Novel *Curvularia* species from clinical specimens

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Key words

*Bipolaris*

*Curvularia*

gpd

ITS

LSU

phylogeny

RPB2

systematics

Abstract  The fungal genus *Curvularia* includes numerous plant pathogens and some emerging opportunistic pathogens of humans. In a previous study we used morphology and sequences of the nuclear ribosomal internal transcribed spacer region (ITS) and the glyceraldehyde-3-phosphate dehydrogenase (gpd) gene to identify species within a set of 99 clinical *Curvularia* isolates from the USA. Seventy-two isolates could be identified while the remaining 27 isolates belonged in three unclassified clades that were tentatively labelled *Curvularia* sp. I, II and III. In the present study, we further assess the taxonomic placement of these isolates using sequences of ITS, gpd, the large subunit rDNA, and the second largest subunit of RNA polymerase II. DNA sequence comparisons with a set of 87 isolates representing 33 *Curvularia* spp. and members of the closely-related genera *Bipolaris* and *Exserohilum* revealed that *Curvularia* sp. I, II and III represent novel lineages in *Curvularia*. These lineages are morphologically different from the currently accepted species. In the phylogenetic tree, *Curvularia* sp. I and sp. III were each split into two distinct lineages. Morphology and phylogeny supported the proposal of five new species, to be named *C. americana*, *C. chlamydospora*, *C. hominis*, *C. muehlenbeckiae* and *C. pseudolunata*. The concatenated 4-locus phylogeny revealed the existence of six clades in *Curvularia*, which are associated with particular morphological features. They were named after representative species, namely *americana*, *eragrostidis*, *hominis*, *lunata*, *spicifera* and *trifoli.*

Article info  Received: 2 February 2014; Accepted: 5 March 2014; Published: 29 July 2014.

**INTRODUCTION**

*Curvularia*, typified by *C. lunata*, is a species-rich genus, which includes numerous grass pathogens and saprobes occurring on plant material, dung and soil (Faurel & Schottet 1965, Sivanesan 1987, Jiang & Zhang 2007). At least eight species of this genus have been reported from opportunistic diseases in humans ranging from mild skin and nail infections to severe invasive disease, depending on route of infection and immune status of the host (Kamalam et al. 1992, Ismail et al. 1993, Lopes & Jobim 1998, Ebright et al. 1999, de Hoog et al. 2000). Morphologically, *Curvularia* is characterised by the production of sylindrical conidiophores with tretic, terminal and intercalary conidigenous cells and elongate, transversely septate conidia with a dark basal scar. Conidia are often curved at an asymmetrically swollen intermediate cell, but species with straight conidia also have been described (Sivanesan 1987). Authors such as Ellis (1971, 1976), de Hoog et al. (2000) and Revankar & Sutton (2010) have described the conidia as truly septate or ‘euseptate’, i.e. composed of a single wall with septa that are formed as inward extensions of that wall (Luttrell 1963). A similar genus is *Bipolaris*, type species *B. maydis*, which traditionally has been distinguished from *Curvularia* by producing conidia which lack an asymmetrically swollen intermediate cell and are ‘distoseptate’ (Domsch et al. 2007, Revankar & Sutton 2010), i.e. they have a common outer wall enclosing more or less spherical cells, each of which is surrounded by an individual wall (Luttrell 1963). The separation of the two genera has been a matter of controversy and many authors have stated that *Curvularia* species also have distoseptate conidia (Alcorn 1983a, Sivanesan 1987, Seifert et al. 2011).

Sexual stages of *Bipolaris* and *Curvularia* were traditionally placed in *Cochliobolus*. Typically, they feature thick-walled, ostiolate ascomata with pseudoparaphyses, and bitunicate asci that give rise to filiform, multiseptate ascospores (Sivanesan 1987, Zhang et al. 2012). The ascospores often appear more or less helically coiled within the ascus. A similar genus, *Pseudocochliobolus*, was segregated from *Cochliobolus* to accommodate species producing ascomata on columnar stromata, with ascospores appearing linearly parallel or loosely coiled within the asci. The asexual stages of *Pseudocochliobolus* species were *Curvularia* and *Bipolaris* species with short, rather straight conidia (Tsuda et al. 1977, Tsuda & Ueyama 1981). Most authors have not accepted *Pseudocochliobolus* as a separate genus because the degree of coiling of the ascospores can vary greatly within a species. Also, the addition of a second genus with *Curvularia* and *Bipolaris* asexual stages would introduce unnecessary complexity into the taxonomy of this group of fungi instead of clarifying it (Alcorn 1983a, 1988, Sivanesan 1987). *Cochliobolus*, *Pseudocochliobolus* and their *Bipolaris* and *Curvularia* asexual morphs were previously considered to be related either to the *Dothideales* (Eriksson 2001) or to the *Pleosporales* (Barr 1979, Sivanesan 1984). Molecular data confirmed their placement in the latter order and more precisely in its largest family, *Pleosporaceae*, along with other important genera of plant pathogens and clinically-relevant fungi such as *Alternaria* and *Exserohilum* (Olivier et al. 2000, Zhang et al. 2009, 2012). Berbee et al. (1999) performed a phylogenetic study to assess the evolutionary relationships of *Cochliobolus*, *Pseudocochliobolus*, *Curvularia* and *Bipolaris*. Their phylogenetic trees, based on the internal transcribed spacer (ITS)
region of the rDNA and the glycereraldehyde-3-phosphate dehydrogenase (gpd) gene, revealed that isolates were distributed mainly in two clades which were named ‘Cochliobolus groups 1 and 2’. Group 1 exclusively encompassed species with Bipolaris asexual morphs, including the type species, B. maydis, agent of southern corn leaf blight, as well as other economically-relevant phytopathogenic species. The sexual morph of B. maydis is Cochliobolus heterostrophus, type species of Cochliobolus (Sivanesan 1987). Group 2 included mostly plant pathogens and saprobes with Bipolaris and Curvularia asexual morphs, including the type species of the latter genus, C. lunata and all species of Pseudocochliobolus.

