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Abstract: *Cladophialophora* is a genus of black yeast-like fungi comprising a number of clinically highly significant species in addition to environmental taxa. The genus has previously been characterized by branched chains of ellipsoidal to fusiform conidia. However, this character was shown to have evolved several times independently in the order *Chaetothyriales*. On the basis of a multigene phylogeny (nucLSU, nucSSU, *RPB*1), most of the species of *Cladophialophora* (including its generic type *C. carrionii*) belong to a monophyletic group comprising two main clades (*carrionii*- and *bantiana*-clades). The genus includes species causing chromoblastomycosis and other skin infections, as well as disseminated and cerebral infections, often in immunocompetent individuals. In the present study, multilocus phylogenetic analyses were combined to a morphological study to characterize phenetically similar *Cladophialophora* strains. Sequences of the ITS region, partial Translation Elongation Factor 1-α and β-Tubulin genes were analysed for a set of 48 strains. Four novel species were discovered, originating from soft drinks, alkylbenzene-polluted soil, and infected patients. Membership of the both *carrionii* and *bantiana* clades might be indicative of potential virulence to humans.

Key words: Biodiversity, bioremediation, *Cladophialophora*, chromoblastomycosis, disseminated infection, MLST, mycetoma.
Taxonomic novelties: *Cladophialophora samoënsis* Badali, de Hoog & Padhye, sp. nov., *Cladophialophora subtilis* Badali & de Hoog, sp. nov., *Cladophialophora mycetomatis* Badali, de Hoog & Bonifaz, sp. nov., *Cladophialophora immunda* Badali, Satow, Prenafeta-Boldú, Padhye & de Hoog, sp. nov.

INTRODUCTION

Cladophialophora is a genus of black yeast-like fungi which are remarkably frequently encountered in human infections, ranging from mild cutaneous lesions to fatal encephalitis. The genus is morphologically characterized by one-celled, ellipsoidal to fusiform, dry conidia arising through blastic, acropetal conidiogenesis, and arranged in branched chains. The chains are usually coherent and conidial scars are nearly unpigmented (Borelli 1980, Ho *et al.* 1999, de Hoog *et al.* 2000). The genus was initially erected to accommodate species exhibiting *Phialophora*-like conidiogenous cells in addition to conidial chains (Borelli 1980) but thus far this feature is limited to the species *C. carrionii*. Teleomorphs have not been found, but judging from SSU rDNA phylogeny data, these are predicted to belong to the ascomycete genus *Capronia*, a member of the order *Chaetothyriales* (Haase *et al.* 1999).

The type species of *Cladophialophora*, *C. carrionii*, is an agent of chromoblastomycosis, a cutaneous and subcutaneous disease histologically characterized by muriform cells in skin tissue. Muriform cells represent the invasive form of fungi causing chromoblastomycosis (Mendoza *et al.* 1993). Infections are supposed to originate by traumatic implantation of fungal elements into the skin and are chronic, slowly progressive and localised. Tissue proliferation usually occurs around the area of inoculation, producing crusted, verrucose, wart-like lesions. The genus has been expanded to encompass several other clinically significant species, including the neurotropic fungi *C. bantiana* and *C. modesta* causing brain infections (Horré & de Hoog 1999), *C. devriesii* and *C. arxii* causing disseminated disease (de Hoog *et al.* 2000) and *C.*

boppii, *C. emmonsii* and *C. saturnica* causing cutaneous infections (de Hoog *et al.* 2007, Badali *et al.* 2008).

Based on molecular data, anamorphs morphologically similar to *Cladophialophora* have been found in other groups of ascomycetes, particularly in the *Dothideales / Capnodiales* (*e.g.*, *Pseudocladosporium*, *Fusicladium*; Crous *et al.* 2007). Distinction between chaetothyrialean and dothidealean / capnodialean anamorphs is now also supported by their teleomorphs. Braun & Feiler (1995) reclassified the dothidealean species *Venturia hanliniana* (formerly *Capronia hanliniana*) as the teleomorph of *Fusicladium brevicatenatum* (formerly *Cladophialophora brevicatenata*). Further distinction between *Chaetothyriales* and *Dothideales / Capnodiales* lies in their ecology, with recurrent human opportunists being restricted to the *Chaetothyriales*. Braun (1998), summarizing numerous statements in earlier literature, separated *Cladophialophora* with *Capronia* teleomorphs (*Herpotrichiellaceae, Chaetothyriales* mostly as opportunistic or pathogens), from the predominantly saprobic or plant associated isolates in the *Dothideomycetes*.

