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A new species, *Phialophora europaea*, causing superficial infections in humans

Eine neue Art, *Phialophora europaea*, als Erreger oberflächlicher Infektionen beim Menschen

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Key words. *Phialophora europaea*, nova species, cutaneous infections, onychomycosis, ITS rDNA sequencing, physiology, diagnostics.

Schlüsselwörter. *Phialophora europaea*, nova species, Hautinfektion, Onychomykose, ITS rDNA-Sequenzierung, Physiologie, Diagnostik.

Summary. A new species, *Phialophora europaea*, member of the *P. verrucosa* complex, is introduced. It is distinguished from existing species by reduced, flaring phialidic collarettes and inability to assimilate melibiose as sole source of carbon. Analysis of ITS1 and 2 rDNA of six strains attributed to the species show it to be clearly individualized. All strains originated from cutaneous and nail infections of humans in North-western Europe. A key to morphologically similar taxa is provided.

Zusammenfassung. Wir beschreiben eine neue Art, *Phialophora europaea*, innerhalb der *P. verrucosa*-Gruppe. Diese Spezies ist durch reduzierte, etwas fransige Collaretten der Phialiden gekennzeichnet und unfähig, Melibiose als einzige Kohlstoffquelle zu verwerten. Analyse der ITS1 und 2 rDNA von sechs Stämmen dieser Art zeigte eine deutliche Abgrenzung zu verwandten Taxa. Alle Stämme wurden von kutanen Läsionen bzw. Nagel-Infektionen bei Menschen aus Nordwest Europa isoliert. Ein Bestimmungsschlüssel für diese und morphologisch ähnliche Pilze wird zur Verfügung gestellt.

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Introduction

The genus *Phialophora* is characterized by melanized hyphae and by the production of slimy, one-celled conidia in heads from phialides with distinct collarattes. The genus is polyphyletic, as it comprises species which belong to different orders of the fungal kingdom [1].

Phialophora-like species causing infections of human skin are frequently referred to as *Phialophora verrucosa* Medlar, which is one of the classical agents of chromoblastomycosis. However, based on rDNA ITS sequencing data, de Hoog *et al.* [1] demonstrated that a number of clearly distinct species are involved. The differences found corresponded well with phenetic characters, both in morphology and in nutritional physiology. The authors introduced two new species, *Phialophora reptans* de Hoog and *Phialophora sessilis* de Hoog, and a third entity was recognized but not yet formally introduced as a new taxon. More strains of the latter taxonomic entity have now been recognized, which all showed the same molecular features and closely similar phenetic characters. All strains originated from human symptomatic cutaneous and nail infections in The Netherlands, Germany and France. Hence we believe that this entity

Table 1. Growth reactions and other physiological tests of *Phialophora europaea* strains

	656.82	218.78	831.91	102391	129.96	101466
D-Glucose	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+
L-Sorbose	+	w	+	w	+	w
D-Glucosamine	+	+	+	w	+	w
D-Ribose	+	+	+	w	+	+
D-Xylose	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+
D-Arabinose	+	+	+	w	+	w
L-Rhamnose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
α,α-Trehalose	+	+	+	+	+	+
methyl-α-D-Glucoside	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+
Salicin	+	+	+	w	+	+
Arbutin	+	+	+	w	+	+
Melibiose	–	–	–	–	–	–
Lactose	–	–	–	–	–	w
Raffinose	+	+	+	+	+	+
Melezitose	+	+	+	+	+	+
Inulin	–	–	–	–	–	–
Soluble starch	+	+	w	w	w	w
Glycerol	+	+	+	+	+	+
meso-Erythritol	+	+	+	+	+	+
Ribitol	+	+	+	+	+	+
Xylitol	+	+	+	+	+	+
L-Arabinitol	+	+	+	+	+	+
D-Glucitol	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+
Galactitol	w	v	w	–	–	–
myo-Inositol	v	+	v	w	w	+
D-Gluconate	+	+	+	+	+	+
D-Glucuronate	+	+	+	+	w	+
D-Galacturonate	v	w	w	–	–	–
DL-Lactate	v	v	v	–	–	–
Succinate	+	v	v	–	w	–
Citrate	w	–	w	–	–	–
Methanol	–	–	–	–	–	–
Ethanol	+	+	v	w	w	+
Nitrate	+	+	+	+	+	+
Nitrite	+	+	+	+	+	+
Ethylamine	+	+	+	+	+	+
L-Lysine	+	+	+	+	+	+
Cadaverine	+	+	+	+	+	+
Creatine	+	w	+	w	+	w
Creatinine	–	w	w	–	–	w
Lugol	+	+	+	+	+	+
Mycosel	+	+	+	+	+	+
Urease	+	+	+	+	+	+
30°C	+	+	+	+	+	+
37°C	w	+	w	+	+	+
40°C	–	–	–	–	–	–
Elastine	–	–	–	–	–	?
Xanthine	–	–	–	–	–	?
Hypoxanthine	–	–	–	–	–	?
Guanine	–	–	–	–	–	?
Testosterone	–	–	–	–	–	?
Keratin	–	–	–	–	–	?
Adenine	–	–	–	–	–	?
Tyrosin	++	+	++	++	++	?
Casein	+	++	–	+	w	?

