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STUDIES IN MYCOLOGY 50: 323–335. 2004.

Paraconiothyrium, a new genus to accommodate the mycoparasite *Coniothyrium minitans*, anamorphs of *Paraphaeosphaeria*, and four new species

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Abstract: *Coniothyrium*-like coelomycetes are drawing attention as biological control agents, potential bioremediators, and producers of antibiotics. Four genera are currently used to classify such anamorphs, namely, *Coniothyrium*, *Microsphaeropsis*, *Cyclothyrium*, and *Cytoplea*. The morphological plasticity of these fungi, however, makes it difficult to ascertain their best generic disposition in many cases. A new genus, *Paraconiothyrium* is here proposed to accommodate four new species, *P. estuarinum*, *P. brasiliense*, *P. cyclothyrioides*, and *P. fungicola*. Their formal descriptions are based on anamorphic characters as seen *in vitro*. The teleomorphs of these species are unknown, but maximum parsimony analysis of ITS and partial SSU nrDNA sequences showed that they belong in the *Pleosporales* and group in a clade including *Paraphaeosphaeria s. str.*, the biocontrol agent *Coniothyrium minitans*, and the ubiquitous soil fungus *Coniothyrium sporulosum*. *Coniothyrium minitans* and *C. sporulosum* are therefore also combined into the genus *Paraconiothyrium*. The anamorphs of *Paraphaeosphaeria michotii* and *Paraphaeosphaeria pilleata* are regarded representative of *Paraconiothyrium*, but remain formally unnamed. *Paraconiothyrium* species are phylogenetically distant from typical members of the other coelomycete genera mentioned above.

Taxonomic novelties: *Paraconiothyrium* Verkley gen. nov., *Paraconiothyrium brasiliense* Verkley sp. nov., *Paraconiothyrium cyclothyrioides* Verkley sp. nov., *Paraconiothyrium estuarinum* Verkley & M. da Silva sp. nov., *Paraconiothyrium fungicola* Verkley & Wicklow sp. nov., *Paraconiothyrium minitans* (W.A. Campb.) Verkley comb. nov., *Paraconiothyrium sporulosum* (W. Gams & Domsch) Verkley comb. nov.
Key-words: Anamorph, biological control, bioremediation, *Cyclothyrium*, *Cytoplea*, fungicolous fungus, *Microsphaeropsis*, molecular systematics, nuclear ribosomal DNA.

INTRODUCTION

Coniothyrium- or *Microsphaeropsis*-like coelomycetes are widely dispersed and commonly isolated from many different habitats. Recently, these fungi have drawn attention as biological control agents (Carisse, El Bassam & Benhamou 2001, Carisse & Bernier 2002a, b, El Bassam *et al.* 2002), potential bioremediators (da Silva *et al.* 2003a, b), and producers of antibiotics (Fukami *et al.* 2000, Seephonkai *et al.* 2002, Tsuda *et al.* 2003). In the genus *Coniothyrium* Corda alone, many hundreds of species have been described on the basis of material found on plants, and most of these species have never been critically re-examined or studied in culture. Their morphology is relatively simple and provides few diagnostic characters, and the taxonomy has been primarily based on the host. Occasionally, species have been described from organisms other than plants or from soil. In this paper, we present four new species, which were preliminarily identified as either *Microsphaeropsis* sp. or *Cyclothyrium* sp. (a *Coniothyrium*-like genus with stromatic fruiting bodies accepted by Sutton 1980). They were each isolated from a single source, one from a heavily polluted estuarine sediment, and the others from basidiocarps of a polypore fungus, a soil sample, and fruit of coffee plants, respectively. No teleomorphs were observed, and since the fructifications in nature are unknown, the species had to be described on the basis of their anamorphic characters as seen *in vitro*. The sequences of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA were found to diverge, and morphological differences that were observed in the fructifications support the idea that the isolates belong to four distinct species.

The generic disposition of these new species posed difficulties. Sutton (1980) upheld four genera for the classification of *Coniothyrium*-like coelomycetes. These taxa, *Coniothyrium*, *Microsphaeropsis* Sacc., *Cyclothyrium* Petr., and *Cytoplea* Bizz. & Sacc., were based on conidiomatal structure, conidiogenesis and conidium morphology. The morphological plasticity of coelomycetes, however, often makes it difficult to determine the most appropriate generic disposition for newly recognized species. In order to gather more reliable information about the affinities of the new species and about *Coniothyrium palmarum* Corda, *Microsphaeropsis olivacea* (Bonord.) Höhn., and *Cyclothyrium juglandis* (Schum.) B. Sutton, the type species of *Coniothyrium*, *Microsphaeropsis*, and *Cyclothyrium*, respectively, we sequenced a part of the nuclear small subunit (SSU, 18S). So far, *Coniothyrium*-like anamorphs have been reported for genera in the *Pleosporales*, viz., *Leptosphaeria* Ces. & De Not., and *Paraphaeosphaeria* O.E. Erikss., as well as recently erected segregates of *Paraphaeosphaeria*, viz., *Neophaeosphaeria* Câmara, M.E. Palm & A.W. Ramaley and *Phaeosphaeriopsis* Câmara, M.E. Palm & A.W. Ramaley (Câmara *et al.* 2001, 2003). Phylogenetic analyses are placing some *Coniothyrium*-like coelomycetes in other orders, e.g., in the *Mycosphaerella*-clade of the *Dothideales*, and in the *Diaporthales* grouping closely to *Cryphonectria* (Sacc.) Sacc. & D. Sacc. and *Endothia* Fr. (Lennox *et al.*, this volume).

The molecular work presented here shows that the new species group in a clade with *Paraphaeosphaeria s. str.* (Câmara *et al.* 2001, 2003), and with *Coniothyrium minitans* W.A. Campb. and *Coniothyrium sporulosum* (W. Gams & Domsch) van der Aa, while the type species of *Coniothyrium* and *Microsphaeropsis* reside in different clades, and that of *Cyclothyrium* is also remote. Muthumeenakshi *et al.* (2001) demonstrated the close relationship between *C. minitans* and *C. sporulosum* on the basis of ITS sequence analyses. *Coniothyrium minitans*, a mycoparasite of world-wide distribution, has been intensively studied and successfully applied as a control agent against the economically important pathogen *Sclerotinia sclerotiorum* (Campbell 1947, Whipps & Gerlagh 1992, Sandys-Winsch *et al.* 1993, Goldstein *et al.* 2000, Grendene *et al.* 2002). From the phylogenetic point of view, none of the available generic names can be used for these fungi. We therefore propose to place them in a new genus, *Paraconiothyrium*.

MATERIAL AND METHODS

Isolations

The strain CBS 972.95 was isolated with a standard dilution plate method, using oatmeal (OA) and Czapek agars (Gams et al. 1998). CBS 109850 was isolated as described by da Silva *et al.* (2003a), and CBS 113269 as described by Holler *et al.* (2002). No data are available pertaining to the isolation of CBS 100299.