Some species attributed to *Cladophialophora* may be found in association with living plants. De Hoog *et al.* (2007) reported a cactus endophyte, *Cladophialophora yegresii*, as the nearest neighbour of *C. carrionii*, which is a major agent of human chromoblastomycosis. The latter fungus was believed to grow on debris of tannin-rich cactus spines, which were also supposed to be the vehicle of introduction into the human body. Crous *et al.* (2007) described several host-specific plant pathogens associated with the *Chaetothyriales*. *Cladophialophora hostae* caused spots on living leaves of *Hosta plantaginea*, *C. proteae* was a pathogen of *Protea*

Table 1. Isolation data of examined strains.

Name	CBS	Status	Other reference	GenBank ITS, *TUB*, *EF1α*	Source	Origin
Cladophialophora carrionii	CBS 114392		UNEFM 82267 = dH 13261	EU137267, EU137150, EU137211	Chromoblastomycosis; leg; female,	Venezuela, Falcon State
	CBS 114393		UNEFM 9801 = dH 13262	EU137268, EU137151, EU137212	Chromoblastomycosis; hand; male	Venezuela, Falcon State
	CBS 114396		UNEFM 2001/1 = dH 13265	EU137269, EU137152, EU137213	Chromoblastomycosis; arm; male	Venezuela, Falcon State
	CBS 114398		UNEFM 2003/1 = dH 13267	EU137271, EU137154, EU137215	Chromoblastomycosis; arm; female	Venezuela, Falcon State
	CBS 260.83		CDC B-1352 = FMC 282 = ATCC 44535	EU137292, EU137175, EU137234	Skin lesion in human	Venezuela, Falcon State
	CBS 160.54	LT	CDC A-835 =(ex-LT of *C. carrionii*) = ATCC 16264	EU137266, EU137201, EU137210	Chromoblastomycosis, human	Venezuela, Falcon State
Cladophialophora yegresii	CBS 114406		UNEFM SgSR1; dH 13275	EU137323, EU137208, EU137263	*Stenocereus griseus* asymptomatic plant	Venezuela, Falcon State
	CBS 114405		UNEFM SgSr3; dH 13276	EU137322, EU137209, EU137262	*Stenocereus griseus* asymptomatic plant	Venezuela, Falcon State
	CBS 114407		UNEFM SgSR1; dH 13274	EU137324, –, U137264	*Stenocereus griseus* asymptomatic plant	Venezuela, Falcon State
Cladophialophora emmonsii	CBS 640.96		CDC B-3634; NCMH 2248; 4991	EU103995, –, U140584	Sub-cutaneous lesion, cat	–
	CBS 979.96		CDC B-3875; NCMH 2247	EU103996, –, U140583	Sub-cutaneous lesion right forearm, human	U.S.A., Virginia
Cladophialophora boppii	CBS 126.86		FMC 292; dH 15357	EU103997, –, U140596	Skin lesion, on limb, male	Brazil
	CBS 110029		det M-41/2001 56893; dH 12362	EU103998, –, U140597	Scales of face, male	Netherlands, Dordrecht
Cladophialophora bantiana	CBS 173.52	T	CBS 100433	EU103989, –, U140585	Brain abscess, male	U.S.A.
	CBS 444.96		–	EU103994, –, U140591	Disseminated infection, dog	South Africa, Pretoria, Onderstepoort
	CBS 678.79		CDC B-3658; NCMH 2249; NIH B-3839	EU103992, –, U140592	Skin lesion, cat	U.S.A., Bethesda
	CBS 648.96		UAMH 3830	EU103993, –, U140587	Liver, dog	Barbados
Cladophialophora saturnica	CBS 109628		dH 12333; IHM 1727	EU103983, –, U140601	Dead tree	Uruguay, Isla Grande del Queguay
	CBS 109630		dH 12335; IHM 1733	FJ385270, –, –	Trunk, cut tree	Uruguay, Isla Grande del Queguay
	CBS 118724	T	157D; dH 12939	EU103984, –, EU140602	Interdigital toe lesion, child	Brazil, Paraná, Curitiba
	CBS 102230		dH 11591; 4IIBPIRA	AY857508, –, EU140600	Litter, vegetable cover/soil	Brazil, Paraná, Curitiba
	CBS 114326		ATCC 200384	AY857507, –, EU140603	Toluene biofilter	Netherlands, Wageningen
Cladophialophora devriesii	CBS 147.84	T	ATCC 56280; CDC 82-030890	EU103985, –, EU140595	Disseminated infection, male	U.S.A., Grand Cayman Island
	CBS 118720		ISO 13F	FJ385275, –, –	Litter, vegetable cover/soil	Brazil, Paraná, Curitiba
Cladophialophora arxii	CBS 306.94	T		EU103986, –, EU140593	Tracheal abscess, male	Germany
Cladophialophora minourae	CBS 987.96		IFM 4701; UAMH 5022	EU103988, –, EU140599	Rotting wood	Japan, Yachimata, Chiba
	CBS 556.83		ATCC 52853; IMI 298056	AY251087, –, EU140598	Decaying wood	Japan, Shiroi