Manamgoda et al. (2012), with a wider sampling of species and based on the analysis of ITS, large subunit (LSU) rDNA, gpd and elongation factor 1-α (EF1-α) genes, applied the one fungus one name concept (Hawksworth et al. 2011) to the species of Pseudocochliobolus. These included important agents of opportunistic infections in vertebrates, such as B. australiensis, B. hawaiiensis and B. spicifera (de Hoog et al. 2000). The last of these had been previously considered a Curvularia species by Boedijn (1933).

Curvularia spp. have been identified mostly based on morphology, but the names applied often do not correlate with DNA sequence-based identifications. Furthermore, the species most commonly reported from humans, C. lunata, appeared to be a species complex (Berbee et al. 1999, Yanagihara et al. 2010). Da Cunha et al. (2013) recently characterised a set of 99 clinical Curvularia strains from the USA using sequences of the ITS region and the gpd gene. They could identify 73.2% of the isolates, including C. aerea, which was the most common species. The remaining isolates were distributed over three different lineages which did not correlate with any known species. In this study we used DNA sequence data of four nuclear loci to further assess the taxonomic position of these isolates.

MATERIALS AND METHODS

Fungal isolates

Twenty-seven clinical Curvularia isolates from the USA were studied (Table 1). These isolates were obtained from the Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, and represent the clades named Curvularia sp. I, II and III in the study by da Cunha et al. (2013). These isolates were compared with ex-type or reference strains of different Curvularia spp. from the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands.

Phenotypic study

Colony morphology and growth rates were studied on potato carrot agar (PCA; 20 g of potatoes, 20 g of carrots, 20 g of agar, 1 L of distilled water) and oatmeal agar (OA; 30 g of filtered oat flakes, 20 g of agar, 1 L of distilled water) after 7 d of incubation at 25 °C in the dark. Microscopic features were studied in lactic acid from colonies on the same media after 10–21 d of incubation. Size ranges in the species descriptions are derived from at least 30 measurements.

Cryo-Scanning Electron microscopy

Relevant areas of fungal cultures were carefully selected by means of a stereo microscope (Nikon SMZ1500, Nikon, Amsterdam, The Netherlands). Small (c. 3 × 5 mm) agar blocks were carefully cut out with a surgical blade (no. 11, Swan-Morton, Sheffield, UK), while disturbing of fungal structures was kept to a minimum during cutting and transferring of the samples to a copper cup (diam 10 mm, height 8 mm). Agar blocks were glued to the copper cup with frozen tissue medium (KP-Cryoblock, Klinipath, Duiven, The Netherlands). The copper cup was placed on an agar surface inside a closed Petri dish to prevent drying of the sample. The sample was quickly frozen in nitrogen slush and immediately transferred to a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan) equipped with an Oxford CT1500 cryostation. The sample was viewed at 2.5 kV and ice was removed by sublimation after heating of the SEM-stage to −85 °C. Then the sample was sputter-coated in the cryostation by means of a gold target for three times 90 s holding the sample at different angles for an optimal coating.

Electron micrographs were acquired with the F3 or F4 scan at 5 kV and contrast levels digitally enhanced in Adobe® Photoshop® Creative Suite v. 6.

Molecular study

DNA extraction of Curvularia spp. I–III was performed with the PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA, USA) as described by da Cunha et al. (2013). DNA extraction of isolates of the other species studied was carried out from colonies growing on malt extract agar (Oxoid, Basingstoke, England) with the UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). Amplification and sequencing of the ITS and RNA polymerase II second largest subunit (RPB2) were performed with primers ITS5 + ITS4 (White et al. 1990) and 5F2 + 7Cr (O’Donnell et al. 2007) following the protocols of Amaradasa et al. (2014). Amplification of the gpd and LSU genes were performed with primers gpd1 + gpd2 (Berbee et al. 1999) and LROR + LRS (Vilgalys & Hester 1990) as described in Manamgoda et al. (2012). The ITS PCR products were purified and sequenced at Macrogen Europe (Amsterdam) using a 3739 XL DNA analyser (Applied Biosystems). The gpd, LSU and RPB2 loci were sequenced at the CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands), using the BigDye terminator sequencing kit v. 3.1 (Applied Biosystems) and an ABI PrismTM 3100 DNA sequencer (Applied Biosystems). The program SeqMan Pro (Lasergene, Madison, WI, USA) was used to obtain consensus sequences from the complementary sequences of each isolate. Sequences of the clinical isolates were aligned with those of a set of 60 isolates representing 33 species of Curvularia, and two phylogenetically related genera of Pleosporaceae, i.e. Bipolaris (nine spp.) and Exserohilum (one sp., used as outgroup) using Clustalv. 1.81 (Thompson et al. 1997), followed by manual adjustments with a text editor. Individual alignments of ITS, LSU, gpd and RPB2 and a concatenated 4-locus dataset were analysed with maximum likelihood (ML) using MEGA5 (Tamura et al. 2011) with partial deletion of gaps, substitution models proposed by this program and 1 000 bootstrap replicates. Bootstrap support values (bs) ≥ 70 % were considered significant. Incongruence among data sets was tested by a visual inspection of all groups with ≥ 70 bs in the partial trees to search for potentially conflicting groups. A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v. 3.1.1 (Ronquist & Huelsenbeck 2003). The best models of nucleotide substitution for each locus for the Bayesian analysis were determined using MrModeltest v. 2.3 (Nylander 2004). Two analyses of four MCMC chains were run from
### Table 1: Isolates included in the phylogenetic study, their origins, and GenBank accession no.