DNA extraction and sequencing

Strains were transferred from agar cultures to 2 mL liquid medium (2 % malt extract) and incubated on a rotary shaker (300 rpm) for 3 wk at room temperature. Liquid cultures were transferred to 2 mL tubes, centrifuged and washed twice with sterile water. DNA was extracted using the FastDNAkit (Omnilabo 6050073, BIO 101, CA) according to the manufacturer's instructions. For ITS sequence analysis a part of the ribosomal RNA gene cluster was amplified by PCR using primer pairs V9G (De Hoog and Gerrits van den Ende 1998) and LR5 (Vilgalys and Hester 1990). Part of the SSU was amplified using primers NS1 and NS24 (White *et al.* 1990), or primer pairs NS1/Oli1, NS3/Oli2, and NS5/NS24 (Hendriks *et al.* 1989, White *et al.* 1990, Hopfer *et al.* 1993). PCR was performed in 50 µL reaction volumes, each reaction containing 10–100 ng of genomic DNA, 25 pM of each primer, 40 µM dNTP, 1 unit Supertaq DNA polymerase and 5 µL 10× PCR buffer (SphaeroQ, Leiden, the Netherlands). PCR was performed in an Applied Biosystems (Foster City, CA) thermocycler with the following programme: 1 min 95 ºC, 30× (1 min 95 ºC, 1 min 55 ºC, 2 min 72 ºC) followed by a final extension of 5 min at 72 ºC PCR products were cleaned with GFX columns (Amersham Pharmacia, NJ, 27-9602-01) and analyzed on a 2 % agarose gel to estimate concentrations. ITS1 and ITS4 (White *et al.* 1990) were used as internal sequencing primers for the ITS region. The SSU region was sequenced using the PCR primers. Sequencing was performed with the BigDye terminator chemistry (Applied Biosystems) following the manufacturer's instructions. The sequencing products were cleaned with G50 Superfine Sephadex columns (Amersham Pharmacia 17-0041-01), and separated and analyzed in ABI Prism 3700 DNA Analyzer (Applied Biosystems). Forward and reverse sequences were matched using SeqMan (DNAstar Inc., WI).

Phylogenetic analyses

Pairwise and global alignments of consensus sequences of the ITS region and partial SSU of the nuclear ribosomal RNA gene array were performed in Bionumerics 3.0 (Applied Maths, Kortrijk, Belgium). Where necessary manual adjustments were made. Parsimony analysis was done with the heuristic search option in PAUP v. 4.0b10 (Swofford 2002), with the following parameter settings: characters unordered with equal weight, random taxon addition, branch swapping with tree bisection-reconnection (TBR) algorithm, branches collapsing if the maximum branch length was zero, maxtrees set at 10 000. Alignment gaps were treated as missing characters in the analysis of the ITS dataset, and as fifth base in the SSU dataset, where they occurred in relatively conserved regions. Parsimony bootstrap analyses were performed using the full heuristic search option, random stepwise addition, and 1000 replicates, with maxtrees set at 100.

BLAST searches in GenBank with SSU sequences of the newly described species revealed highest similarity to *Letendraea helminthicola* (Berk. & Broome) Weese, *Bimuria novae-zelandiae* D. Hawksw., Chea & Sheridan, *Helminthosporium* spp. and *Paraphaeosphaeria* spp. Additional Pleosporalean taxa were found in BLAST searches using the SSU sequences of the following type species of relevant genera, *Microsphaeropsis olivacea*, *Coniothyrium palmarum*, and *Cyclothyrium juglandis*, and these were also added to the SSU dataset. The range of species selected for the SSU dataset was too diverse for alignment of the ITS region. BLAST searches with the ITS sequences of the newly described species revealed highest similarity to species of *Paraphaeosphaeria*, some *Coniothyrium* spp., and also *Leptosphaeria bicolor*, *L. taiwanensis*, and *Helminthosporium* spp. In total 23 sequences were included in the ITS dataset. Unambiguous alignment for all sequences was only possible for the 5.8 S gene and most of ITS 2, and the initial analysis was based on those genes only, using *Massarina lacustris* (AF250831) as outgroup. In a second analysis, the complete ITS region was included for taxa in the *Paraconiothyrium/Paraphaeosphaeria* clade, using *Helminthosporium velutinum* Link as outgroup (15 sequences). GenBank accession numbers and corresponding taxon names are given in Figs 1 and 2. GenBank accession numbers of sequences generated in this study are given in Table 1. A strain of *Helminthosporium velutinum* was defined as outgroup for the ITS dataset, while a sequence of *Peziza echinospora* P. Karst. (as *P. sylvestris* in Harrington *et al.* 1999) was used as outgroup for the SSU dataset. The alignments and trees were lodged in TreeBase (accession SN2133).

Culture studies and morphological analyses

The strains were studied on OA, 3 % malt extract (MEA, Oxoid), and cornmeal (CMA), and potato-dextrose (PDA) agars. Media were prepared according to Gams *et al.* (1998). Plates were incubated in the laboratory in diffuse daylight (ddl, 20 ºC), or in an incubator under n-UV light (12 h light, 12 h dark) at 15 ºC. Growth characteristics were studied on MEA in the dark in series of incubators set at different temperatures (range 6–36 ºC, 3º intervals). Colony diameter in cultural descriptions was measured at 20 ºC. Colours were described according to Rayner (1970). Sporulating structures were mounted in water and examined microscopically. Digital images were recorded with Nikon Coolpix 995.

RESULTS

Phylogenetic analyses

ITS sequences: The alignment of the ITS region comprised in total 572 characters. In the initial analysis, 37 characters within insertions/deletions or with ambiguous position homology were excluded from this analysis, as were all further constant characters, so that in total 64 (11 %) characters were included. The heuristic search resulted in two most parsimonious trees (MPT) of 107 steps (consistency index (CI) = 0.785, redundancy index (RI) = 0.924, rescaled consistency index (RC) = 0.725, homoplasy index (HI) = 0.215), one of which is depicted in Fig. 1. The other MPT was identical with the strict consensus tree, and only differed from the tree in Fig. 1 in that *Paraphaeosphaeria pilleata* Kohlm., Volkm.-Kohlm. & O.E. Erikss. and its sister group collapsed into a trichotomy. Bootstrap supports over 50 % are indicated in the tree above the branches. The included *Coniothyrium* species, the newly described *Paraconiothyrium* species, and *Paraphaeosphaeria* species grouped in a well-supported clade (97 %). Within this *Paraconiothyrium/Paraphaeosphaeria*-clade, three main groups were found. The first group comprised two strains of *Coniothyrium sporulosum* and CBS 132.26, identified as *Coniothyrium fuckelii* Sacc., as well as AK9629 *Coniothyrium* sp., and was supported in 87 % of the replications. There was 100 % ITS sequence homology among these four strains, which most likely all belong to *C. sporulosum*. A bootstrap support of 88 % was obtained for a group containing three *Coniothyrium minitans* strains which all had identical sequences. CBS 972.95 and N119 ‘*Paraphaeosphaeria* sp.’ differed from each other by two base positions, and grouped together with 80 % support. CBS 109850 was the closest sister taxon to these two strains, and together they formed a highly supported clade (86 %). CBS 109850 differed by 5 base positions from CBS 972.95 and N119. *Paraphaeosphaeria michotii* (Westend.) O.E. Erikss. and *Paraphaeosphaeria pilleata* did not group together in a single clade. Two isolates with very distinct ITS sequences, CBS 113269 and 100299, did not group closely with the other taxa studied. For example, CBS 113269 differed from CBS 972.95 at 38 base positions, and from CBS 100299 at 37 base positions (plus at an eight-base insertion in the ITS1 region of CBS 100299). The *Leptosphaeria* and *Helminthosporium* spp. grouped in a single sister-clade with maximal bootstrap support (100 %). In the second analysis, the entire ITS region was included for 15 taxa. The internal topology of the *Paraconiothyrium/Paraphaeosphaeria* clade remained unresolved, but the main groups were the same as in the first analysis. The bootstrap values over 50 % obtained in the second analysis are indicated below the branches of the same groups in Fig. 1.

SSU sequences: The alignment of the SSU of 58 taxa comprised 1727 characters. In the maximum parsimony analysis, 175 (10 %) informative characters were included, while constant and autapomorphic characters were excluded. The heuristic search yielded 1821 MPT’s of 428 steps (CI = 0.523, RI = 0.851, RC = 0.445, HI = 0.477). The strict consensus tree with bootstrap values of the clades over 50 % is given in Fig. 2.

