-protuberant, and neither thickened nor darkened conidiogenous loci. The conidia are variable in shape, occasionally didymosporous or phragmosporous, but usually minutely cheiroid at first and composed of a subcylindrical to obconical, often inflated, straight or curved basal cell that bears a subglobose lateral cell and a short row of 2–4 cells. The apical cell of the developing conidium curves and fuses with the lateral and adjacent cells once in contact to eventually forming a tightly appressed, subglobose to broadly clavate conidium. Conidial secession is schizolytic, but the conidial basal cells often show minute marginal frills and darkened hila. Phylogenetic analyses using DNA sequences from the nuclear ribosomal large subunit and internal transcribed spacer region also supported *Curviclavula* as distinct and placed it within the Helotiales (Leotiomycetes, Ascomycota) with strong affinity to the family Hyaloscyphaceae.

Keywords Airborne fungi · Microfungi · Phylogeny · Taxonomy

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Introduction

During a routine analysis of culturable air samples received in our laboratory for indoor air quality examination, a colony of an unusual fungus was recovered and isolated. The source sample was collected outdoors at the townsite of Los Alamos, New Mexico, where the surrounding vegetation is a lower montane coniferous forest dominated by the widespread Ponderosa pine (*Pinus ponderosa* P. Lawson & C. Lawson)/Gambel oak (*Quercus gambelii* Nutt.) community type (Dick-Peddie 1993; Morrow 1995). The fungus was first tentatively identified as a species of *Cheiromycella* Höhn. based on the presence of a variable conidial morphology that includes didymosporous, phragmosporous, and minute cheirosporous conidia (Ellis 1971). After careful observations, however, the isolate showed an unusual combination of morphological features, particularly certain aspects of conidial development, which are considerably different from any previously known taxa of hyphomycetous anamorphs. In order to further confirm the fungus identity and to explore its possible phylogenetic and taxonomic relationships, DNA sequences of the nuclear ribosomal large subunit (LSU) and internal transcribed spacer (ITS) region were obtained and analyzed. Molecular data also supported the recognition of a novel taxon, and, therefore, a new genus and species is introduced here to accommodate it based on morphological and molecular characters.

Material and methods

Isolation and morphological studies

The original colony was recovered from an air sample collected with an Andersen sampler on an agar plate (Andersen 1958) after seven days of incubation at 25 °C. It was transferred aseptically to 2 % Malt Extract Agar (MEA: Healthlink,

Jacksonville, FL) and incubated as previously described. Single-spore cultures were obtained following Choi et al. (1999) and colony features were observed and measured after 21 days. Color codes in parentheses are from Kornerup and Wanscher (1978). Microscopic slides were mounted in lactocotton blue and microphotographs were taken using an Olympus BX-45 compound microscope. A total of 100 measurements were made at 1,000× magnification for each fungal structure, and minimum, maximum, fifth and 95th percentile values were calculated using Microsoft Office Excel 2007, with outliers shown in parentheses. A line drawing was made using a drawing tube (Carl Zeiss, Oberkochen, Germany). A culture plate inoculated with the fungus was sent to the Advanced Microscopy Facility of the University of Victoria, British Columbia, Canada for scanning electron microscopy (SEM), where the sample was processed following a standard microwave fixation protocol and dried with Hexamethyldisilazane (E. Humphrey, personal communication), sputter-coated with gold-palladium, and examined using a Hitachi S-4800 field emission scanning electron microscope at 0.5–1.0 kV. Selected images of light and SEM microscopy were edited in Adobe Photoshop Elements 7.0. The type specimens, in the form of dried cultures and semi-permanent slides, are deposited in the Herbarium of the US National Fungus Collections (BPI) and the Illinois Natural History Survey Fungarium (ILLS). A living culture is also deposited in the Centraalbureau voor Schimmelcultures (CBS).