| Taxon                                | Isolate no.1/2 | Source                                                                 | ITS/LSU/gpd/KPB2 |
|--------------------------------------|----------------|------------------------------------------------------------------------|-----------------|
| Bipolaris chloridis                  | CBS 242.77B    | Chorisia gayana, Australia                                            | HSF934028       |
| B. cynodontis                         | CBS 285.51     | Cymodon transvaalensis, Kenya                                          | HSF934029       |
| B. maydis                            | CBS 305.64     | Cymodon dacylon, USA                                                   | HSF934030       |
| C. graminicola                       | CBS 130.26     | Zaeza mays, Japan                                                      | HSF934037       |
| C. heteropogonis                     | CBS 307.64     | Zaeza mays, USA                                                        | HSF934026       |
| C. microlaenae                       | CBS 280.91     | Microlaena stipoides leaf, Australia                                  | HSF934033       |
| C. oryzae                            | CBS 195.54     | Oryza sativa grain, Indonesia                                          | HSF934031       |
| C. sorghicola                        | CBS 249.60     | Sorghum rugosum var. sudanense, Locality unknown                      | HSF934032       |
| C. tripogonis                        | CBS 140.31     | Substrate unknown, Japan                                               | HSF934035       |
| C. heteropogonis                     | CBS 145.32     | Triticum durum, Locality unknown                                       | HSF934034       |
| C. zeae                              | CBS 127716     | Unknown                                                                | HGF779890       |
| C. zeicola                           | CBS 316.64     | Zaeza mays, USA                                                        | HSF934038       |
| C. tuberculata                       | CBS 317.64     | Zaeza mays, USA                                                        | HSF934027       |
| C. affinis                           | CBS 294.61     | Air, Brazil                                                            | HSF934010       |
| C. hominis                           | CBS 154.34     | Manihot utilissima, Java                                               | HGF779891       |
| C. akiai                             | CBS 185.49     | Manihot utilissima, Java                                               | HGF779892       |
| C. coccis                            | CBS 318.86     | Substrate unknown, Japan                                               | HGF934021       |
| C. cymbopoganis                      | CBS 172.57     | Oryza sativa seed, Vietnam                                             | HGF934012       |
| C. braschyspora                      | CBS 186.50     | Sol, India                                                             | HGF779883       |
| C. carica-papayae                     | CBS 139.51     | Carica papaya leaf, India                                              | HGF779894       |
| C. coicis                            | CBS 192.29     | Coix lacrima-jobi var. typica, Japan                                  | HGF934017       |
| C. cymbopoganis                      | CBS 419.78     | Yucca sp. leaf, Netherlandlands                                      | HGF934085       |
| C. australisism                       | CBS 193.65     | Air, Pakistan                                                          | HGF934013       |
| C. eragrostidis                       | CBS 189.48     | Sorghum seed, Java                                                     | HGF779886       |
| C. gladioli                          | CBS 210.79     | Gladiolus sp. leaf, Romania                                            | HGF779887       |
| C. graminicola                       | BRP 23186      | Aristida ingrata, Australia                                           | JN192376        |
| C. hawaiitennis                       | CBS 173.57     | Oryza sativa, Hawaii                                                   | HGF779898       |
| C. intermedi                         | CBS 484.72     | Salt-marsh soil, Kuwait                                                | HGF934096       |
| C. heteropogonis                     | CBS 727.96     | Substrate unknown, USA                                                 | HGF779890       |
| C. heteropogonis                     | CBS 284.91     | Heteropogon contortus leaf, Australia                                  | HGF934019       |
| C. intermedia                        | CBS 511.91     | Heteropogon contortus leaf, Australia                                  | HGF934018       |
| C. ischaemii                         | CBS 334.82     | Avena versicolor, China                                                | HGF779891       |
| C. ischaemii                         | CBS 185.49     | Manihot utilissima, Java                                               | HGF779892       |
| C. lacrima-jobi                      | CBS 169.53     | Oryza sativa seed, Vietnam                                             | HGF934006       |
| C. ovariicola                        | CBS 285.91     | Ergatosis parviflora, Australia                                       | HGF779893       |
| C. perdidos                          | CBS 143.64     | Ergatosis parviflora, Australia                                       | HGF779894       |
| C. prasadi                           | CBS 350.90     | Perotis rara, Australia                                                | HGF779895       |
| C. protuberata                       | CBS 144.61     | Jasminum sambac, India                                                | HGF779896       |
| C. pulcherrima                       | CBS 376.65     | Substrate unknown, England                                             | HGF779897       |
| C. ravenelii                         | CBS 127709     | Unknown                                                                | HGF779899       |
| C. robusta                           | CBS 624.65     | Dichanthium annulatum leaf, USA                                       | HGF779900       |
| C. sudanense                         | CBS 148.71     | Substrate unknown, Nigeria                                            | HGF779901       |
| C. spinifera                         | CBS 199.31     | Capsicum annuum, China                                                | HGF934015       |
| C. trifoli                           | CBS 173.55     | Trifolium repens, USA                                                 | HGF779023       |
| C. tripogonis                        | BRP 12375      | Tripogon jacquemonti, India                                           | JN192388        |
| C. tuberculata                       | CBS 146.63     | Zaeza mays, leaf, India                                                | HGF934007       |
| C. uncinata                          | CBS 221.52     | Oryza sativa leaf, Vietnam                                            | HGF779024       |
| C. verruciformis                     | CBS 537.70     | Oryza sativa seeds, Denmark                                           | HGF779025       |
| C. verruculosa                       | CBS 149.63     | Lobisia sp. feather, New Zealand                                      | HGF934090       |
| C. austeni                           | CBS 150.63     | Punica granatum, leaf, India                                          | HGF934008       |
| C. mucilagenes                       | CBS 144.83     | Muehlenbeckia sp. leaf, India                                         | HGF779002       |
| C. tripogonis                        | CBS 08.2905    | Chest, USA                                                             | HGF779005       |
| C. tuberculata                       | CBS 07.2971    | Comnea, USA                                                            | HGF779003       |
| C. tuberculata                       | CBS 07.3105    | Nasal sinus, USA                                                       | HGF779004       |
| C. tuberculata                       | CBS 07.3114    | Nasal sinus, USA                                                       | HGF779005       |
| C. tuberculata                       | CBS 07.3581    | Nail, USA                                                              | HGF779006       |
| C. tripogonis                        | CBS 08.849     | Eye, USA                                                               | HE861836        |
| C. tuberculata                       | CBS 130.29     | Nail, USA                                                              | HGF779007       |
| C. tripogonis                        | CBS 08.2418    | Bronchial wash, USA                                                    | HGF779008       |
| C. tripogonis                        | CBS 09.464     | Comnea, USA                                                            | HGF779011       |
| C. tripogonis                        | CBS 08.2517    | Foot, USA                                                              | HGF779009       |
| C. tuberculata                       | CBS 08.3737    | Bronchial wash, USA                                                    | HGF779010       |
| C. tripogonis                        | CBS 09.1692    | Nasal sinus, USA                                                       | HGF779012       |
| C. tripogonis                        | CBS 09.2542    | Nasal sinus, USA                                                       | HSF554856       |
| C. tripogonis                        | CBS 09.2532    | Nasopharynx, USA                                                       | HGF779013       |
| C. tripogonis                        | CBS 09.3403    | Unknown tissue, USA                                                    | HGF779014       |
random trees for 4,598 100 generations and sampled every 100 generations, resulting in 45,961 trees, of which 25% were discarded as the burn-in phase. Posterior probabilities (pp) were determined from the remaining trees. The sequences generated during this study and the alignments used in the phylogenetic analyses were deposited in GenBank (Table 1) and TreeBASE (submission ID http://purl.org/phylo/treebase/study/TB2:S14881), respectively.