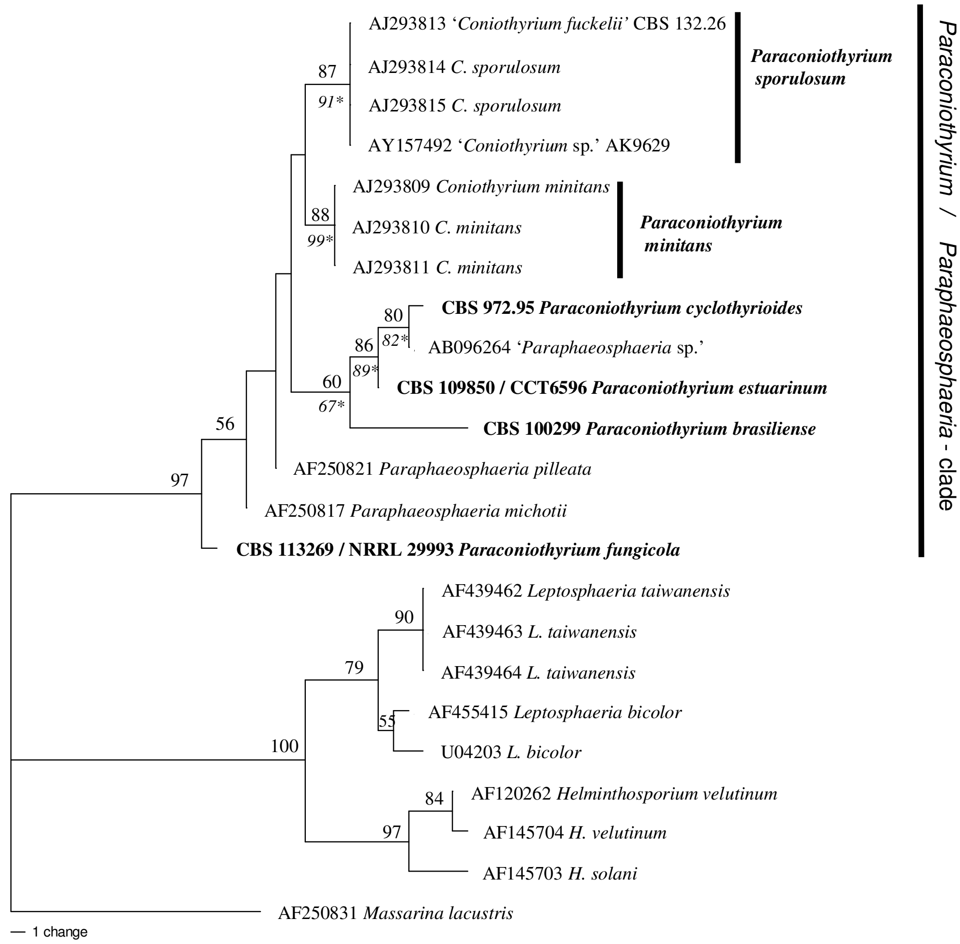


Fig. 1. One of two most parsimonious trees (MPT) of 107 steps (consistency index (CI) = 0.785, redundancy index (RI) = 0.924, rescaled consistency index (RC) = 0.725, homoplasy index (HI) = 0.215), obtained in PAUP using a heuristic search of the 5.8 nrDNA- ITS2 region. Numbers above the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50 %. Numbers with asterisk below the branches in the *Paraconiothyrium/Paraphaeosphaeria* clade are bootstrap values (1000 replications) obtained in a heuristic search of ITS1-5.8S-ITS2, in which only the taxa of this clade were included, using *Helminthosporium velutinum* (AF145704) as outgroup (see text).

All four new *Paraconiothyrium* species grouped with *Coniothyrium minitans* and *Paraphaeosphaeria michotii* and *Paraphaeosphaeria pilleata* in a *Paraconiothyrium/Paraphaeosphaeria* clade with 99 % bootstrap support. Their sequences were almost 100 % homologous. These taxa were nested within a highly supported clade (98 %) with *Letendraea helminthicola*, *Bimuria novae-zelandiae*, and the *Helminthosporium* species. A *Microsphaeropsis* clade could also be identified (96 %), comprising *M. olivacea* strains (CBS 401.81, 442.83), representing the type species of the genus *Microsphaeropsis*, and two mutually morphologically indistinguishable strains of *Coniothyrium insitivum* Sacc., CBS 157.37 and 100453. These *C. insitivum* isolates may actually represent two different taxa, as both SSU and ITS sequences showed differences (at 4 and 12 base positions, respectively). The strains representing *Coniothyrium palmarum*, the type species of the genus *Coniothyrium*, CBS 400.71 and 758.73, had 100 % identical ITS and SSU sequences, and grouped together with pleosporalean taxa considered to belong to various families (*Pleosporaceae*, *Phaeosphaeriaceae*, *Melanommataceae*, *Leptosphaeriaceae*). CBS 194.49 is a sterile strain that was originally identified as *Thyridaria rubronotata* (Berk. & Br.) Sacc., but most likely is a species of *Neophaeosphaeria* or a closely related genus. *Thyridaria rubronotata* (anamorph: *Cyclothyrium juglandis*, type species of the genus *Cyclothyrium*) strains CBS 385.39 (SSU) and 419.85, of which the ITS sequences were identical (no SSU sequence available for 419.85) also fall within the *Pleosporales*. The *Pleosporales* clade obtained maximal bootstrap support (100 %) in our analysis, but the SSU sequence of CBS 385.39 was incomplete.