Symbols used: +, growth; w, weak; –, no growth.

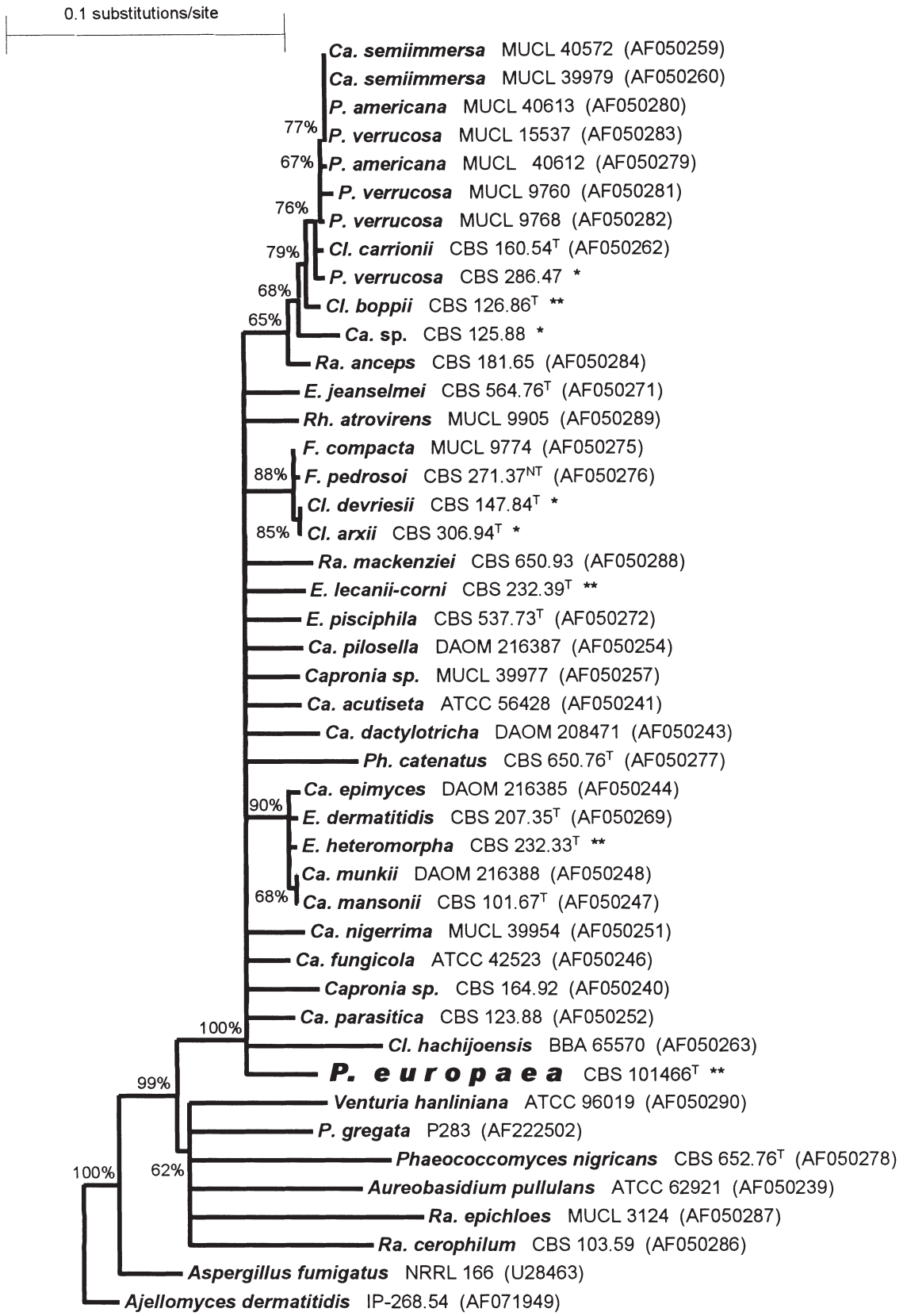

Figure 1. Rooted phylogenetic tree of selected members of Herpotrichiellaceae inferred by the neighbour-joining algorithm using 445 aligned positions of the 5′ prime end of the nuclear LSU gene. *Ajellomyces dermatitidis* was used as outgroup. Bootstrap ($n = 100$) values $> 60\%$ are indicated at the respective nodes. Abbreviations used: *Ca*, *Capronia*; *Cl*, *Cladophialophora*; *Ra*, *Ramichloridium*; *Rh*, *Rhinocladiella*; ( ), Genbank accession number of the respective sequence; T, ex-type strain.* sequence data from Masclaux *et al.* [14].** sequences obtained in this study.

deserves description as a new species in *Phialophora*.

Materials and methods

Fungal strains

The strains studied are listed in Table 1. Stock cultures were maintained on slants of 2% malt extract agar (MEA) and oatmeal agar (OA) at 24°C. For morphological observation, slide cultures were made of strains grown on MEA and mounted in Melzer's reagent or lactic acid cotton blue.

DNA extraction

About 1 cm^2 mycelium of 30-day-old cultures was transferred to a 2 ml Eppendorf tube containing 300 µl cetyltrimethylammonium bromide (CTAB) buffer and about 80 mg of a silica mixture (Silica Gel H, Merck (Darmstadt, Germany), 7736/Kieselguhr Celite 545, (Macherey & Nagel, Düren, Germany), 2 : 1, w/w). Cells were disrupted mechanically in a tight-fit sterile pestle for approximately 1 min. Subsequently 200 µl CTAB buffer was added, the mixture was vortexed and incubated for 10 min at 65°C. After addition of 500 µl chloroform, the solution was mixed and centrifuged for 5 min at 16 000 g. and the supernatant transferred to a new tube with 2 vol cold 96% ethanol. The DNA was allowed to precipitate for 30 min at −20°C and then centrifuged again for 5 min at 14 000 r.p.m. Subsequently, the pellet was washed with cold 70% ethanol. After drying at room temperature it was resuspended in 97.5 µl TE-buffer [2] plus 2.5 µl