DNA extraction, PCR amplification, and sequencing

Detailed protocols for DNA extraction, PCR amplification, and Sanger sequencing are described in Promputtha and Miller (2010). A DNeasy® Mini Plant extraction kit (Qiagen Inc., Valencia, CA) was used to extract DNA from the culture following the manufacturer's protocols, except tissues were not ground in liquid nitrogen and final elution volume was 25 μL. The complete ITS region and partial LSU of nrDNA was amplified using two overlapping primer sets: ITS1F–LR3 and LROR–LR6 (Gardes and Bruns 1993; Rehner and Samuels 1995; Vilgalys and Hester 1990; White et al. 1990) and sequenced with the PCR primers along with ITS4 and LR3R on an Applied Biosystems 3730XL high-throughput capillary sequencer (Applied Biosystems, Foster City, CA) at the W. M. Keck Center at the University of Illinois Urbana-Champaign.

Phylogenetic analyses

Two independent datasets were compiled and analyzed, a LSU dataset 903 bp in length consisting of 31 taxa representing nine families in the Helotiales based on Baschien et al. (2013), Hyde et al. (2011), and Réblová and Gams (2011), and a more exclusive ITS dataset 595 bp in length consisting of 17 taxa in the Hyaloscyphaceae and incertae sedis Helotiales. GenBank accession numbers are shown on the phylogenetic tree after species names. Alignments of each region were created manually by eye in Sequencher 5.1 and ambiguous regions were removed using the online version of Gblocks v. 0.91b (Castresana 2000). The settings were set for less stringent selection by allowing smaller final blocks, gap positions within the final blocks, and less strict flanking positions. The Akaike Information Criterion (AIC), implemented in jModelTest 2.1.1 (Guindon and Gascuel 2003; Darriba et al. 2012) with default parameters, was used to select the best model of evolution, which was determined to be the GTR+I+G model for each region. Maximum likelihood (ML) analyses using PhyML (Guindon and Gascuel 2003) in Seaview 4.2 were conducted under the GTR substitution model with six rate classes and invariable sites optimized. An unrooted BioNJ starting tree was constructed and the best of nearest neighbor interchange (NNI) and subtree pruning and regrafting (SPR) tree improvement was implemented during the heuristic search. Nonparametric bootstrap support (BS) (Felsenstein 1985) was determined with 100 replicates. Clades were considered significant and highly supported when BS≥70 % (Hillis and Bull 1993).

Bayesian inference employing a Markov Chain Monte Carlo (MCMC) algorithm was performed using MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001) on the CIPRES Science Gateway Teragrid (Miller et al. 2010) as an additional means of branch support. The GTR+I+G model with six rate classes was employed. Four independent chains of MCMC were run for 100 million generations with every 1000th tree sampled to ensure that trees were not trapped in local optima. Effective sample size (ESS) was estimated using Tracer v. 1.5 (Rambaut and Drummond 2009), which suggested removing the first 10,000 trees as burn-in. Posterior probabilities were determined from a 95 % consensus tree generated in PAUP 4.0b10 (Swofford 2002) with the remaining 90,000 trees. Clades with Bayesian posterior probability (BPP)≥95 % were considered significant and highly supported (Alfaro et al. 2003).

Results

A single sequence (1,666 bp) containing both the LSU and the ITS (KM503089) was obtained and deposited in GenBank. Phylogenetic analyses show that *Curviclavula* belongs within the Helotiales (Leotiomycetes) with the ML analysis of the LSU sequences yielding a single most-likely tree (Fig. 1). *Curviclavula* groups in a single branch with *Hyalodendriella betulae* Crous with poor bootstrap support value (50 %) but high posterior probability (>95 %) and forms an unsupported sister clade with members of Hyaloscyphaceae sensu lato that includes *Cistella spicicola* Huhtinen & Söderh, *Hyphodiscus*