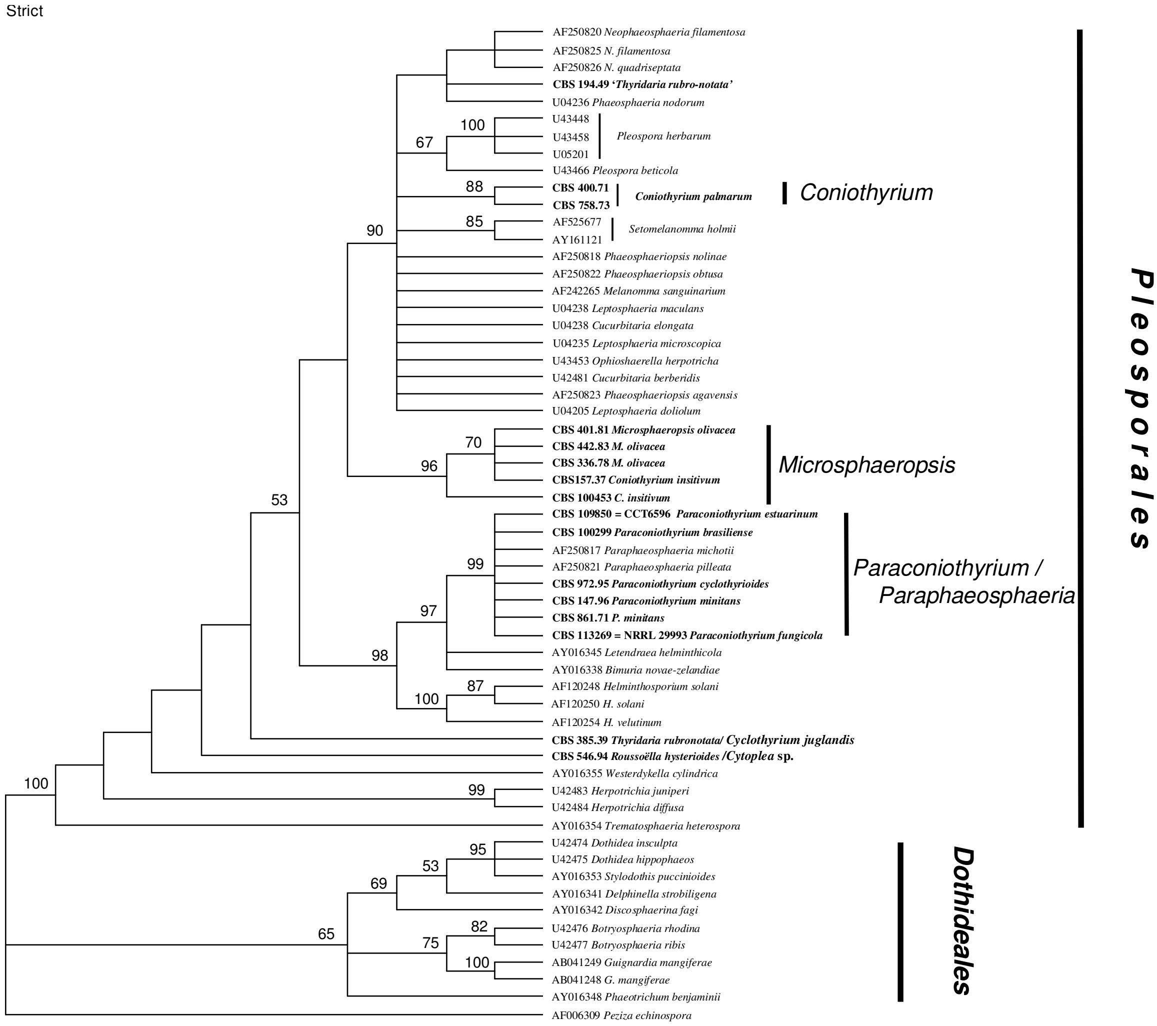


Fig. 2. Strict consensus tree of 1681 MPT's of 428 steps (CI = 0.523, RI = 0.851, RC = 0.445, HI = 0.477), obtained in PAUP using a heuristic search of partial SSU nrDNA. Numbers at the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50 %.

The lower internal nodes of the *Pleosporales* clade were not well-supported. Analysis of an alignment of 5.8 S rDNA and adjacent part of ITS 2 (no tree shown) confirmed the position of *Thyridaria/Cyclothyrium* as found in the analyses of the SSU. None of the *Coniothyrium*-like taxa investigated here showed affinities to any of the *Dothideales* included in the analysis.

Taxonomic part

Paraconiothyrium Verkley, **anam. gen. nov.** MycoBank MB500080.

Conidiomata eustromatica, simplicia vel complexa, raro pycnidialia, *cellulae conidiogenae* discretae vel integratae, phialidicae, raro semel ad ter percurrentes; *conidia* aseptata, interdum uniseptata, tempore liberationis hyalina, deinde fusca, glabra vel minute asperata, teleomorphosis *Paraphaeosphaeriae*.
Typus: *Paraconiothyrium estuarinum* Verkley & M. da Silva sp. nov.

Conidiomata eustromatic, simple or complex, rarely pycnidial, *conidiogenous cells* discrete or integrated, phialidic, sometimes percurrent, *conidia* aseptate, sometimes 1-septate, thin-walled, smooth-walled or minutely warted, hyaline when liberated, later brown, teleomorph in the genus *Paraphaeosphaeria*.

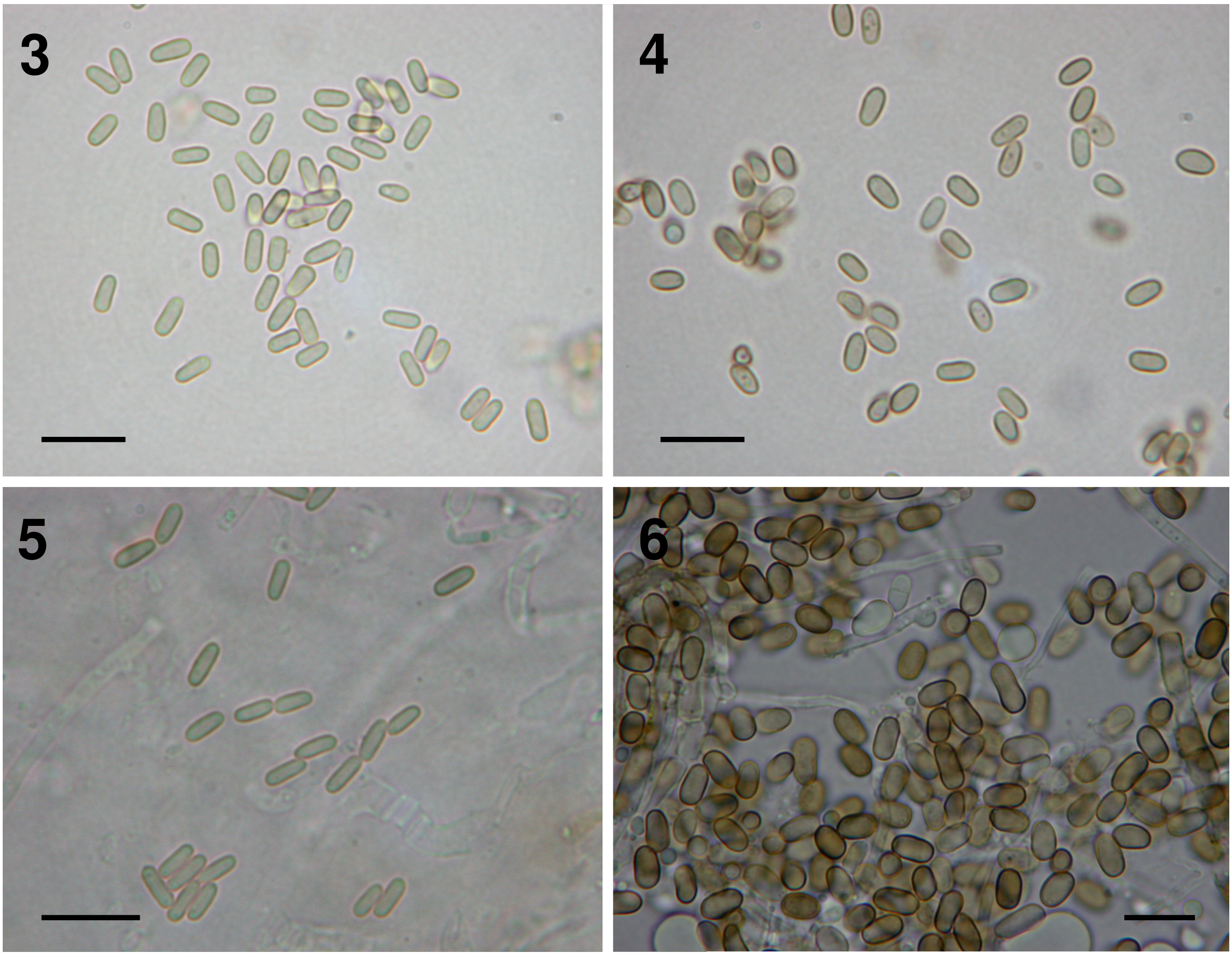
Paraconiothyrium estuarinum Verkley & M. da Silva, **sp. nov.** MycoBank MB500081. Figs 3, 7, 8.

Conidiomata eustromatica, 0.2–0.5(–1) mm diametro. *Cellulae conidiogenae* hyalinae, phialidicae, raro semel percurrentes, 4–6.5 × 2.5–3.5(–4) µm. *Conidia* anguste ellipsoidea vel breviter cylindrica, hyalina, continua, tempore liberationis hyalina, deinde olivacea vel luteo-fusca, (3–)3.2–4(–6) × 1.4–1.7(–2) µm (agaro 'oatmeal').

Conidiomata mostly submerged in the agar, but also superficial and in the aerial mycelium, eustromatic, globose or flattened, dark brown to black, 0.2–0.5(–1) mm diam, with several merging cavities, ostioles absent, opening by dissolution of upper cells; *co-*

nidiomatal wall composed of a 30–45 µm thick outer layer of isodiametric or more flattened cells with hyaline to reddish brown walls thickened up to 1.5 µm, lined by a 35–60(–75) µm thick inner layer of *textura angularis* with cells 3–10 µm diam with hyaline walls thickened up to 0.5 µm. The surface of the conidiomatal wall often covered under brown entangling hyphae. *Conidiogenous cells* discrete, assembled into protruding masses of cells, or integrated in very compact conidiophores, ampulliform to subcylindrical, hyaline, indeterminate, phialidic with an inconspicuous periclinal thickening and collarette, later often with a single percurrent proliferation, mostly 4–6.5 × 2.5–3.5(–4) µm. *Conidia* narrowly ellipsoidal or short-cylindrical, straight or slightly curved, rounded at both ends, 1-celled, with one or two small, polar guttules, and with thin and smooth walls that are hyaline at secession, but soon become olivaceous- or yellowish brown, on OA (3–)3.2–4(–6) × 1.4–1.7(–2) µm, on MEA (3–)3.2–4.2(–5.8) × 1.4–2 (–2.2) µm (all in diffuse daylight).