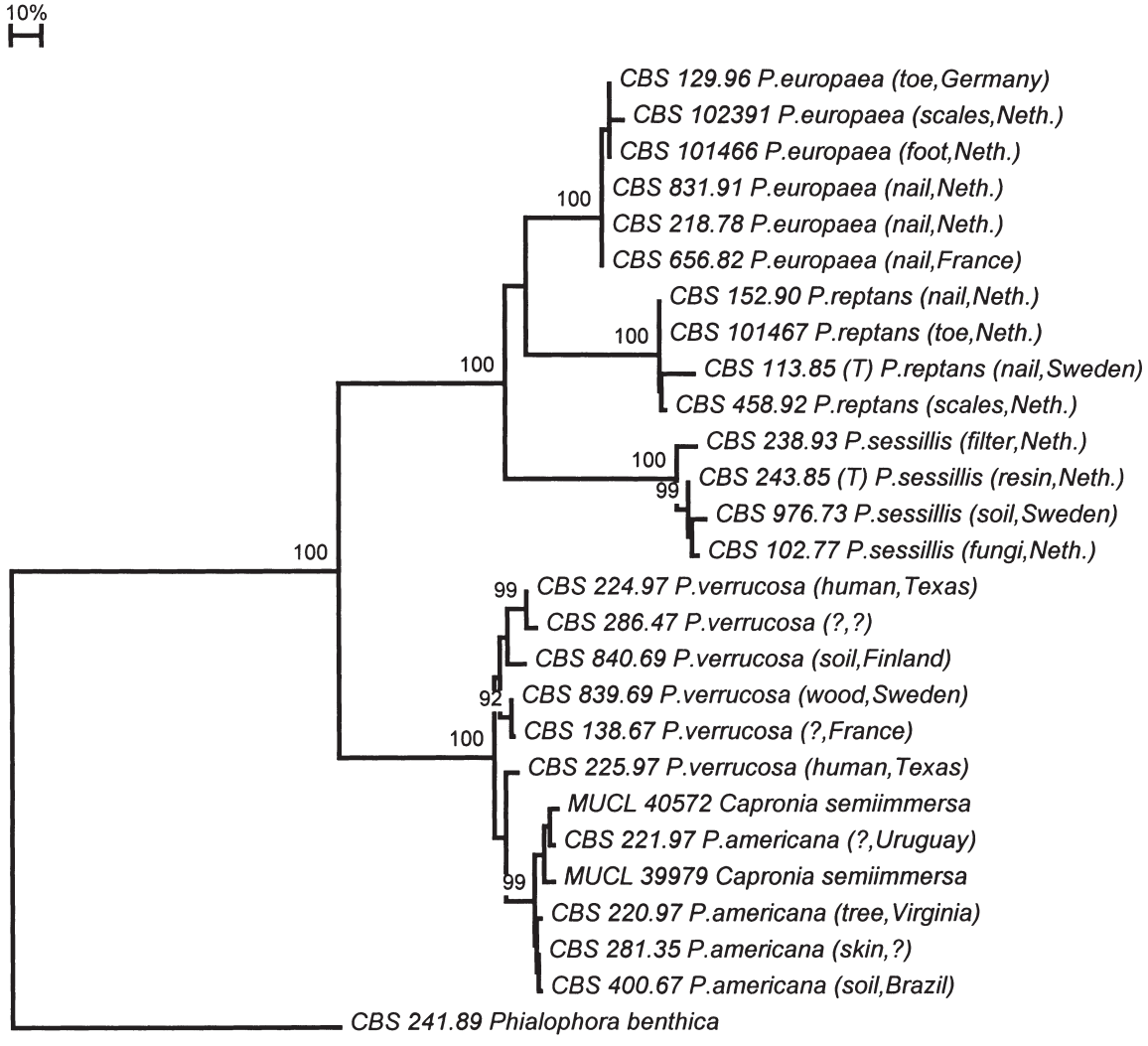

Figure 2. Phylogenetic tree of *Phialophora verrucosa* and similar species based on confidently aligned positions of the rDNA ITS domain. The tree was generated with the TREECON package using the neighbour-joining algorithm and Kimura correction. *Phialophora benthica*, CBS 241.89 was used as outgroup. The tree was subjected to 100 bootstrap replications; only values >90 are shown. Abbreviations used: Neth., Netherlands; ?, source or geography unknown.

RNAse 20 U ml^{-1} and incubated for 5 min at 37°C.

Sequencing

Amplicons were generated by PCR using primers V9D and LS266 [2] and cleaned with Microspin S-300 HR columns (Pharmacia, Rosendaal, The Netherlands). Sequencing was performed on an ABI 310 automatic sequencer with primers ITS2, ITS3, ITS4 and ITS5. The 5′ end of the nuclear large-subunit LSU rDNA gene was amplified and sequenced using the procedure described by Kurtzman and Robnett [3] with primers NL-1, NL-2 A, NL-3 A and NL-4.

Alignment and phylogenetic analysis

Sequences were assembled using the SeqMan package (DNAStar Inc., Madison, Wisconsin, USA). A preliminary automatic alignment was generated using BioNumerics version 1.50 (Applied Maths, Kortrijk, Belgium) and adjusted by eye. The TREECON package [4] was applied to generate a distance tree using the neighbour-joining algorithm with Kimura correction; only unambiguously aligned positions (127 out of 198 ITS1, all 151 of 5.8S, 111 out of 177 ITS2) were taken into account. The LSU alignment contained 445 informative positions. A total of 100 bootstrap replicates were used for analysis. The topology was verified using several algorithms (Parsimony, Ward's averaging, UPGMA).

Nutritional physiology

Growth and fermentative abilities were tested twice in duplicate in liquid media using the methodology summarized by Yarrow [5] with adaptations for filamentous fungi according to de Hoog *et al.* [6]. For assimilation tests, tubes were incubated in a nearly horizontal position at 25°C, aerated with slow rocking (approx.