RESULTS

Phylogenetic study

After removing ambiguously aligned regions, we obtained ITS, LSU, gpd and RPB2 alignments of 533, 830, 434, and 793 positions of which 64 (12%), 39 (4.69%), 111 (25.57%) and 259 (32.66%) were variable, respectively. MEGA5 proposed a K2 + G + I model for the ITS and RPB2 loci, K2 + I for LSU, T92 + G for gpd and GTR + G + I for the concatenated 4-locus dataset. These models were used in the ML analyses. Partial trees (not shown) were congruent except for the following clades: Curvularia gladioli CBS 210.79 grouped with C. ischaemi CBS 630.82 (93% bs) in the ITS tree, but in the RPB2 tree the former isolate grouped with Curvularia trifolii CBS 173.55 (77% bs), while the CBS 630.92 grouped with Curvularia coicus CBS 192.29 (100% bs). These incongruencies affected species that are not closely related to Curvularia sp. I–III of da Cunha et al. (2013) and therefore the four loci were used. Partial trees revealed that RPB2 was the most informative locus with 35 clades with significant bs, followed by gpd with 23. ITS and LSU both showed only 10 clades with significant bs. The ITS and LSU ML trees provided good support for a clade representing the genus Bipolaris, but Curvularia species appeared in several clades, some of which had low bootstrap support. The gpd ML tree separated Bipolaris and Curvularia as two clades with 93% and 70% bs, respectively, whereas these clades showed 99% and 95% bs in the RPB2 tree. In the concatenated 4-locus ML tree (not shown) the Bipolaris and Curvularia clades had 100% and 97% bs, respectively. For Bayesian analysis, MrModeltest proposed a SYM + I + G model for the ITS locus and GTR + I + G for LSU, gpd and RPB2. These models were incorporated in the analysis. The consensus tree obtained from the Bayesian analysis (Fig. 1) agreed with the topology of the ML tree (not shown) for the 4-locus dataset.

The 4-locus tree (Fig. 1) revealed that C. carica-papayae, listed as a synonym of C. aeria by Sivanesan (1987), is a phylogenetically distinct species. The concatenated tree also corroborated that isolates in *Curvularia* spp. I–III of da Cunha et al. (2013) are different from accepted species of this genus represented in the CBS collection (Fig. 1). However, *Curvularia* spp. I and III were each split into two lineages that are sufficiently distant from each other to represent different species. These lineages were named here Ia, Ib and IIa, IIb, accordingly. One of them, IIb, shows considerable genetic variation, but is treated here as a single taxon because its complex topology does not seem to suggest a clear separation of species within it. The ITS and LSU ML trees did not provide enough resolution to separate lineages within *Curvularia* spp. I and III, but showed 87% and 99% bs for *Curvularia* spp. II. The gpd ML tree gave 80% bs to *Curvularia* sp. II and separated lineages Ia (98% bs) and Ib (52% bs) of *Curvularia* sp. I, but did not separate the two lineages of *Curvularia* sp. III. The RPB2 ML tree gave 100% bs to *Curvularia* sp. II and provided enough resolution to separate lineages Ia, Ib, IIa and IIb with bs > 75%. *Curvularia* sp. Ia, Ib, IIa and IIb are morphologically and phylogenetically different from other members of the genus and therefore are proposed here as new taxa. These species were respectively named *C. muehlenbeckiae*, C. hominis, *C. americana*, C. chlamydospora and *C. pseudolunata* and described in alphabetical order in the Taxonomy section.

Within the *Curvularia* clade, six well-supported lineages were associated with certain combinations of morphological features. These lineages were named the *american*- eragrostidis-, *hominis*- lunata-, *spicifera*- and *trifoli*-clades (Fig. 1). The eragrostidis-clade (82% bs, 1 pp) was formed by *C. eragrostidis*, *C. graminicola* and *C. intermedia*, and is characterised by producing inconspicuously distoseptate (i.e. the two cell wall layers within the conidium are difficult to distinguish in mature conidia), straight to somewhat unequal-sided, 4-celled conidia, associated with certain combinations of morphological features. These species appeared

| Taxon | Isolate no. | Source | GenBank accession no. |
|-------|-------------|--------|-----------------------|
| Curvularia sp. II | UTHSC 08-3414 | Ankle, USA | HE861833 HG779056 HG565488 HG779200 |
| (= C. americana sp. nov.) | UTHSC 07-2649 | Toe tissue, USA | HE861834 HG779054 HG565486 HG779196 |
| UTHSC 08-84 | Nasal sinus, USA | HE779015 HG779069 HG779115 HG779197 |
| UTHSC 08-278 | Peritoneal dialysis fluid, USA | HE861832 HG779055 HG565487 HG779198 |
| UTHSC 08-2697 | Leg, USA | HG779016 HG779079 HG779117 HG779199 |
| UTHSC 09-2907 | Nose nail, USA | HG779017 HG779071 HG779114 HG779201 |
| UTHSC 09-2806 | Bone marrow, USA | HG779018 HG779072 HG779112 HG779202 |
| UTHSC 09-2803 | Bronchial wash, USA | HG779019 HG779073 HG779113 HG779203 |
| UTHSC 10-1276 | Maxillary sinus, USA | HG779020 HG779074 HG779116 HG779204 |
| UTHSC 08-2906 | Ear, USA | HG779021 HG779075 HG779151 HG779205 |
| UTHSC 08-1283 | Cervical lymph node, USA | HG779022 HG779076 HG779152 HG779206 |
| Curvularia sp. III Lineage A | UTHSC 07-2764 | Nose, USA | HG779023 HG779076 HG779152 HG779206 |
| (= C. chlamydospora sp. nov.) | UTHSC 08-1283 | Nasal sinus, USA | HG779024 HG779076 HG779152 HG779206 |
| Curvularia sp. III Lineage B | UTHSC 09-2092 | Nasal sinus, USA | HG779025 HG779076 HG779152 HG779206 |
| (= C. pseudolunata sp. nov.) | UTHSC 09-2092 | Nasal sinus, USA | HG779026 HG779076 HG779152 HG779206 |

Exserohilum turicum CBS 330.64 Zea mays, USA | HF934950 HF934887 HG779153 HF934852 |
The isolate number.