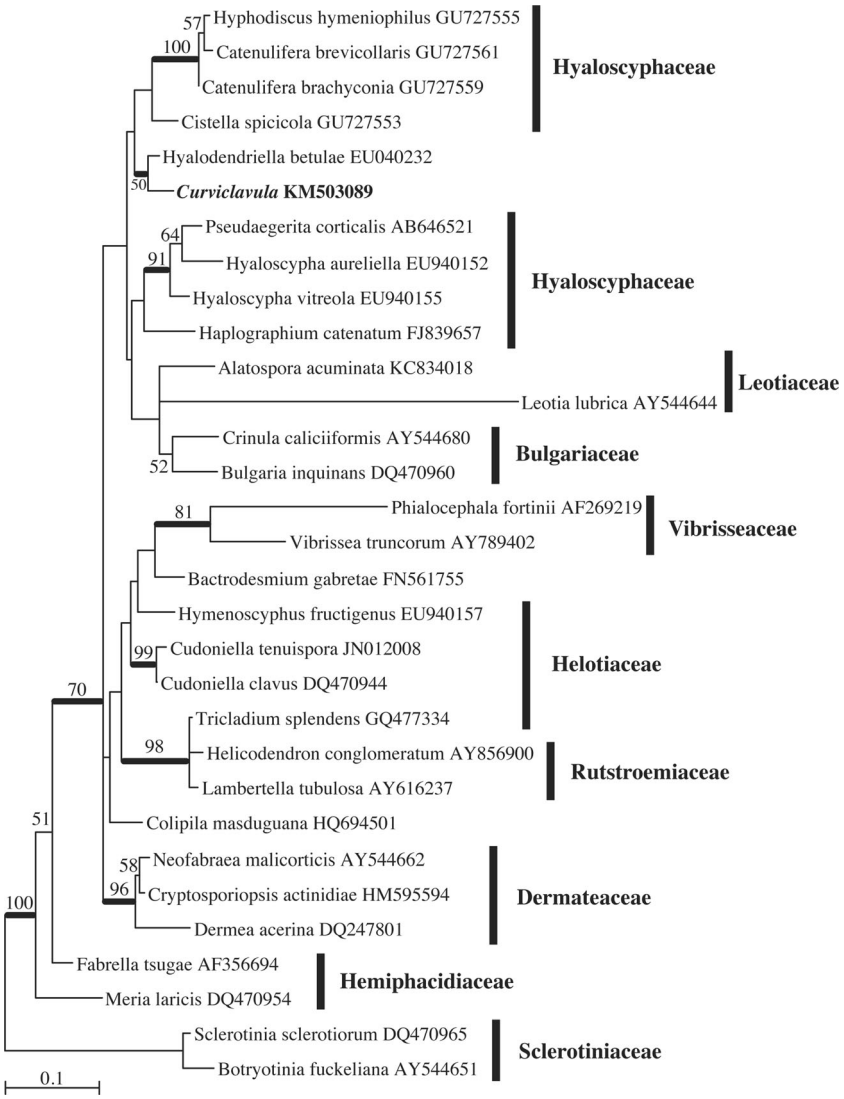

Fig. 1 Phylogenetic tree generated after ML analysis of the LSU nrDNA sequence data showing the position of *Curviclavula* among Helotiales. Numbers above or below branches are bootstrap values≥ 50 % and thickened branches are Bayesian posterior probabilities≥ 95 %

hymeniophilus (P. Karst.) Baral and *Catenulifera* anamorphs. The ML analysis of the ITS data also generated a single tree (not shown) where *Curviclavula* clustered most closely with *Chalara* species belonging to Helotiales and again to *H. betulae*.

Taxonomy

Curviclavula G. Delgado, F.A. Fernández & A.N. Mill., gen. nov.

MycoBank MB 809670

Etymology: from the Latin *curvus*=curved, bent and *clavula*=small club, referring to the conidial shape.

Colonies on MEA moderately slow growing, velvety, dark gray. Mycelium immersed and superficial. Conidiophores macronematous, mononematous, repeatedly dichotomously branched few times, septate, brown. Conidiogenous cells monoblastic or polyblastic, integrated, terminal or intercalary, indeterminate, sympodial, cylindrical or geniculate; conidiogenous loci flattened, inconspicuous. Conidial secession schizolytic. Conidia acropleurogenous, complanate, pale brown, smooth, variable in shape, didymosporous, phragmosporous, minutely cheiroid at first or eventually subglobose to broadly clavate, the apical cell curving and fusing with other conidial cells once in contact forming a closely appressed conidium; basal cell with truncate, darkened hilum and minute marginal frill.

Type species: *Curviclavula anemophila* G. Delgado, F.A. Fernández & A.N. Mill.