Table 1. (Continued).

Name	CBS	Status	Other reference	GenBank ITS, *TUB*, *EF1α*	Source	Origin
Cladophialophora immunda	CBS 110551		dH15250	FJ385274, EU137207, EU137261	Gasolin-station soil	Netherlands, Apeldoorn
	CBS 109797		dH 11474	FJ385271, EU137206, EU137260	Biofilter inoculated with soil	Germany, Kaiserslautern
	CBS 834.96	T	CDCB-5680; de H.10680	EU137318, EU137203, EU137257	Sub-cutaneous phaeohyphomycosis, male	U.S.A., Georgia, Atlanta
	CBS 102227		dH 11588	FJ385269, –, EU137259	Syagrum romanzoffianum, stem	Brazil, Paraná, Colombo
	CBS 102237		dH 11601	FJ385272, EU137205, EU137285	Decaying cover vegetable	Brazil, Paraná, Sarandi
Cladophialophora samoënsis	CBS 259.83	T	CDC B-3253; dH 15637	EU137291, EU137174, EU137233	Chromoblastomycosis skin lesion, male	U.S.A., Samoa
Cladophialophora subtilis	CBS 122642	T	dH 14614	FJ385273, –, –	Ice tea	Netherlands, Utrecht
Cladophialophora mycetomatis	CBS 454.82	T	dH 15898	EU137293, EU137176, EU137235	Culture contaminant	Netherlands
	CBS 122637	T	dH 18909	FJ385276, –, –	Eumycetoma, male	Mexico, Jicaltepec
Fonsecaea monophora	CBS 289.93	T	dH 15691	AY366925, EU938554, –	Lymphnode, aspiration-biopsy	Netherlands (zoo)
	CBS 102238		dH 11602, 1PLE	AY366927, EU938546, –	Soil	Brazil
	CBS 102248		dH 11613	AY366926, EU938550, –	Chromoblastomycosis, male	Brazil
Fonsecaea pedrosoi	CBS 271.37	T	ATCC 18658; IMI 134458; dH 15659	AY366914, EU938559, –	Chromoblastomycosis, male	South America
	CBS 272.37		dH 15661	AY366917, –, –	Chromoblastomycosis, male	–
Cladophialophora australiensis	CBS 112793		CPC 1377	EU035402, –, –	Sports drink	Australia
Cladophialophora chaetospira	CBS 491.70		–	EU035405, –, –	Roots of *Picea abies*	Denmark
Cladophialophora potulenturum	CBS 112222		CPC 1376; FRR 4946	EU035409, –, –	Sports drink	Australia
	CBS 114772		CPC 1375; FRR 4947	EU035410, –, –	Sports drink	Australia

Abbreviations used: ATCC = American Type Culture Collection, Manassas, U.S.A.; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DH = G.S. de Hoog private collection; IFM = Research Institute for Pathogenic Fungi, Chiba, Japan; IHM = Laboratory of Mycology, Faculty of Medicine, Montevideo Institute of Epidemiology and Hygiene, Montevideo, Uruguay; IMI = International Mycological Institute, London, U.K.; IWW = Rheinisch Westfälisches Institut für Wasserforschung, Mülheim an der Ruhr, Germany; GHP = G. Haase private collection; MUCL = Mycotheque de l'Université de Louvain, Louvain-la-Neuve, Belgium; NCMH = North Carolina Memorial Hospital, Chapel Hill, U.S.A.; RKI = Robert Koch Institute, Berlin, Germany; UAMH = Microfungus Herbarium and Collection, Edmonton, Canada; UTHSC = Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center at San Antonio, U.S.A.; UTMB = Medical Mycology Research Center, Galveston, U.S.A.; UNEFM = Universidade Nacional Experimental Francisco de Miranda, Coro, Falcon, Venezuela. *T = ex-type culture; LT = ex-lectotype culture;*