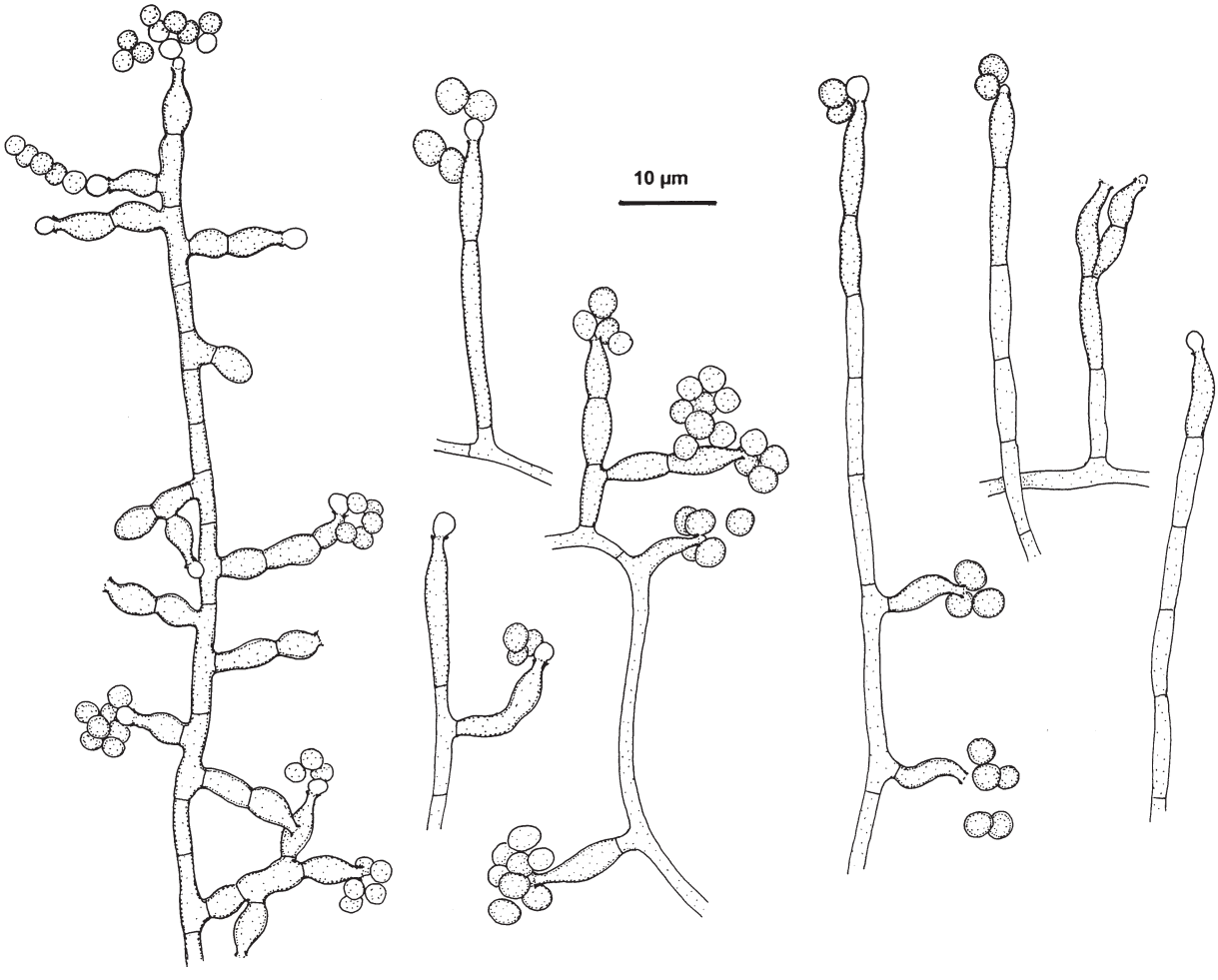

Figure 3. Morphology of *Phialophora europaea* grown on PDA. (a), CBS 101466, well developed conidial apparatus; (b), CBS 129.96, degenerate conidial apparatus. Bar = 10 µm.

50 r.p.m.), and examined weekly for 21 days. The abilities were scored by rating growth by comparison with positive and negative controls. The fermentation of glucose was tested using Durham tubes incubated in an upright position at 25°C; the tubes were kept stationary apart from a brief manual shaking prior to each reading, and were examined every 2 days for 14 days. Arbutin tests were performed on Petri dishes according to Yarrow [5] and also in liquid medium, where growth and discoloration were scored separately.