Curviclavula anemophila G. Delgado, F.A. Fernández & A.N. Mill., sp. nov. (Figs. 2 and 3)

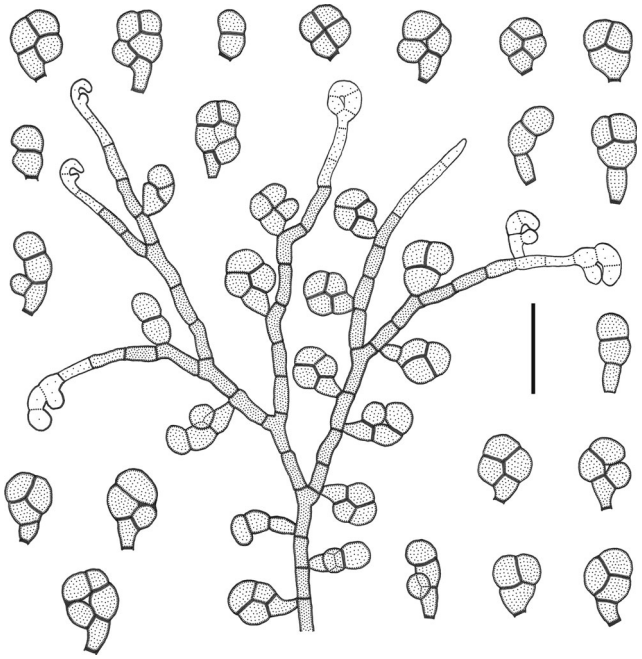

Fig. 2 *Curviclavula anemophila* (from holotype). Line drawings of a branched conidiophore, conidiogenous cells and conidia. Scale bar= 15 μm

MycoBank MB 809671

Etymology: from the Greek *ánemos*=wind and *philein*=to love, referring to the air, where the fungus was isolated.

Colonies on MEA moderately slow growing, reaching 23–28 mm diam. after 21 days at 25 °C, velvety, dark gray or blackish gray (22 F1/22G1), becoming dark brown (6 F7) in age, with cottony, light gray (22D1) aerial mycelium in the center, margin narrow, entire or slightly undulate, reverse pale yellow (2A3), often with concentric rings, sporulation abundant. Mycelium immersed and superficial, composed of branched, septate, smooth or finely rough, hyaline to light brown hyphae, 1–2 μm wide. Conidiophores arising intercalary or terminally from the aerial hyphae, macronematous, mononematous, erect, flexuous, smooth, septate, slightly attenuated at the septa, repeatedly dichotomously branched 1–5 times, brown, length indeterminate, 2–3 μm wide; branches usually formed below a septum, flexuous, somewhat geniculate, brown, paler toward the apex, up to 120 μm long. Conidiogenous cells monoblastic or polyblastic, integrated, terminal or intercalary, indeterminate, irregularly sympodial, cylindrical, subcylindrical or sometimes geniculate, with 1–2 conidiogenous loci per cell; conidiogenous loci flattened, inconspicuous, apical, lateral or on shoulders formed by sympodial proliferation, non-protuberant, neither thickened nor darkened. Conidial secession schizolytic. Conidia holoblastic, solitary, dry, acropleurogenous, complanate, pale brown, smooth, composed of 2–5 (6) cells, each individual cell 3–7×3–6 μm, septate, constricted at the septa, variable in shape, occasionally simple, didymosporous or phragmosporous and subglobose, oblong or broadly obovoid, but usually minutely cheiroid at first and consisting of a subcylindrical to obconical, often inflated, straight or curved basal cell bearing a single, subglobose lateral cell and a short row of 2–4 cells, the apical cell of the developing conidium curves and fuses with the lateral and remaining cells once in contact eventually forming a closely appressed, subglobose to broadly clavate conidium, (7) 8–13 (15)×(4) 6–9 (10) μm, 4–6 μm thick; basal cell with a truncate, darkened hilum, 2–2.5 μm wide, often showing a minute marginal frill. Teleomorph unknown.