Cultural characteristics: *Colonies* on OA reaching 90 mm diam within 14 d, spreading, with an even, glabrous, colourless margin; immersed mycelium becoming pale mouse-grey, later darkening to olivaceous, the surface with a diffuse to fairly dense mat of finely felted to woolly-floccose aerial mycelium, which is also greyish and near the margin almost pure white, but later becomes olivaceous-buff throughout the colony surface; reverse mouse-grey, in the centre becoming olivaceous-black; complex conidiomata developing from the centre in radiating rows or in a more scattered pattern after 5–7 d.



Figs 3–6. Conidia from oatmeal agar cultures. 3**.** *Paraconiothyrium estuarinum*, CBS 109850. 4. *P. brasiliense*, CBS 100299. 5. *P. cyclothyrioides*, CBS 972.95**.** 6. *P. fungicola*, CBS 113269. Scale bars = 10 µm

Colonies on CMA reaching 90 mm diam within 14 d, spreading, with an even, glabrous, colourless margin; immersed mycelium olivaceous-grey to grey-olivaceous, aerial mycelium as on OA; reverse olivaceous-grey to olivaceous-black; scarce, scattered simple to complex conidiomata which are similar in structure as those on OA developing from 5–7 d..

Colonies on MEA reaching 80 mm diam in 14 d, spreading, with an even, colourless to buff, almost glabrous margin; colony surface almost entirely covered by a dense mat of woolly aerial mycelium, which is pale olivaceous-grey to olivaceous-grey, in the centre olivaceous-black, and near the margin paler to almost pure white; reverse in the centre mostly chestnut to sepia, surrounded by umber, cinnamon and ochreous areas or zones; Conidiomata as on OA

Growth characteristics: Optimum 27 ºC, maximum 33 ºC.

Holotype: **Brazil**, São Paulo State, Cubatão, Piaçaguera River, isolated from an estuarine sediment polluted with industrial discharges, isolate da Silva CCT6596 = CBS 109850, living culture; Herbarium CBS H-10528, dried culture on oatmeal agar, **holotype**; also kept metabolically inactive in frozen and dried state.

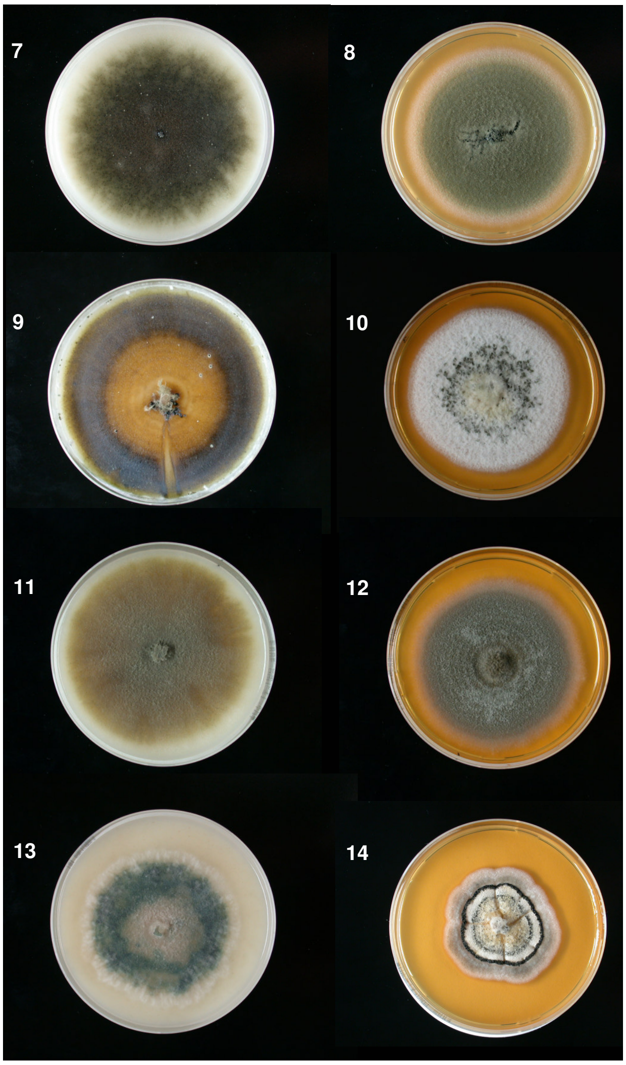
Additional strains examined: Brazil, São Paulo State, Cubatão, Piaçaguera River, isolated from an estuarine sediment polluted with industrial discharges, da Silva INCQS 40202, 40203, 40204, 40205, 40206, 40207.

Notes: In screening for ability to degrade polycyclic aromatic hydrocarbons, the type strain proved a potent bioremediator, degrading phenanthrene, pyrene, anthracene and benzo[a]pyrene in relatively high levels (da Silva *et al.* 2003 b, 2004).

Paraconiothyrium brasiliense Verkley, **sp. nov.** MycoBank MB500082. Figs 4, 9, 10.

Conidiomata eustromatica, simplicia, vulgo complexa, (0.2–)0.5–2(–3) mm diametro. *Cellulae conidiogenae* hyalinae vel pallide luteae, phialidicae, 4–6 × 3.5–5 µm. *Conidia* ellipsoidea vel breviter cylindrica, in agaro 'MEA' interdum obpyriformia, continua, tempore liberationis hyalina, deinde olivacea, (3–)3.4–4.6(–5) × (1.8–)2–2.3(–2.5) µm (agaro 'oatmeal').

Conidiomata superficial or immersed in the agar, eustromatic, dark brown to black, (0.2–)0.5–2 (–3) mm diam, with a single cavity, more often complex with several merging cavities, ostioles absent, opening by dissolution of upper cells; *conidiomatal wall* composed of a 10–20(–25) µm thick outer layer of texture angularis with relatively thin, dark brown walls, the cells 4.5–10 µm diam, lined by a 10–35(–45) µm thick inner layer of *textura angularis–globulosa*, the cells 4–12 µm diam with thin, pale yellow to hyaline walls; surface of the conidiomatal wall covered by 2–3 µm wide hyphae with dark-brown, smooth walls. *Conidiogenous cells* discrete or assembled into protruding masses, indeterminate, phialidic, formed from the inner cells all over the conidiomatal wall, hyaline to pale yellow, broadly ampulliform to globose, with distinct periclinal thickening, collarette absent, 4–6 × 3.5–5 µm; *conidia* ellipsoid to short-cylindrical, rounded at both ends, on CMA and MEA also obpyriform (narrowing towards the base), 1-celled, with thin and smooth walls that are hyaline at secession, but soon become olivaceous, contents minutely granular or with a few small polar guttules, conidial mass dark brown to black; conidia on CMA (3.2–)3.4–4.6(–5.3) × (2–)2.2–3(–3.6);



Figs 7–14. Cultures on oatmeal (OA) and malt extract agar (MEA) (diffuse daylight, unless indicated otherwise). **7, 8.** *P. estuarinum*. **7.** CBS 109850, 14 d old culture on OA; **8.** CBS 109850, 14 d old culture on MEA. **9, 10.** *P. brasiliense*. **9.** CBS 100299, 19 d old culture on OA. **10.** CBS 100299, 14 d old culture on MEA. **11, 12.** *P. cyclothyrioides*. **11.** CBS 972.95, 14 d old culture on OA. **12.** CBS 972.95, 14 d old culture on MEA. **13, 14.** *P. fungicola*. **13.** CBS 113269, 21 d old culture on OA. **14.** CBS 113269, 7 wk old culture on MEA grown under 12 hrs nUV (12 hrs dark).

on OA (3–)3.4–4.6(–5) × (1.8–)2–2.3(–2.5) µm, on MEA (2.8–)3.2–4(–4.5) × (1.8–)2–2.4(–3) (all diffuse daylight).