Species proposed in this study are shown in the coloured boxes. Ex-type and ex-neotype isolates for each species are indicated with a ‘T’ or ‘NT’, respectively.

The average number of substitutions per site. Bootstrap values ≥ 0.95 (in italics) are given near the internodes. The new species proposed in this study are shown in the coloured boxes. Ex-type and ex-neotype isolates for each species are indicated with a ‘T’ or ‘NT’, respectively, after the isolate number.

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Fig. 1 Bayesian consensus tree obtained from the combined ITS, LSU, gpd and RPB2 alignment of Curvularia and related genera. The scale bar represents the average number of substitutions per site. Bootstrap values ≥ 70 % and posterior probabilities ≥ 0.95 (in italics) are given near the internodes. The new species proposed in this study are shown in the coloured boxes. Ex-type and ex-neotype isolates for each species are indicated with a ‘T’ or ‘NT’, respectively, after the isolate number.
in Fig. 1 as two distinct species separated by relatively long branches. The hominis-clade included two new species, *C. hominis* and *C. muehlenbeckiae*. One isolate of the latter taxon, CBS 144.63, had been labelled 'C. lunata' in the CBS collection, but in this study it proved to be phylogenetically quite distant from the ex-neotype strain of that species, CBS 730.96. The lunata-clade was formed by *C. aeria*, *C. brachyspora*, *C. carica-papayae*, *C. chlamydospora*, *C. lunata*, *C. prasadii* and *C. pseudolunata*. Accentuated septa can be observed in all members of this clade and elongate blackish stromata have been reported in *C. carica-papayae* and *C. aeria* (Mathur & Mathur 1959, Ellis 1966, 1971, Sivanesan 1987). This kind of stromata is also produced by old cultures of the ex-neotype strain of *C. lunata*, CBS 730.96 (unpubl. data). Isolates of *C. chlamydospora* and *C. pseudolunata* can produce aggregates of brown chlamydospores in culture (Fig. 3k and 6i). The spicifera-clade (98 % bs, 1 pp) was formed by *C. australiensis*, *C. ellisii*, *C. hawaiensis*, *C. perotidis* and *C. spicifera*. Members of this clade produce conspicuously distoseptate conidia that are straight in all species, except in *C. ellisii* which produces both straight and curved conidia (Sivanesan 1987). Three taxa of this clade are agents of opportunistic infections in humans, i.e. *C. australiensis*, *C. hawaiensis* and *C. spicifera* (McGinnis et al. 1986, de Hoog et al. 2000). The trifoli-clade (95 % bs, 1 pp) included *C. akaii*, *C. heteropogonis*, *C. gladioli* and *C. trifolii*. These species produce 4-celled, usually curved, inconspicuously distoseptate conidia which, in contrast to those seen in the other clades discussed here, show a strongly protruding hilum (Sivanesan 1987, Boerema & Hamers 1989, Alcorn 1990). Two other species in our study produce conidia with a protruding hilum, i.e. *C. cymbopogonis* and *C. protuberata*. Their conidia, however, are 5-celled (Sivanesan 1987).

Not all *Curvularia* species were included in the six clades previously mentioned, and other well-supported lineages were
observed. *Curvularia oryzae* and *C. tuberculata*, for example, appeared as sister taxa with 99 % bs and 1 pp. These species are morphologically very different, i.e. the conidia of *C. oryzae* are 3-distoseptate and smooth while those of *C. tuberculata* are 3–8-distoseptate and tuberculate at maturity (Sivanesan 1987). We preferred not to name morphologically heterogeneous lineages because future studies including more taxa might reveal more homogeneous groupings within such lineages.

**Taxonomy**

*Curvularia americana* Da Cunha, Madrid, Gené & Cano, sp. nov. — MycoBank MB806052; Fig. 2

*Etymology.* The name refers to the continent where this species was found.

*Vegetative hyphae* septate, branched, subhyaline to brown, smooth to asperulate, 1.5–4 μm wide, anastomosing. *Conidiophores* semi- to macronematous, mononematous, septate, usually simple, slightly geniculate, subhyaline to dark brown, smooth to asperulate, with cell walls often thicker than those of the vegetative hyphae, 60–299 × 2–5 μm. *Conidiogenous cells* terminal or intercalary, polytretic, proliferating sympodially, subcylindrical to slightly swollen, 8–22 × 4–8 μm. Conidia 4(–5)-celled, straight to slightly curved, 13–28 × 7–15 μm, usually with the third cell unequally sided and larger than the others, second and third cells pale brown to brown, apical and basal cell subhyaline, apical cell smooth-walled, intermediate smooth (slightly verruculose under SEM), basal cell often verruculose; hilum non-protruding, flat, darkened and thickened, 1.5–3 μm wide. *Microconidiation* sometimes present, forming 1-celled, pale brown, globose conidia 5–6 μm wide. *Chlamydospores* not observed. *Sexual morph* not observed.

*Culture characteristics* — Colonies on OA and PCA attaining 62 and 69 mm diam, respectively, in 7 d at 25 °C, funiculose and greenish grey to dark green at the centre, effuse and greyish white towards the periphery, with a fimbriate margin; reverse olive to dark green.