Specimen examined: UNITED STATES. New Mexico, Los Alamos Co., Los Alamos, 35°53′16″ N, 106°18′56″ W, 2,200 m asl., from outdoor air, isol. F.A. Fernández, 24.1.2013. Holotype (BPI 892883), Isotype (ILLS 71919), ex-holotype culture (CBS 138123).

Discussion

Certain aspects of conidial morphology and development make *Curviclavula* unique among previously described genera of hyphomycetes (Seifert et al. 2011). The most significant developmental feature involves the curving and sequential fusing of the apical cell with other elements of the conidium, first with the lateral cell and later with the remaining conidial cells during maturation. Initially, the conidial filament elongates while the tip bends to one side, and the apical cell is then delimited by a thin septum. The bottom and now basal cell produces a small bud laterally somewhat below the septum, which also elongates forming a lateral cell after being delimited by a second septum. At this point the conidium initial shows a resemblance to a tiny hand in side view. Meanwhile, a further septum is formed at the conidial filament delimiting a subapical cell. More or less synchronously with the formation and delimitation of the lateral cell, the apex of the conidial filament curves and grows towards it. Once both cell tips are in contact, they press together or even fuse but remain delimited by the cell walls as a new, additional septum. Subsequent swelling of the filament causes the walls of the lateral and subapical cell to also fuse delimiting another new septum. The final conidium is composed of four cells and five septa, one longitudinal and four oblique, and is more or less broadly clavate in shape, often showing a tiny space in the center as a result of incomplete fusing.

The pattern described above is the most common, but there are variations among cheiroid conidia (Figs. 2 and 3d). Occasionally only the lateral walls of the apical, subapical, and lateral cells press together while the cell tips remain free. Some conidia have only four cells tightly appressed and arranged side by side forming a cross. Sometimes, it is the suprabasal cell of the row and not the basal cell that elongates forming a lateral cell after being delimited by a septum. The final conidium is then composed of five cells and six septa