Cultural characteristics: *Colonies* on OA reaching 90 mm diam in 14 d, spreading, with an even, glabrous, colourless margin; immersed mycelium becoming honey to amber with some citrine or pure yellow, showing a concentric and radiating pattern, later darkening to olivaceous or dark brick, lacking aerial mycelium after 7 d, but later developing some diffuse, felty grey aerial mycelium; reverse honey to vinaceous-buff, later also becoming greyish sepia, where conidiomata develop soon dark brick to brown-vinaceous; complex conidiomata developing from the centre in radiating rows after 5–7 d, black, globose or flattened, 0.5–2(–3) mm diam, glabrous. *Colonies* on CMA reaching of 80–85 mm diam in 14 d, spreading, with an even, glabrous, colourless margin; immersed mycelium with radiating and concentric pattern of umber to rust on an amber to pale luteous background, later darkening to predominantly olivaceous and umber, aerial mycelium diffuse, scarce, pale olivaceous-grey to greenish olivaceous, and some scattered larger pure white tufts; reverse dark brick to sepia, surrounded by hazel to isabelline and honey zones; scattered, simple to complex conidiomata developing from 7 d, later numerous also at the edge of the Petri dish, black, 0.2–1(–1.5) mm diam, bearing numerous grey to white undifferentiated hyphae on the surface. *Colonies* on MEA (3 %, Oxoid) reaching a diam of 72–74 mm in 14 d, spreading, with an even, colourless to buff, glabrous margin; colony surface almost entirely covered by a dense mat of woolly-floccose aerial mycelium that remains pure white except in the centre, where it becomes olivaceous-buff; reverse mostly ochreous, but with fulvous zones around a rust centre; numerous complex conidiomata developing from 5–12 d onwards, visible as scattered black dots on either side of the plate, later developing also in a submarginal ring, black, covered with a thin layer of undifferentiated greyish hyphae.

Growth characteristics: Optimum 24 ºC, maximum 33 ºC.

Holotype: **Brazil**, Patrocinio-Minas Gerais, isolated from fruit of *Coffea arabica*, 'M. Taniwaki 163', living culture CBS 100299 (CBS, kept metabolically inactive in freeze-dried state).

Notes: In *Coniothyrium coffeae* P. Henn., conidiomata *in planta* (twigs) have a well-developed ostiolum, while the conidia are 5–7.5 × 2.5–3 µm, formed from cylindrical conidiogenous cells (5–7 × 1.5 µm, Petrak & Sydow 1927).

Paraconiothyrium cyclothyrioides Verkley, **sp. nov.** MycoBank MB500083. Figs 5, 11, 12.

Conidiomata eustromatica, simplicia, vulgo complexa, 0.3–1.2(–1.6) mm diametro. *Cellulae conidiogenae* hyalinae, phialidicae, interdum semel vel bis percurrentes, 4.5–8 × 2.5–4 µm. *Conidia* breviter cylindrica, continua, tempore liberationis hyalina, deinde luteo-fusca, (2.5–)3–4.2(–5) × (1–)1.2–1.5(–1.8) µm (agaro 'oatmeal').

Conidiomata submerged in the agar, or superficial, eustromatic, with a single cavity, mostly complex, irregularly globose or flattened, reddish brown to black, 0.3–1.2(–1.6) mm diam, with several merging cavities, ostioles absent or poorly differentiated; *conidiomatal wall* composed of a 30–75 µm thick outer layer of isodiametric and irregular cells with reddish brown walls thickened up to 2 µm, and a 25–50(–65) µm thick inner layer of *textura angularis* with cells 3–10 µm diam with hyaline walls thickened up to 1 µm. The surface of the conidiomatal wall sometimes clothed by a diffuse network of brown entangling hyphae. *Conidiogenous cells* integrated in very compact conidiophores, rarely discrete in masses of cells protruding into the cavity, ampulliform to subcylindrical, hyaline, indeterminate, phialidic, periclinal thickening and collarette indistinct, sometimes with one or two percurrent proliferations, mostly 4.5–8 × 2.5–4 µm. *Conidia* 1-celled, short-cylindrical, straight or slightly curved, rounded at both ends, with one or two very small, polar guttules, with thin and smooth walls which are hyaline at secession, but soon become yellowish brown, on OA (2.5–)3–4.2(–5) × (1–)1.2–1.5(–1.8) µm, (2.5–)3–4.8(–6) × (1–)1.2–1.6(–2) µm on MEA (all diffuse daylight).

Cultural characteristics: *Colonies* on OA reaching 90 mm diam within 14 d, spreading, with an even, glabrous, colourless margin; immersed mycelium becoming homogeneously ochreous-amber fading to pale luteous towards the margin, later darkening to umber, the surface provided with a very diffuse, finely felty greyish aerial mycelium, occassionally in sectors darker, Umber to grey-olivaceous; reverse honey to hazel, underneath above mentioned sectors sepia or darker. *Colonies* on CMA reaching a diam of 90 mm within 14 d, spreading, with an even, glabrous, colourless margin; immersed mycelium with radiating and concentrical patterns of isabelline to olivaceous over a greenish olivaceous to honey or pale luteous background, later becoming darker umber, or larger areas less pigmented, first pale honey to pale luteous, and later becoming ochreous to fulvous, aerial mycelium diffuse, moderately developed, woolly-floccose in the central area, pale olivaceous-grey; reverse pale hazel to isabelline or honey. *Colonies* on MEA reaching a diam of 70–72 mm in 14 d, spreading, with a somewhat ruffled, colourless to buff, glabrous margin;

immersed mycelium olivaceous to olivaceous-black, covered by a well-developed, woolly-floccose, pale olivaceous grey aerial mycelium; reverse mostly bay, dark brick and brown-vinaceous with irregular to concentric patterns, abruptly fading to a cinnamon pale ocreous marginal zone. Conidiomata developing as single pycnidia or in complexes in aggregations near the centre, releasing conidial slime in clear droplets after 10–14 d.

Growth characteristics: Optimum 27 ºC, maximum 33 ºC.

Holotype: **Papua New Guinea**, Central Province, Varirata National Park near Port Moresby, ex soil sample A. Aptroot, 'A 430', X.1995, living culture CBS 972.95 (CBS, kept metabolically inactive in frozen and freeze-dried state).

Notes: Sequence AB096264 in GenBank of the isolate 'N 119' (Tsuda *et al.* 2003) was almost identical to the ITS sequence of CBS 972.95, indicating that it might be representative of the same species. N119 was isolated from 'horse mussel'. We did not receive any further information regarding this isolate.

Paraconiothyrium fungicola Verkley & Wicklow, **sp. nov.** MycoBank MB500084. Figs 6, 13, 14.

Conidiomata eustromatica, simplicia vel complexa, raro ostiolis papillatis, 0.3–1(–1.5) mm diametro. *Cellulae conidiogenae* hyalinae, phialidicae, interdum semel ad ter percurrentes, 5–7(–9) × 3–5 µm. *Conidia* ovoidea, ellipsoidea vel breviter cylindrica, continua vel uniseptata, tempore liberationis hyalina, deinde rubro-brunnea, continua (4.2–)4.4–6.2(–7) × (2.7–)3–3.4(–3.6) µm, uniseptata 7 × 3 µm µm (agaro 'oatmeal').