![Fig. 3 Curvularia chlamydospora (a–d, h–k: CBS 136984; e–g: FMR 11040). a, b. Colonies on OA and PCA, respectively, at 25 °C after 7 d; c–g, i, j. conidiophores and conidia; h. microconidiation; k. chlamydospore. — Scale bars: c, i, k = 20 μm; d–h = 10 μm; j = 5 μm.](image-url)
Sexual morph conidia 1–2-celled, pale brown, globose to subglobose, 4–6 μm wide. Hilum non-protruding, flat, darkened and thickened, 1.5–3 μm wide, anastomosing. Vegetative hyphae septate, branched, subhyaline to brown, smooth to slightly asperulate 1.5–5 μm wide, anastomosing. Conidiogenous cells semi- to macronematous, mononematous, septate, usually simple, geniculate or bent at the apex, brown to dark brown, smooth to asperulate, 22–323 × 2–5 μm. Conidiogenous cells terminal or intercalary, polytretic, proliferating sympodially, subcylindrical to irregularly shaped, 7–18 × 5–10 μm. Conidia 4-celled, mostly slightly curved, 16–25 × 7–12 μm wide in the broadest part, smooth-walled (basal cell verruculose under SEM), usually with the central septum appearing slightly accentuated, the third cell from the base slightly larger and unequal sided, second and third cells darker than the others, brown to dark brown, end cells paler, hilum non-protruding, flat, darkened and thickened, 1.5–3 μm wide. Chlamydospores present, initially as intercalary chains but later forming clusters of swollen cells, 13–80 μm, smooth to verruculose and thick-walled. Microconidiation present, forming conidia 1–2-celled, pale brown, globose to subglobose, 4–6 μm diam. Sexual morph not observed.

Notes — Curvularia americana is similar to C. lunata and C. prasadii in conidial morphology. However, the conidia of C. lunata are slightly narrower, up to 13 μm wide (Manamgoda et al. 2012) and, in contrast to C. americana, all septa in conidia of C. prasadii are accentuated and up to 2.4 μm wide (Mathur & Mathur 1959, Ellis 1966, 1971). The phylogenetic study placed C. lunata and C. prasadii in the lunata-clade, a lineage relatively distant from C. americana. The 4-locus tree indicated that C. americana is the sister taxon of C. verruculosa, but these species were separated by a considerable genetic distance (Fig. 1). The conidia of C. verruculosa are slightly larger (20–40 × 12–17 μm) than those of C. americana and show distinctly verruculose intermediate cells (Tandon & Bilgrami 1962, Ellis 1966, 1971, Sivanesans 1987).

Curvularia chlamydospora Madrid, Da Cunha, Gené & Guarro, sp. nov. — MycoBank MB 806053, Fig. 3

Etymology. The name refers to the presence of chlamydospores.

Vegetative hyphae septate, branched, subhyaline to brown, smooth to slightly asperulate 1.5–5 μm wide, anastomosing. Conidiophores semi- to macronematous, mononematous, septate, usually simple, geniculate or bent towards the apex, subhyaline to brown, smooth to asperulate, with cell walls often thicker than those of the vegetative hyphae, 55–325 × 2–5 μm wide. Conidiogenous cells terminal or intercalary, polytretic, proliferating sympodially, subcylindrical to irregularly shaped, 6–26 × 4–9 μm; conidigenous loci usually somewhat thickened and darkened. Conidia 4–5-celled, slightly curved, 18–30 × 7–14 μm wide in the broadest part, with the third cell from the base often larger and unequal sided, intermediate cells usually verruculose and darker than the others, brown, end cells subhyaline to pale brown and smooth-walled; hilum non-protruding, flat, darkened and thickened, 1.5–3 μm wide. Microconidiation and chlamydospores were not observed. Sexual morph not observed.

Culture characteristics — Colonies on OA and PCA attaining 70–72 mm diam in 7 d at 25 °C, fuscilucose and dark green at the centre, floccose and olive to white towards the periphery, with a fimbriate margin; reverse olive to dark green.

Specimens examined. USA, Florida, culture from cornea (human), 2009, D.A. Sutton (holotype CBS H-21467, culture ex-type FMR 11539 = UTHSC 09-464 = CBS 136985); Arkansas, culture from nasal sinus (human), 2007, D.A. Sutton (FMR 11172 = UTHSC 07-3184); Louisiana, culture from eye (human), 2008, D.A. Sutton (FMR 11688 = UTHSC 08-849); Minnesota, culture from nail (human), 2007, D.A. Sutton (FMR 11698 = UTHSC 07-3581); Minnesota, culture from nasal sinus (human), 2009, D.A. Sutton (FMR 11527 = UTHSC 09-2197); Ohio, culture from nasal sinus (human), 2009, D.A. Sutton (FMR 11535 = UTHSC 09-1692); Texas, culture from nasal sinus (human), 2009, D.A. Sutton (FMR 11704 = UTHSC 07-3105); Texas, culture from nail (human), 2008, D.A. Sutton (FMR 11683 = UTHSC 08-1296); Texas, culture from bronchial wash (human), 2008, D.A. Sutton (FMR 11680 = UTHSC 08-2418); Texas, culture from bronchial wash (human), 2008, D.A. Sutton (FMR 11542 = UTHSC 08-3737); Texas, culture from foot (human), 2008, D.A. Sutton (FMR 11678 = UTHSC 08-2517); Texas, culture from naopharynx (human), 2009, D.A. Sutton (FMR 11521 = UTHSC 09-2532); Texas, culture from tissue (human), 2009, D.A. Sutton (FMR 11509 = UTHSC 09-3403); Utah, culture from cornea (human), 2007, D.A. Sutton (FMR 11708 = UTHSC 07-2791).