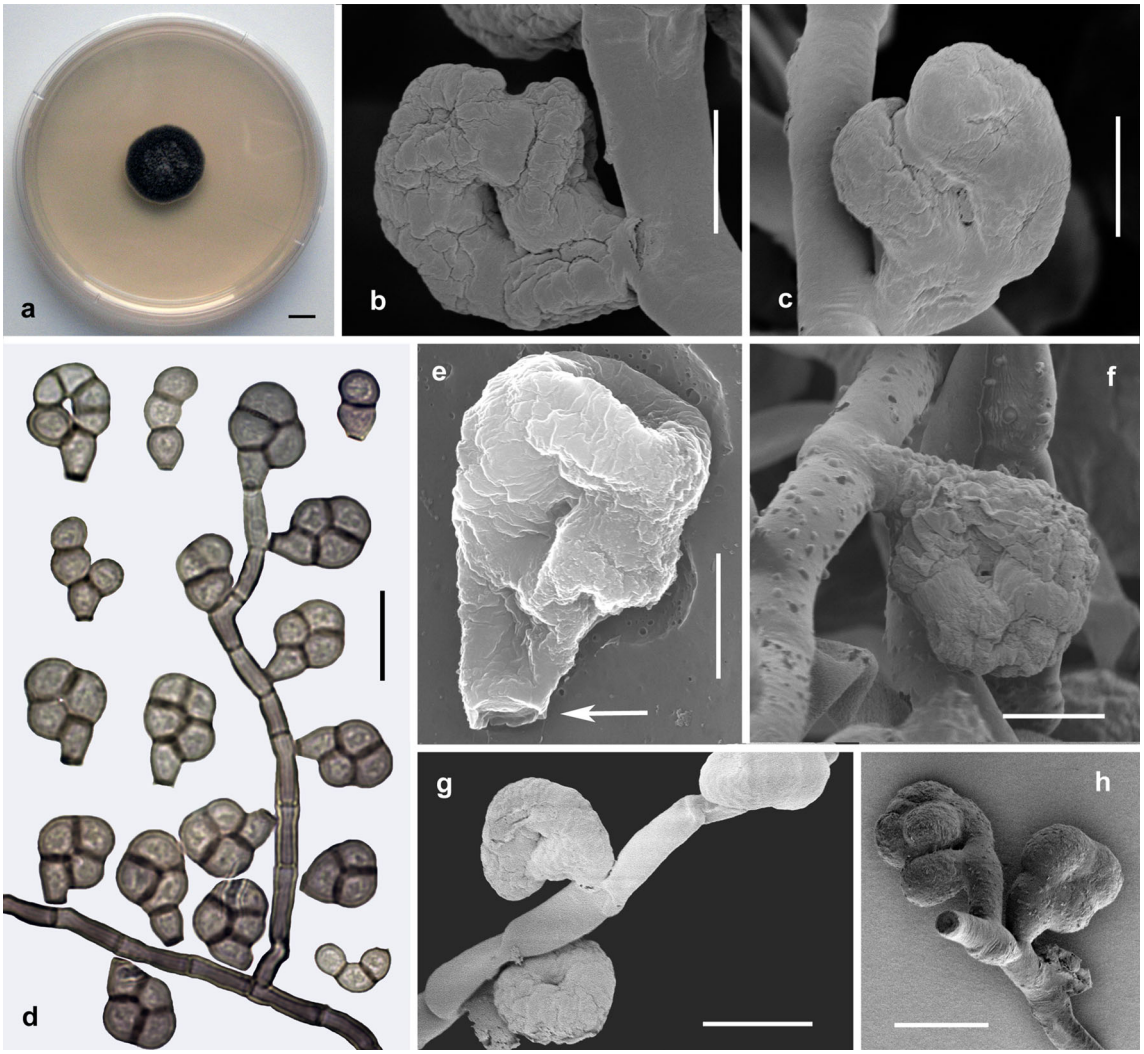

Fig. 3 *Curviclavula anemophila* (from holotype) (**a**) Colony on MEA after 21 days incubation at 25 °C. Conidiophores, conidiogenous cells and conidia (**b**–**c**), (**e**–**h**) SEM (**b**) Seceding conidium showing irregular tear of the outer conidiogenous cell wall (**e**) single conidium. Arrow indicates the irregular marginal frill left after schizolytic secession (**d**) light microscopy. Scale bars a=1 cm, b-c, e-f=3 μm, g-h=5 μm, d= 10 μm

once the apex curves and fuses. Often, the basal cell is inflated and no delimiting septum is laid down. A lateral cell is, therefore, not formed and the apical cell fuses with one side of the basal cell forming a subglobose conidium made of three tightly appressed cells and three septa more or less arranged in a "Y". A variation of this pattern occurs when the inflated basal cell enlarges laterally without forming a delimiting septum. The enlarged side then contacts the tip or the lateral wall of the apical cell and the resulting conidium is made up also of three cells and three septa arranged in a "Y", often with a tiny space in the center. A similar variation occurs when the suprabasal cell is the one that is inflated or enlarges laterally, and the conidium then possesses four cells, a subcylindrical or obconical basal cell and three distal cells disposed in the same "Y" septal arrangement. Rarely, the basal cell produces a lateral cell delimited by a septum, but also the suprabasal cell enlarges laterally forming a second lateral cell. When the apical cell curves, it then contacts this second upper lateral cell, which is located in the middle between both the apical and the bottom lateral cell. The final conidium possesses six cells and up to nine septa with different longitudinal, transverse, or oblique orientation. Another variation occurs when the suprabasal cell enlarges laterally but no septum is laid down to form a second lateral cell. That enlargement is then surrounded by the apical cell, which curves to contact the tip of the lateral cell, resulting in a five-celled conidium with seven septa also in different orientations.

This peculiar conidial morphology and development is in combination with other features such as repeatedly dichotomously branched conidiophores and terminal or intercalary, irregularly sympodial conidiogenous cells bearing one or two loci per cell. Conidial secession is schizolytic, but the conidial basal cells often bear minute, hardly visible, marginal frills under the light microscope. SEM microscopy revealed

that the lytic zone between the wall of the conidiogenous cell and the conidium basal septum is relatively broad and often thickened and the seceding conidium tears the surface of the supporting conidiogenous cell rather irregularly (Fig. 3b, f). Once released, the conidium carries around the base a small portion of the ruptured outer wall as a frill of irregular margin and leaves a more or less circular scar on the conidiogenous cell. An ambiguous conidial feature was wall ornamentation, smooth under the light microscope but finely rough to wrinkled under SEM microscopy, and possibly the result of sample preparation artifacts.