Isolates were tested for the production of extracellular, starch-like compounds after 21 days of growth on all carbon and nitrogen assimilation media. Cultures were examined microscopically in Melzer's reagent [7]; blue and purple colour reactions were observed in the medium of strains that were positive for amyloidity. The hydrolysis of urea to ammonia was tested using Christensen's urea broth [5]. Cultures were observed at regular intervals for 21 days. Temperature/growth relationships were tested on slants of 4% MEA incubated for 2 week at 24, 30, 37 and 40°C.

Decomposition of adenine, guanine, hypoxanthine, xanthine, tyrosine, elastin, keratin, testosterone [8], and hydrolysis of casein [9] were performed on agar-plates and incubated for 4 weeks at 30°C.

Results and discussion

The strains under consideration were initially identified on morphological grounds as 'atypical *Phialophora verrucosa*' (e.g. [10]: CBS 129.96), which suggests a close phylogenetic coherence with that species. However, using 18S rDNA sequencing, Haase *et al.* [11] found that, although both entities belonged to the same family of black yeasts, the Herpotrichiellaceae, a considerable distance was apparent. Alignment of the partial LSU rDNA gene of six of these 'atypical' strains revealed an infraspecific variability of three nucleotides. The inferred LSU tree (Fig. 1) shows that the species belongs to the Herpotrichiellaceae clade and shows no conspecificity with any *Capronia* teleomorph species. However, since members of the Herpotrichiellacaea seem to be in an early stage of early evolutionary radiation [11] leading to poorly resolved trees, and since an insufficient number of related fungi have as yet been studied, precise assignment to any main group is difficult. The species clustered near the origin of the clade (Fig. 1) and so a final placement is not possible, whereas *P. verrucosa* is an unambiguous member of the Herpotrichiellaceae.

Furthermore, with ITS rDNA the two entities are clearly apart [1]. The ITS differences with other species compared were significant; the phylogenetic tree (Fig. 2) was robust, showing the same topology with several algorithms (data not shown). In part these differences concerned incongruent blocks in the alignment, having the appearance of larger evolutionary events rather than isolated point mutations [1]. Given the large distances between the two taxa, they are anyway clearly apart from each other. A new taxon should therefore be described for the strains of '*Phialophora* tax. sp. 1' [1].

The classical *Phialophora* species, *P. verrucosa* and *Phialophora americana* (Nannf.) Conant, resemble each other in their ITS sequences [12], although they are morphologically clearly different by having funnel- versus vase-shaped collarettes. *Phialophora americana* is likely to be the anamorph of *Capronia semiimmersa* Candousseau [13]. 'Taxonomic species 1' [1] is morphologically different by having flaring but very short collarettes, and by its inability to assimilate melibiose (Table 1). A positive Lugol reaction was consistentely noted with cadaverine, mostly also with L-lysine, occasionally with some other N-source but never with any C-source. As a morphological criterion, the conidia are subspherical rather than ellipsoidal, subhyaline, and often occur in chains. The phialides are usually part of short side branches, whereas in *P. verrucosa* and *P. americana* they are sessile directly on hyphae. The latter character is, however, only observed in part of the strains.

Phialophora europaea *de Hoog, Mayser & Haase* ***sp. nov.*** – *Fig. 3*

Coloniae olivaceae; phialides cum collarettis fusis et excectis, brevis; conidiis subsphaeroideis, 1.8–2.5 µm latis. Teleomorphis ignotus. Typus CBS 10146 ex laesione humana, vivus et exsiccatus, in herbariorum CBS depositus.

The following description is based on the extype strain CBS 101466 on PDA at 22°C.

Colonies attaining 10 mm diam in 1 month, olivaceous black. Hyphae pale olivaceous brown, 1.5–2 µm wide, regularly septate about every 10–20 µm, with numerous anastomoses. Fertile hyphae bearing phialides either directly, or with one to two on slightly swollen subtending cells of about 2–3 µm wide; subtending cells occasionally arranged in chains containing two to eight cells. Phialides flask-shaped to elongate, somewhat narrowed towards the tip, often with a

nearly cylindrical apical portion, 6–9 μm long. Collarettes very short, flaring, somewhat darkened, phialide opening 1 μm wide, producing conidia in fragile chains or in heads. Conidia subhyaline (sub)spherical, 1.8–2.5 μm. Chlamydospores absent. Teleomorph unknown. Physiological properties listed in Table 1.