cynaroides, and *C. scillae* caused leaf spots on *Scilla peruviana*. Finally, Davey & Currah (2007) described *Cladophialophora minutissima* from mosses collected at boreal and montane sites in central Alberta, Canada. Within the *Chaetothyriales*, most of these plant-associated species are found at relatively large phylogenetic distance from the main clade of *Cladophialophora* comprising most of the opportunistic species.

The core of the genus *Cladophialophora* does comprise a number of environmental saprobes. *Cladophialophora minourae* and *C. chaetospira* occur in plant litter. Badali *et al.* (2008) described *C. saturnica* from plant debris in the environment, but the species was also found causing an interdigital infection in a Brazilian child with HIV infection. The species *C. australiensis* and *C. potulentorum* were found in soft drinks (Crous *et al.* 2007). If environmental species are able to provoke opportunistic infections, the question arises whether members of *Chaetothyriales* isolated from food products might imply a health risk. Understanding of the phylogeny and ecology of *Cladophialophora* is therefore essential. Many review articles incorrectly mention that black yeast-like fungi are commonly found on decomposing plant debris and in soil. In fact, Chaetothyrialean members are difficult to isolate from the environment as they seem to have quite specific, hitherto undiscovered ecological niches (Satow *et al.* 2008, Vicente *et al.* 2008). For their isolation, selective methods are required, *e.g.* by the use of high temperatures (Sudhadham *et al.* 2008), a mouse vector (Gezuele *et al.* 1972, Dixon *et al.* 1980), alkyl benzenes (Prenafeta-Boldú *et al.* 2006) or isolation via mineral oil (Satow *et al.* 2008, Vicente *et al.* 2001, 2008). An association with assimilation of toxic monoaromatic compounds has been hypothesised. Black yeasts and their filamentous relatives in the *Chaetothyriales* are potent degraders of monoaromatic compounds and tend to accumulate in industrial biofilters (Cox *et al.* 1997, Prenafeta-Boldú *et al.* 2001, de Hoog *et al.* 2006). This might be a clue to dissecting their dual behavior as rare environmental oligotrophs as well as invaders of human tissue containing aromatic neurotransmitters.

The present paper combines ecological information with phylogenetic and taxonomic data, and interprets them in the light of potential health hazards of seemingly saprobic species that may occur in food products. We applied multilocus sequence analysis and phenetic characterization to distinguish novel *Cladophialophora* species from various sources.

Table. 2. Primer sequences for PCR amplification and sequencing.

Gene	PCR primers	Sequencing primers	References
ITS rDNA	V9G[a], LS266[b]	ITS1[c], ITS4[c]	[a] de Hoog *et al.* (1998) [b] Masclaux *et al.* (1995) [c] White *et al.* (1990)
TUB	Bt2a, Bt2b	Bt2a, Bt2b	Glass & Donaldson (1995)
EF1-α	EF1-728F, EF1-986R	EF1-728F, EF1-986R	Carbone & Kohn (1999)
SSU rDNA	NS1[a], NS24[b]	(BF83, Oli1, Oli9, BF951, BF963, BF1438, Oli3, BF1419)[c]	[a] White *et al.* (1990) [b] Gargas & Taylor (1992) [c] de Hoog *et al.* (2005)

MATERIALS AND METHODS

Fungal strains

Strains used in this study were obtained from the Centraalbureau voor Schimmelcultures (Table 1). Stock cultures were maintained on slants of 2 % malt-extract agar (MEA, Difco) and oatmeal agar (OA, Difco) and incubated at 24 °C for two weeks (Gams *et al.* 1998). All cultures in this study are maintained in the culture collection of CBS (Utrecht, The Netherlands) and taxonomic information for new species was deposited in MycoBank (www.MycoBank.org).