Notes — Although all isolates of this fungus were obtained from humans, the species might also be common in the environment. Curvularia hominis resembles other species of the genus with 4-celled conidia and an asymmetrically swollen, darkened cell, such as C. aerea, C. carica-papayae, C. lunata and C. prasadii, but differs from them in producing conidia with verruculose intermediate cells (Figs. 4e–i). The latter four species are members of the lunata-clade, whereas C. hominis and C. muehlenbeckiae form a distinct lineage, the hominis-clade (Fig. 1).
Curvularia muehlenbeckiae Madrid, Da Cunha, Gené, Guarro & Crous, sp. nov. — MycoBank MB806055; Fig. 5

**Etymology.** The name refers to the substrate from which the ex-type strain was obtained, Muehlenbeckia sp.

Vegetative hyphae septate, branched, subhyaline to brown, smooth-walled, 1.5–5 µm wide, anastomosing. Conidiophores semi- to macronematous, mononematous, septate, simple to branched, straight or flexuous, geniculate towards the apex, subhyaline to dark brown, smooth to asperulate with cell walls often thicker than those of the vegetative hyphae, 21.5–398 × 2–5 µm with subnodulose and nodulose intercalary swellings up to 9.5 µm wide, swellings coinciding with conidiogenous loci. Conidiogenous cells integrated, terminal and intercalary, subcylindrical to irregularly shaped, mono- to polytretic, proliferating sympodially; intercalary conidiogenous cells 5–18 µm long, terminal conidiogenous cells 5–25 µm long. Conidia 4-celled, asymmetrical to more or less curved at the third cell from base, 17–26 × 8.5–12 µm, intermediate cells dark brown and usually verruculose, end cells paler and smooth-walled or less ornamented than central cells. Chlamydospores and microconidiation not observed. Sexual morph not observed.

**Culture characteristics** — Colonies on OA attaining 76 mm diam in 7 d at 24 °C, cottony to funiculose, pale grey at the centre, dark olive towards the periphery, with a fimbriate margin; reverse olivaceous-black. Colonies on PCA attaining 40 mm diam at the same temperature and period of incubation, radiate, funiculose, dark olive with a slightly fimbriate margin; reverse concolorous with surface.

**Specimens examined.** INDIA, from Muehlenbeckia sp. leaf, 1962, K.S. Bilgrami (holotype CBS H-10451, culture ex-type CBS 144.63). – USA, Utah, culture from chest (human), 2008, D.A. Sutton (UTHSC 08-2905 = FMR 11671 = CBS 136986).

**Notes** — This species is the sister taxon of C. hominis, which has slightly larger conidia (18–30 × 7–14 µm) with a similar ornamentation consisting of small but conspicuous warts. Some Curvularia species outside the hominis-clade produce conidia ornamented with warts, e.g. C. tuberculata, C. verruculosa and C. verruciformis. The first two species produce larger conidia, i.e. 23–52 × 13–20 µm and 20–40 × 12–17 µm, respectively.
and the third one differs from members of the *hominis*-clade by having mostly 5-celled, more strongly ornamented conidia (Jain 1962, Agarwal & Sahni 1963, Ellis 1966, Sivanesan 1987). *Curvularia* species with warted conidia appear in different clades, suggesting that this kind of ornamentation evolved several times in *Curvularia*.

*Curvularia pseudolunata* Da Cunha, Madrid & Gené, sp. nov.
— MycoBank MB806056; Fig. 6

Etymology. The name refers to the morphological resemblance and phylogenetic closeness of this species to *Curvularia lunata*.

Vegetative hyphae septate, branched, subhyaline to brown, smooth-walled, 1.5–5 μm wide. *Conidiophores* macronematous, mononematous, septate, unbranched, geniculate near the apex, brown, smooth-walled, 100–350 × 2–4.5 μm. *Conidiogenous cells* mostly terminal, polytretic, proliferating sympodially, subcylindrical, subglobose to irregularly shaped, 4.5–30 × 6–10 μm. *Conidia* 4-celled, mostly curved, 20–27 × 8–12 μm, with the third cell from base usually unequally sided, larger and darker than the others, brown, second and end cells subhyaline to pale brown, smooth-walled, basal cell often verruculose; hilum non-protruding, flat, darkened and thickened, 1.5–2.5 μm wide. *Chlamydospores* abundant, initially as intercalary chains, later forming clusters of swollen cells, up to 60 μm diam, smooth- and thick-walled. *Microconidiation* not observed. *Sexual morph* not observed.

Fig. 5 *Curvularia muehlenbeckiae* (CBS 144.63). a, b. Colonies on OA after 7 d and on PCA after 5 d, respectively, at 25 °C; c–h. conidiophores and conidia. — Scale bars: c–g = 10 μm; h = 5 μm.
Culture characteristics — Colonies on OA attaining 71 mm diam in 7 d at 25 °C cottony to lanose, greenish grey, with a fimbriate margin; reverse dark green. Colonies on PCA attaining 78 mm diam at the same temperature and time of incubation, lanose at the centre, floccose towards the periphery, greyish green, with a fimbriate margin; reverse olive green.

Specimen examined. USA, California, culture from nasal sinus (human), 2009, D.A. Sutton (holotype CBS H-21468, cultures ex-type FMR 11529 = UTHSC 09-2092 = CBS 136987).

Notes — Curvularia pseudolunata is morphologically similar to C. lunata and these taxa grouped together in the 4-locus phylogeny (Fig. 1). However, the conidia of C. lunata are slightly larger (21–31 × 9–13 µm) and this species is separated from C. pseudolunata by a considerable genetic distance.

DISCUSSION

Traditionally, Curvularia and Bipolaris have been distinguished by conidial features, i.e. euseptate and typically curved at a swollen intermediate cell in Curvularia, but straight to slightly curved and distinctly distoseptate in Bipolaris (Kwon-Chung & Bennett 1992, de Hoog et al. 2000, Revankar & Sutton 2010). We agree with the view of authors like Alcorn (1983b), Sivanesan (1987) and Seifert et al. (2011) that both genera have distoseptate conidia. Phylogenetic studies (Berbee et al. 1999, Manamgoda et al. 2012) have demonstrated that species with conspicuously distoseptate conidia previously placed in Bipolaris actually belong in Curvularia, e.g. members of the spicifera-clade (Fig. 1). Furthermore, in the new species described herein, two wall layers were often evident in young conidia (Fig. 2f, 4d, 5f) and septa are already visible at this stage; however, in mature conidia the layers may appear so close to one another that the conidia may look euseptate under the light microscope (Fig. 2g, 4i, 5e). A recently described pleosporalean genus, Porocercospora, the causal agent of buffalograss false-smut disease, also shows two-layered conidial cell walls, but mature conidia often seem to have both eu- and distosepta, depending on how closely together the two cell wall layers cohere near the septa. This genus is phylogenetically closely related to Bipolaris and Curvularia and is similar to them in having tretic conidiogenesis and darkly pigmented mycelium. However, Porocercospora has conidiospores without a geniculate rachis, conidiogenous cells with inconspicuous, non-darkened conidiogenous loci and long, obclavate to cylindro-obclavate conidia (Amaradasa et al. 2014).