Conidiomata superficial or immersed in the agar, eustromatic, dark brown to black, clothed with white hyphal projections, 0.3–1(–1.5) mm diam, simple, or complex with several merging cavities, sometimes with papillate ostioles, releasing dark brown to black droplets of conidial slime; c*onidiomatal wall* covered by brown etangling 2–4 µm wide hyphae, composed of a single tissue of *textura angularis-globulosa*, 30–125 µm thick, between cavities also with more elongated hyphal cells, the cells 3–6 µm diam with hyaline to pale yellow walls up to 0.5 µm thick. *Conidiogenous cells* discrete, rarely assembled into protruding masses, determinate, phialidic, occassionally indeterminate, proliferating percurrently 1–3 times (only on PDA dominating), formed from the inner cells all over the conidiomatal wall, hyaline, subglobose, or broadly to narrowly ampulliform, sometimes with a relatively wide elongated neck, with an indistinct periclinal thickening, collarette absent, 5–7(–9) × 3–5 µm; *conidia* one-celled, ovoid, ellipsoid to short-cylindrical, broadly rounded at both ends or slightly tapering towards one end, some constricted in the middle, or two-celled, constricted around the euseptum, with up to 0.4 µm thick, smooth walls which are hyaline at secession, but soon become reddish-brown, contents vinaceous to olivaceous, minutely granular with a few small guttules near the poles, conidial mass dark brown to black; conidia on OA 1-celled (4.2–) 4.4–6.2(–7) × (2.7–)3–3.4(–3.6) µm, 2-celled 7 × 3 µm; on MEA 1-celled (4–)5–6(–7) × (2.7–)3–3.7(–4.8), 2-celled 6–8 × 4.5–5.2 µm (all ddl); on PDA 1-celled (4–)4.5–6(–7) × (3–)3.2–4(–4.3) µm, two-celled not observed.

Colonies on OA reaching a diam of 65 mm in 21 d, spreading, with an irregularly undulating or somewhat ruffled, glabrous, colourless margin; colony surface with a diffuse coverage of pure white, low, finely felty or floccose aerial mycelium, immersed mycelium becomes distinctly glaucous blue-green to dark herbage green in large concentrical zones or irregular patches, the remainder buff to rosy-buff; reverse concolourous, in the centre distinctly rosy-buff to vinaceous buff. Pycnidia developing on the surface of the colony from 10 d onwards. *Colonies* on PDA reaching a diam of 60–63 mm in 21 d, spreading, with a somewhat ruffled, glabrous,and colourless margin; colony surface with a diffuse, pure white, finely felted aerial mycelium, but around the centre becoming first citrine, then olivaceous buff to greenish olivaceous; immersed mycelium long colourless, but in the centre gradually becoming olivaceous buff to olivaceous; reverse for the most buff, but in the centre becoming honey, and then isabelline to hazel. *Colonies* on MEA reaching a diam of 35–38 mm in 21 d; restricted and already elevated in the centre up to 5 mm after 14 d, with an even or slightly undulating, glabrous buff margin; colony surface covered by a dense mat of woolly, pure white to honey or straw to primrose (pale yellow) aerial mycelium bearing numerous clear to somewhat yellowish water droplets, reverse brick at the centre, surrounded by cinnamon and ochreous zones. Pycnidia developing around the centre of the colony after 10–14 d.

Growth characteristics: Optimum 21–24 ºC, maximum 30 ºC.

Holotype: **U.S.A.**, Georgia, Dougherty Co., swamp area in Albany Nursery of the Department of Natural resources, colonist of a resupinate polypore fungus on a branch of dead hardwood collected by B. W. Horn, isolated by D. T. W., NRRL 29993 = CBS 113269, living culture; CBS, kept metabolically inactive in frozen state.

Notes: Bioassay-guided fractionation of the ethyl acetate extract of solid-substrate fermentation cultures of NRRL 29993 afforded a new isopimarane diterpenoid glucoside and a mycoparasitic acid analog, both of which showed potent antifungal activity in disk assays against *Aspergillus flavus* Link NRRL 6541 and *Fusarium verticillioides* (Sacc.) Nirenberg NRRL 25457 (N. H. Lee, J. B. Gloer, D.T. Wicklow, unpubl.).

Paraconiothyrium minitans (W.A. Campb.) Verkley, **comb. nov.** MycoBank MB500085.
Basionym: *Coniothyrium minitans* W.A. Campb., Mycologia 39: 191. 1947.

In *Paraconiothyrium minitans* conidiomata are thin-walled pycnidia, the conidiogenous cells are discrete or integrated (small protruding masses of cells), enteroblastic, phialidic with a minute periclinal thickening, but often also percurrently proliferating once or twice over a small distance, to form inconspicuous annellations (OA, CBS 861.71).

Paraconiothyrium sporulosum (W. Gams & Domsch) Verkley, **comb. nov.** MycoBank MB500086.
Basionym: *Coniothyrium fuckelii* var. *sporulosum* W. Gams & Domsch, Nova Hedwigia 18: 9. 1969.
≡ *Coniothyrium sporulosum* (W. Gams & Domsch) van der Aa, Verh. Kon. Ned. Akad. Wet., tweede sect., 68: 3. 1977.

DISCUSSION

The SSU and ITS data show that the four new *Paraconiothyrium* species are part of a distinct phylogenetic lineage within the pleosporalean ascomycetes. They share this lineage with the genus *Paraphaeosphaeria s. str.* as emended by Câmara *et al.* (2003), the anamorphs of which need not be formally named but are considered here as representative of *Paraconiothyrium*. The shared evolutionary history of these fungi is also reflected in phenotype, as the *Paraconiothyrium* anamorphs (including those of *Paraphaeosphaeria s. str.*) show a combination of morphological characters by which they can be distinguished from typical *Coniothyrium*, *Microsphaeropsis*, and *Cyclothyrium* species. In *Paraconiothyrium*, the conidiomata generally are complex, eustromatic and relatively thick-walled. They may appear as simple pycnidia, but then they usually lack a well-differentiated ostiola. In *Coniothyrium*, the conidiomata are true pycnidia, which may merge *in vitro* but then always produce well-developed, sometimes even papillate ostiola. The most distinctive *Coniothyrium* feature is the conidiogenous cells, which are annellidic, i.e., percurrently proliferating after the secession of each conidium. *Coniothyrium* conidia are thick-walled and verruculose, with a truncate base and sometimes a basal frill (Sutton 1980). *Microsphaeropsis* species are also pycnidial, but their conidiogenous cells are discrete, *Phoma*-like phialides, which only rarely proliferate percurrently. *Microsphaeropsis olivacea*, the type species, has pale brown, 1-celled, thin- and smooth-walled conidia. Sutton (1980) also included species with thick-walled, asperate or verrucose conidia in *Microsphaeropsis*. In *Cyclothyrium juglandis*, the type species of the genus *Cyclothyrium* and the anamorph of *Thyridaria rubronotata*, the conidiomata are eustromatic and the conidiogenous cells phialidic as in *Paraconiothyrium*. However, in *Cyclothyrium* the conidiogenous cells are more elongated than in most species of *Paraconiothyrium*, whilst the conidia are almost truncate at the base, or at least are much less rounded at the base than are conidia of *Paraconiothyrium*. When Petrak proposed the genus *Cyclothyrium*, he also transferred *Coniothyrium incrustans* (Sacc.) Petr., and *Coniothyrium ulmigenum* (Berk.) Petr. to this genus (Petrak 1923). Both names, however, were later included in the synonymy of *Cyclothyrium juglandis* (Petrak & Sydow 1927), while *Cyclothyrium* itself was reclassified as a subgenus of *Cytoplea* Bizz. & Sacc. No strain is available of the type species of *Cytoplea*, *Cytoplea arundinacea* (Sacc.) Petr. & Syd. (basionym *Coniothyrium arundinaceum* Sacc.). Sutton (1980) studied the holotype of *C. arundinaceum* and accepted the genus *Cytoplea* for species with eustromatic, multiloculate conidiomata and consistently discrete, *Phoma*-like phialides, which produce oval to ellipsoid, verrucose to warty, aseptate conidia. *Cytoplea* is linked to *Roussoëlla* Sacc. of the *Didymosphaeriaceae* (Hyde *et al.* 2000), and the 18S and ITS data for the type species of *Roussoëlla*, *Roussoëlla hysterioides* (Ces.) Höhn. (CBS 546.94, neotype strain, syn. *R. nitidula* Sacc. & Paol.), indicate that this genus is not closely related to *Paraconiothyrium* (ITS 1 completely unalignable). This ITS sequence of *R. hysterioides* was more similar to a sequence of *Cytoplea hysterioides* (AF009811), and both 18S and ITS sequences suggest a relatively close relationship to *Cyclothyrium/Thyridaria*. Barr (2003) transferred the genus *Thyridaria* to the *Didymosphaeriaceae*, but the phylogenetic status of that family is also still uncertain (Eriksson, Myconet Note 3903, 2004). The conidiogenous cells of *Paraconiothyrium fungicola* can form elongated necks, a character suggestive of *Cyclothyrium*, while the papillate ostioles of *P. fungicola* appear to point more towards *Coniothyrium* and *Microsphaeropsis*. The new genus *Prosopidicola*, which Lennox *et al.* (this volume) propose for a *Coniothyrium*-like fungus isolated from leguminous weed *Prosopis* in North America, differs from *Paraconiothyrium* and all other *Coniothyrium*-like genera in its branched conidiophores with percurrently or sympodially proliferating, green-brown conidiogenous cells provided with an irregular, wart-like, green-brown apical region. Genetically it is also distinct, having affinities with taxa of the *Diaporthales* (Lennox *et al.*, this volume).