Type. CBS 101466, ex foot of human patient, H.M.E. Frénay, Dordrecht, The Netherlands (herb. CBS, holotype).

Additional strains examined. CBS 129.96, ex cutaneous infection of toe of human patient, P. Mayser, Gießen, Germany [10]; CBS 218.78, ex nail file-dust, Central Bacteriological Laboratory, Rotterdam, The Netherlands; CBS 656.82, ex human nail, M.D. Linas, Toulouse, France; CBS 831.91, ex onychomycosis mixed with yeasts, Mycology Lab GGD, Haarlem, The Netherlands; CBS 102391 (dH 11436), ex cutaneous infection of 9-year-old female patient, Canisius Hospital, Nijmegen, The Netherlands.

Some infraspecific variation is noted. Strain CBS 101466 shows abundant sporulation with flask-shaped phialides, which are often borne by a subtending cell; the cells of the conidial apparatus are slightly wider than the undifferentiated hyphae, and are often somewhat inflated. In contrast, CBS 129.96 has more elongate, nearly tubular, less differentiated phialides and slightly larger conidia. The six strains analysed were found to have base substitutions in two positions in ITS1 and a single substitution in ITS2.

The feature that is shared by all strains attributed to *P. europaea* is the collarette, which is slightly darkened and flaring, as in *P. verrucosa*, but they are significantly shorter and much reduced. The four older strains of *P. europaea* were maintained in the CBS culture collection under the name *P. verrucosa*, but invariably with a remark on the reduced collarette. A morphological key to *P. verrucosa*-like taxa is presented below.

It is remarkable that all strains of *P. europaea* were isolated from skin scales and nails of human patients in North-western Europe. Most strains analysed were sent for identification to the Centraalbureau voor Schimmelcultures over a period of 20 years. A documented case report for a patient from Germany is that of Mayser *et al.* [10], where a traumatic infection during a holiday on Cape Verde was supposed. Some elongate cells were seen in tissue and hence, assuming that the aetiologic agent was close to *P. verrucosa*, the mycosis was interpreted as an early case of chromoblastomycosis. However, only hyperkeratosis was observed, whereas acanthosis, which probably is the clinical hallmark for chromoblastomycosis, remained absent. The case of CBS 102391 concerned a cutaneous nodule on the cheek of a 9-year-old girl.

Judging from available clinical data, *P. europaea* seems mainly involved in superficial infections, having a low degree of virulence. Nail infection, and, if on skin, some hyperkeratosis may be characteristic for *P. europaea* infections, and as such the species is clinically different from all other *Phialophora* species. In the absence of well-described clinical cases, mostly due to possibly incorrect identification, this predilection cannot be established with certainty. It is remarkable that most strains of its nearest neighbour *Phialophora reptans*, also originate from human superficial locations (compare [1]). In the light of the taxonomic problems highlighted in this article, it might be useful to analyse published cases by *P. verrucosa* in retrospect.

Key to human-pathogenic Phialophora *species and some morphologically similar taxa*

1a. Phialides stiff, tubular, spine-like, dark brown. 2
1b. Phialides flask-shaped, pale olivaceous brown, or the collarettes are directly borne on undifferentiated hyphae. 3
2a. Conidia all hyaline, elongate. *Phaeoacremonium*
2b. Conidia of two types: brown and spherical, and elongate and hyaline. *Phialophora richardsiae*
3a. Phialides funnel-or vase-shaped, clearly darker than the rest of the phialide. 4
3b. Phialides inconspicuous, not clearly darker than the rest of the phialide. 6
4a. Phialides intercalary, collarettes directly on undifferentiated hyphae. *Phialophora sessilis*
4b. Phialides flask-shaped. 5
5a. Collarettes funnel-shaped. *Phialophora verrucosa*
5b. Collarettes vase-shaped. *Phialophora americana*
6a. Phialides often aggregated in dense, penicillate branches. *Phialophora repens*
6b. Phialides dispersed, not in densely branched structures. 7
7a. Chlamydospores present. *Phialophora bubakii*
7b. Chlamydospores absent. 8
8a. Collarettes mostly directly on hyphal cells, narrow, 1.5–2.5 μm deep. *Phialophora reptans*
8b. Collarettes nearly always on subcylindrical or flask-shaped phialides, short and shallow, less than 1 μm deep. *Phialophora europaea*

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