Although Bipolaris and Curvularia cannot be distinguished based on the morphology of their conidial septa, other morphological features seem to be of diagnostic value. None of the species of Bipolaris s.str. included in this study (Fig. 1) and in previous works (Berbee et al. 1999, Manamgoda et al. 2012) has conidia curved at an intermediate swollen cell. As described by Berbee et al. (1999) for “Cochliobolus group 1”, the conidia of Bipolaris s.str. can show a gentle curve that continues along the whole length of the conidium. Conidia ornamented with small to coarse warts are produced by some Curvularia species, e.g., C. tuberculata, C. verruciformis and C. verruculosa, but this kind of ornamentation has not been reported in Bipolaris s.str. (Jain 1962, Tandon & Bilgrami 1962, Agarwal & Sahni 1963, Ellis 1966, Sivanesan 1987). Another helpful character is the morphology of the hilum. None of the species in Bipolaris s.str. has conidia with a strongly protruding hilum, but it is observed in several members of Curvularia s.str., such as C. cymbopogonis and C. protuberata, as well as all species in the trifolii-clade (Sivanesan 1987). A protruding hilum is also observed in a closely related genus, Exserohilum, which also includes clinically relevant and plant-pathogenic species (McGinnis et al. 1986, de Hoog et al. 2000). Members of this genus sometimes form curved conidia, but the hilum is different from those seen in Curvularia spp. In Exserohilum...
the hilum appears as a protrusion of the cell wall that is not delimited by a septum and that often appears double-walled, with the outer wall forming an enveloping collar of 'hilar bubble' around it (Alcorn 1983b, 1988). In Curvularia, by contrast, when the hilum protrudes, it appears single-walled in light microscopy and is delimited by a septum (Nelson & Hodges 1965, Sivanesan 1987, Zang et al. 2004). Conidial size might also be helpful to distinguish Bipolaris s.str. from Curvularia s.str. Among the species falling in the Bipolaris clade in Berbee et al. (1999) and Manamgoda et al. (2012), the longest conidia are those of B. zeae, up to 225 μm long (Sivanesan 1987). Conidia of Curvularia s.str. tend to be shorter. Among species in the Curvularia clade (Berbee et al. 1999, Manamgoda et al. 2012), the longest conidia are produced by C. tripogonis and are up to 130 μm long (Sivanesan 1987).

Boedijn (1933) divided Curvularia into three groups of species, i.e. groups Maculans, Lunata and Geniculata. The Maculans group was characterised by producing 4-celled, straight or somewhat asymmetrical conidia with the central cells darker and larger than the end cells. This group included C. maculans (currently considered a synonym of C. eragrostidis), C. cesatii (this species was transferred to the genus Endophragmiella as E. cesatii by Hughes in 1979), C. intermedia and C. spicifera, all of which were unable to produce stromata in culture. The Lunata group included species with 4-celled, more or less curved conidia in which one of the intermediate cells is enlarged and darker than the others. Some of its members were C. lunata, C. ramosa and C. trifolii. This group was reported to produce subcylindrical stromata in culture. The Geniculata group was proposed for species with 5-celled conidia which often produced stromata, such as C. geniculata, C. affinis, C. fallax and C. falcata (this species was synonymized with C. senegalensis by Sivanesan in 1987). In our phylogenetic study, three species of Boedijn’s Maculans group were included, i.e. C. eragrostidis, C. intermedia and C. spicifera. The group is polyphyletic since only the former two species grouped together in the eragrostidis-clade, a lineage characterised by rather straight, inconspicuously distoseptate, 4-celled conidia. This lineage also included C. graminicola (Fig. 1). Curvularia spicifera clustered in a different clade with other species whose conidia show evident distosepta. No DNA sequences have as yet been analysed for Endophragmiella cesatii (this species was transferred to the genus Endophragmiella currently considered a synonym of Curvularia as yet been analysed for Endophragmiella cesatii (this species was transferred to the genus Endophragmiella as currently considered a synonym of Curvularia as yet been analysed for Endophragmiella cesatii (this species was transferred to the genus Endophragmiella as currently considered a synonym of Curvularia as yet been analysed for Endophragmiella cesatii (this species was transferred to the genus Endophragmiella as currently considered a synonym of Curvularia as yet been analysed for Endophragmiella cesatii (this species was transferred to the genus Endophragmiella as currently considered a synonym of Curvularia anemone. Proceeding of the Royal Society of Queensland 107: 1-4.

Among the 20 species falling in the Bipolaris clade in Berbee et al. (1999); this clade (Fig. 1) revealed more than double the percentage of variable sites as those of Bipolaris clade (Berbee et al. 1999, Manamgoda et al. 2012), in which an ITS tree gave <70% bs for clades representing C. spicifera and C. hawaiiensis, two of the main clinically relevant members of the genus. Other authors have found limited species resolution in ITS phylogenies of other members of the Pleosporales (Pryor & Gilbertson 2000, de Hoog & Horré 2002, Pryor & Bigelow 2003, Park et al. 2008, Brun et al. 2013), indicating that additional genes need to be used for reliable species identification in this group of fungi. Protein-coding loci have been reported to be phylogenetically more informative than rDNA in Ascomycota (Schoch et al. 2009) and this is confirmed here in Curvularia. In our work, species discrimination improved with the gpd and RPB2 loci, which revealed more than double the percentage of variable sites seen in ITS. These protein-coding loci are promising markers for future phylogenetic studies in Curvularia and related genera.

Acknowledgements We thank the technical staff, Arien van Iperen (cultures) and Janneke Bloem (DNA isolation, amplification and sequencing of some of the isolates studied) for their invaluable assistance. The study has been economically supported in part by the Spanish Ministry of ‘Economía y Competitividad’, grant CGL 2011-27165.

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