Câmara *et al.* (2001) evaluated morphological data in relation to ITS sequences for nine species of *Paraphaeosphaeria*, and identified three lineages, which were later also confirmed by SSU data (Câmara *et al.* 2003). They found that only one species, *Paraphaeosphaeria pilleata*, was congeneric with the type species *Paraphaeosphaeria michotii*. Thus, only two species were retained in *Paraphaeosphaeria s. str.* For the other species the genera *Phaeosphaeriopsis* and *Neophaeosphaeria* were erected. Câmara *et al.* (2001) gave detailed descriptions of the *Coniothyrium* '*sensu lato*' anamorphs, all of which are formally unnamed, and found that differences in conidiogenesis correlated to a certain degree with divergences in the sequence similarities among *Paraphaeosphaeria*-like ascomycetes. The anamorphs of *Paraphaeosphaeria s. str.* were noted as 'typical of the genus *Microsphaeropsis*', producing smooth-walled, pale brown conidia from inconspicuous phialides with some periclinal thickening (Câmara *et al.* 2003). The anamorphs of the genus *Neophaeosphaeria* were described as '*Coniothyrium*-like', with pigmented aseptate conidia produced from holoblastic, percurrently proliferating conidiogenous cells with conspicuous annellations. The anamorphs of *Phaeosphaeriopsis* were less homogeneous, producing either brown conidia from percurrently proliferating, inconspicuously annellate conidiogenous cells (*Ph. glaucopunctata* (Grev.) Câmara, M.E. Palm & A.W. Ramaley, *Ph. obtusispora* (Speg.) Câmara, M.E. Palm & A.W. Ramaley, and *Ph. nolinae* (A.W. Ramaley) Câmara, M.E. Palm & A.W. Ramaley), or hyaline, bacillar conidia from simple phialides (*Ph. amblyspora* A.W. Ramaley, *Ph. agavensis* (A.W. Ramaley, M.E. Palm & M.E. Barr) Câmara, M.E. Palm & A.W. Ramaley). Conidiogenesis in the new species of *Paraconiothyrium* agrees with *Paraphaeosphaeria s. str.*, which is congruent with our results obtained in the ITS analysis.

Recently, Boerema (2003) mentioned that *Coniothyrium* species with *Phoma*-like phialidic pycnidia are better placed in *Microsphaeropsis* and, as an 'example', he formally proposed the new combination *Microsphaeropsis fuckelii* (Sacc.) Boerema for the anamorph of *Leptosphaeria coniothyrium* (Fuckel) Sacc., which was originally described in *Coniothyrium*. Muthumeenakshi *et al.* (2001) suggested that *C. sporulosum* and *C. fuckelii* could be conspecific, and cited Domsch *et al.* (1980), who had noted that the conidia of these species were indistinguishable. We did not sequence any strains of *C. fuckelii*. The idea that these species are synonymous needs to be tested in a study using mutiple strains. It has already been shown that *Leptosphaeria* is polyphyletic (Morales *et al.* 1995, Dong *et al.* 1998, Muthumeenakshi *et al.* 2001, Câmara *et al.* 2002). It seems likely that many species described in *Coniothyrium* and even some placed in *Phoma* may actually be akin to (or conspecific with) *Microsphaeropsis olivacea*. Apart from reinvestigating taxa by means of examining cultures, it is important to also sequence before proposing name changes. *Coniothyrium minitans* is a case in point. Based on ITS sequences, Muthumeenakshi *et al.* (2001) demonstrated the close relation between *C. minitans* (48 strains with 100 % ITS sequence homology) and *C. sporulosum* (CBS 358.75a) and, in a distant clade, also between *C. cerealis* and *Ampelomyces quisqualis* Ces. and other *Phaeosphaeriaceae*. No further *Coniothyrium*-like fungi were included in their study. From a morphological perspective, the placement of *C. minitans* in *Coniothyrium* is clearly unsatisfactory. On strict morphological grounds, one could also refer it to *Microsphaeropsis* because of its pycnidial fruitbodies and phialidic conidiogenous cells. This, however, would strongly conflict with the phylogenetic data. Although the conidiomatal structure and the asperate conidia of *C. minitans* are aberrant in relation to what is seen in other species in the *Paraconiothyrium/Paraphaeosphaeria* clade, we are obliged to combine *C. minitans* into *Paraconiothyrium*, and thus to adopt a wide morphological concept for this new genus. *Coniothyrium sporulosum* predominantly forms pycnidial fruitbodies, but otherwise conforms more consistently with the other *Paraconiothyrium* spp. Additional molecular work will further our understanding of the phylogeny within the *Paraconiothyrium/Paraphaeosphaeria* clade, and this could ultimately lead to a segregation of more anamorph genera. *Paraconiothyrium brasiliense*, *P. estuarinum* and *P. cyclothyrioides* were not well-supported as separate species in the ITS analyses, even when ITS1 was included. The ITS region contains no strong phylogenetic signal, but in view of the fact that the widely distributed *C. minitans* is invariable in ITS sequence (Muthumeenakshi *et al.* 2001), the variation seen in ITS sequences among the type strains of the new *Paraconiothyrium* species supports that idea that they are specifically distinct. Nonetheless, these species were described primarily on the basis of phenotypic characters.

The extent of morphological variation in the *Coniothyrium*-like anamorphs is still only partly known for the various groups within the *Pleosporales* which contain such anamorphs. The exact phylogenetic relationships among these groups are still not resolved, and the delimitation of the anamorph genera is also far from settled at this point. This situation discourages scientists from formally describing interesting new species. The molecular data presented here are a first important step towards improving the classification of these coelomycetes. By introducing a new generic name now for *Coniothyrium*-like anamorphs of the *Paraconiothyrium/Paraphaeosphaeria* clade, we hope to stimulate the formal description of more species within this interesting and potentially beneficial group of fungi. Researchers in applied fields who work with different isolates will benefit from an improved predictive value for their identifications, and will be able to exchange information more effectively.

ACKNOWLEDGEMENTS

B.W. Horn and A. Aptroot are kindly thanked for collecting samples. Richard C. Summerbell is kindly thanked for critical reading of the manuscript, and Arien van Iperen for isolation and Mieke Starink-Willemse for sequencing strains.

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