Phylogenetic analyses suggest that *Curviclavula anemophila* belongs to the Helotiales with strong affinity to the family Hyaloscyphaceae. The LSU phylogeny shows a relationship supported only in Bayesian analysis with *Hyalodendriella betulae*, a morphologically unrelated helotialean anamorph characterized by limoniform to ellipsoid, aseptate conidia in short chains of 2–3 and dimorphic conidiophores with conidiogenous cells bearing a single or numerous, aggregated conidiogenous loci (Crous et al. 2007). Helotiales is a large and polyphyletic order in the class Leotiomycetes that includes several anamorphic genera distributed within eleven teleomorphic families, but phylogenetic relationships at familial level are still unresolved for many other genera like *Hyalodendriella* (Hyde et al. 2011). ITS sequence data also showed a relationship with *H. betulae* and with species of the morphologically dissimilar, phialidic genus *Chalara* belonging to Helotiales such as *C. recta* Koukol, *C. longipes* (Preuss) Cooke, and *C. piceae-abietis* Hol.-Jech. (Koukol 2011). A more extensive taxon sampling and additional molecular data will be necessary to clarify the phylogenetic affinities of *Curviclavula* with these and other helotialean genera.

Both *Curviclavula* and *H. betulae* form an unsupported sister clade with members of the family Hyaloscyphaceae sensu lato (Han et al. 2014) including phialidic anamorphs in the genus *Catenulifera* such as *C. brevicollaris* (W. Gams) Bogale & Unter. and *C. brachyconia* (W. Gams) Bogale & Unter., and the teleomorphs *Hyphodiscus hymeniophilus* and *Cistella spicicola*. *Curviclavula* also groups closer to a clade consisting of Hyaloscyphaceae sensu stricto (Han et al. 2014) that includes *Hyaloscypha vitreola* (P. Karst.) Boud., the type species of the family, *Pseudaegerita corticalis* (Peck) J.L. Crane & Schokn., the anamorph of *H. spiralis* (Velen.) J.G. Han, Hosoya, H.D. Shin (=*H. lignicola* Abdullah & J. Webster) (Abdullah et al. 2005; Han et al. 2014), and *H. aureliella* (Nyl.) Huhtinen, the teleomorph of *Cheiromycella microscopica* (P. Karst.) S. Hughes (Huhtinen 1989). The family Hyaloscyphaceae includes a morphologically and ecologically diverse group of anamorphic genera that ranges from hyphomycetous or rarely coelomycetous anamorphs with holoblastic or phialidic conidiogenesis living as saprobes in terrestrial environments or in freshwater habitats as aero-aquatic or aquatic (Hyde et al. 2011). The relationship of *Curviclavula* with Hyaloscyphaceae could be further supported by its morphological similarity with *Cheiromycella*, particularly *Ch. microscopica* (Braun et al. 2009; Ellis 1971; Sutton 1985). They share the presence of macronematous, repeatedly branching conidiophores with monoblastic or polyblastic conidiogenous cells and occasionally simple, didymosporous or phragmosporous but also branched cheiroid conidia that are variable in shape and usually with two arms arising from a basal cell and constricted at the septa. Matsushima (1981) illustrated a specimen of *Ch. microscopica* isolated on culture media from the inside of dead bark of *Fagus crenata* Blume in Japan with conidia very similar to those in *Curviclavula*. Conidiophores in *Cheiromycella*, however, are short and irregularly branched, conidiogenous cells are swollen, determinate, clavate, doliiform, sphaerical or subsphaerical in shape, sometimes connected to one another by narrow isthmi, and conidia are aggregated in firm slimy masses, secede schizolytically without bearing a basal marginal frill, and have divergent and flexuous arms with a greater number of cells that never fuse (Ellis 1971).

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Conflict of interest The authors declare that they have no conflict of interest.

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