DNA extraction

The fungal mycelia were grown on 2 % (MEA) plates for 2 wks at 24 °C (Gams *et al.* 1998). A sterile blade was used to scrape off the mycelium from the surface of the plate. DNA was extracted using an Ultra Clean Microbial DNA Isolation Kit (Mobio, Carlsbad, CA 92010, U.S.A.) according to the manufacturer's instructions. DNA extracts were stored at −20 °C prior to use.

Amplification and sequencing

Four genes were amplified: the internal transcribed spacer region (ITS), the translation elongation factor 1 alpha (*EF1-α*), the partial beta tubulin gene (*TUB*), and the small subunit of the nuclear ribosomal RNA gene (nucSSU). The primers used for amplification and sequencing are shown in Table 2. PCR reactions were performed on a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA) in 50 µL volumes containing 25 ng of template DNA, 5 µL reaction buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M KCl, 15 mM $MgCl_2$, 0.1 % gelatine, 1 % Triton X-100), 0.2 mM of each dNTP and 2.0 U Taq DNA polymerase (ITK Diagnostics, Leiden, The Netherlands). Amplification of ITS and nucSSU was performed with cycles of 2 min at 94 °C for primary denaturation, followed by 35 cycles at 94 °C (45 s), 52 °C (30 s) and 72 °C (120 s), with a final 7 min extension step at 72 °C. Annealing temperatures used to amplify EF1α and *TUB* genes were 55 and 58 °C, respectively. Amplicons were purified using GFX PCR DNA and gel band purification kit (GE Healthcare, Ltd., Buckinghamshire U.K.). Sequencing was performed as follows: 95 °C for 1 min, followed by 30 cycles consisting of 95 °C for 10 s, 50 °C for 5 s and 60 °C for 2 min. Reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequence data obtained in this study were adjusted using the SeqMan of Lasergene software (DNAStar Inc., Madison, Wisconsin, U.S.A.).

Alignment and phylogenetic reconstruction

Phylogenetic analyses were carried out on three different datasets. The first dataset included a taxon sampling representative of the order *Chaetothyriales* (94 taxa) and the three genes nucLSU, nucSSU and *RPB*1. This dataset was used in order to assess the phylogenetic placement of diverse species of *Cladophialophora* within the *Chaetothyriales*. For this first analysis, sequences of nucSSU obtained from investigated strains of *Cladophialophora* were added to an existing dataset representing the *Chaetothyriales* (Gueidan *et al.* 2008). Alignments were done manually for each gene

using MacClade 4.08 (Maddison & Maddison 2003) with the help of amino acid sequences for protein coding loci. Ambiguous regions and introns were excluded from the alignments. The program RAxML-VI-HPC v.7.0.0 (Stamatakis *et al.* 2008), as implemented on the Cipres portal v.1.10, was used for the tree search and the bootstrap analysis (GTRMIX model of molecular evolution and 500 bootstrap replicates). Bootstrap values equal or greater than 70 % were considered significant (Hillis & Bull 1993).

The second and third datasets focused on two main monophyletic clades nested within the core group of *Cladophialophora* (Clade I and Clade II, Fig. 1). They included three genes, ITS, EF1α and *TUB*. The goal of these two analyses was to assess the delimitation of species of *Cladophialophora*. The second dataset (Clade I or *carrionii*-clade) comprised 15 taxa, and the third dataset (Clade II or *bantiana*-clade) 33 taxa. Phylogenetic reconstructions and bootstrap values were first obtained for each locus separately using RAxML (as described above). The congruence between loci was assessed using a 70 % reciprocal bootstrap criterion (Mason-Gamer & Kellogg 1996). The loci were then combined and analysed using RAxML (as described above). The phylogenetic trees were edited using Tree View v.1.6.6.

Morphological identification and cultural characterisation

Strains were cultured on 2 % MEA and OA and incubated at 24 °C in the dark for two wks (Gams *et al.* 1998). Identification was based primarily on macroscopic and microscopic morphology. Microscopical observations were based on slide culture techniques using potato dextrose agar (PDA) or OA because these media readily induce sporulation and suppress growth of aerial hyphae (de Hoog *et al.* 2000). Mounts of two-wk-old slide cultures were made in lactic acid or lactophenol cotton blue and light micrographs were taken using Nikon Eclipse 80*i* microscope with a Nikon digital sight DS-Fi1 camera.

Physiology

Cardinal growth temperatures of strains were determined on 2 % MEA (Difco) (Crous *et al.* 1996). Plates were incubated in the dark for two wks at temperatures of 6–36 °C at intervals of 3 °C; in addition, growth at 40 °C was recorded. Experiments consisted of three simultaneous replicates for each isolate; the entire procedure was repeated once.

Muriform cells

All strains were tested for the production of muriform cells, *i.e.*, the meristematic tissue form of agents of human chromoblastomycosis. Strains were incubated at 25 and 37 °C for one wk in a defined medium with low pH containing 0.1 mM/L calcium chloride. The basal medium was prepared by adding the following components to 1 L deionized distilled water: 30 g glucose, 3 g $NaNO_3$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.265 g NH_4Cl, 0.003 g thiamin and 1 mM $CaCl_2$; the pH was adjusted to 2.5 with HCl for all experiments (Mendoza *et al.* 1993).

RESULTS

Phylogeny

Chaetothyriales dataset

Fig. 1 shows that, in the order *Chaetothyriales*, species of *Cladophialophora* belong to at least four different lineages: one lineage corresponding to the family *Herpotrichiellaceae* (lineage 1, Fig. 1), and three basal lineages including mostly rock-inhabiting strains (lineages 2, 3 and 4, Fig. 1). Most of the human-opportunistic species (including the most virulent ones) belong to two well-supported clades within the *Herpotrichiellaceae*. Clade I (*carrionii*-clade, Fig. 1, 2) includes the pathogenic *Cladophialophora* species *C. carrionii* and *C. boppii*, as well as two pathogenic species of *Phialophora* (*P. verrucosa* and *P. americana*). Clade II (*bantiana*-clade, Fig. 1, 3) includes the pathogenic species *C. bantiana*, *C. arxii*, *C. immunda*, *C. devriesii*, *C. saturnica, C. emmonsii,* and *C. mycetomatis*, as well as the pathogenic genus *Fonsecaea* (*F. pedrosoi* and *F. monophora*). All the plant associated species of *Cladophialophora* belong to lineages 2, 3 and 4. Lineage 2 includes rock-inhabiting strains and the opportunistic pathogen *C. modesta*. Lineage 3 includes rock-inhabiting strains, the two opportunistic pathogens *P. europaea*, and *C. laciniata*, and three plant associated species of *Cladophialophora* (*C. proteae, C. hostae,* and *C. scillae*). Lineage 4 includes exclusively rock-inhabiting strains and plant leaving strains (C. *minutissima, C. humicola,* and *C. sylvestris*).

Carrionii-clade dataset

Phylogenetic reconstructions of Clade I (*carrionii*-clade; Fig. 2) were first carried out for each gene separately (ITS: 542 characters, *EF1-α*: 117 characters, *TUB*: 402 characters). Topological conflicts were detected for all genes within both *C. carrionii* and *C. yegresii*. This incongruence involving only below species-level relationships, and therefore – in agreement with the genealogical recognition species concept (Taylor *et al.* 2000) –, the conflicts were ignored and the three loci were combined. In this combined analysis, *C. boppii*, an agent of cutaneous infection, was taken as an out-group for clade I (Fig. 2). The combined analysis shows that both the pathogenic species *C. carrionii* (represented by 6 strains) and the environmental species *C. yegresii* (represented by 3 strains) are monophyletic and well supported (100 % bootstrap, Fig. 2). The two species of *Phialophora* (*P. verrucosa* and *P. americana*) are sister taxa (100 % bootstrap, Fig. 2), and are nested among species of *Cladophialophora*. Two newly investigated strains (CBS 122642, isolated from soft drink; CBS 259.83, isolated from a patient with chromoblastomycosis) are shown to be phylogenetically distinct from other members of Clade I, and are described below as new species (*C. samoënsis* and *C. subtilis*).

Bantiana-clade dataset

Phylogenetic reconstructions of Clade II (*bantiana*-clade, Fig. 3) were first carried out for each gene separately (ITS: 495 characters, *EF1-α*: 133 characters, and *TUB*: 355 characters). As topological incongruence was detected only within the species *C. saturnica* (relationships obtained with ITS differed from relationships obtained with EF1- α or *TUB*), the loci were combined. The resulting tree was rooted using *C. mycetomatis*, a species newly described here. A phylogenetic analysis at larger scale (Fig. 1) shows that this taxon is phylogenetically distinct from other species of *Cladophialophora*, and sister to all the other members of the *bantiana*-clade. The three species *C. immunda*, *C. devriesii* and *C. saturnica* all form
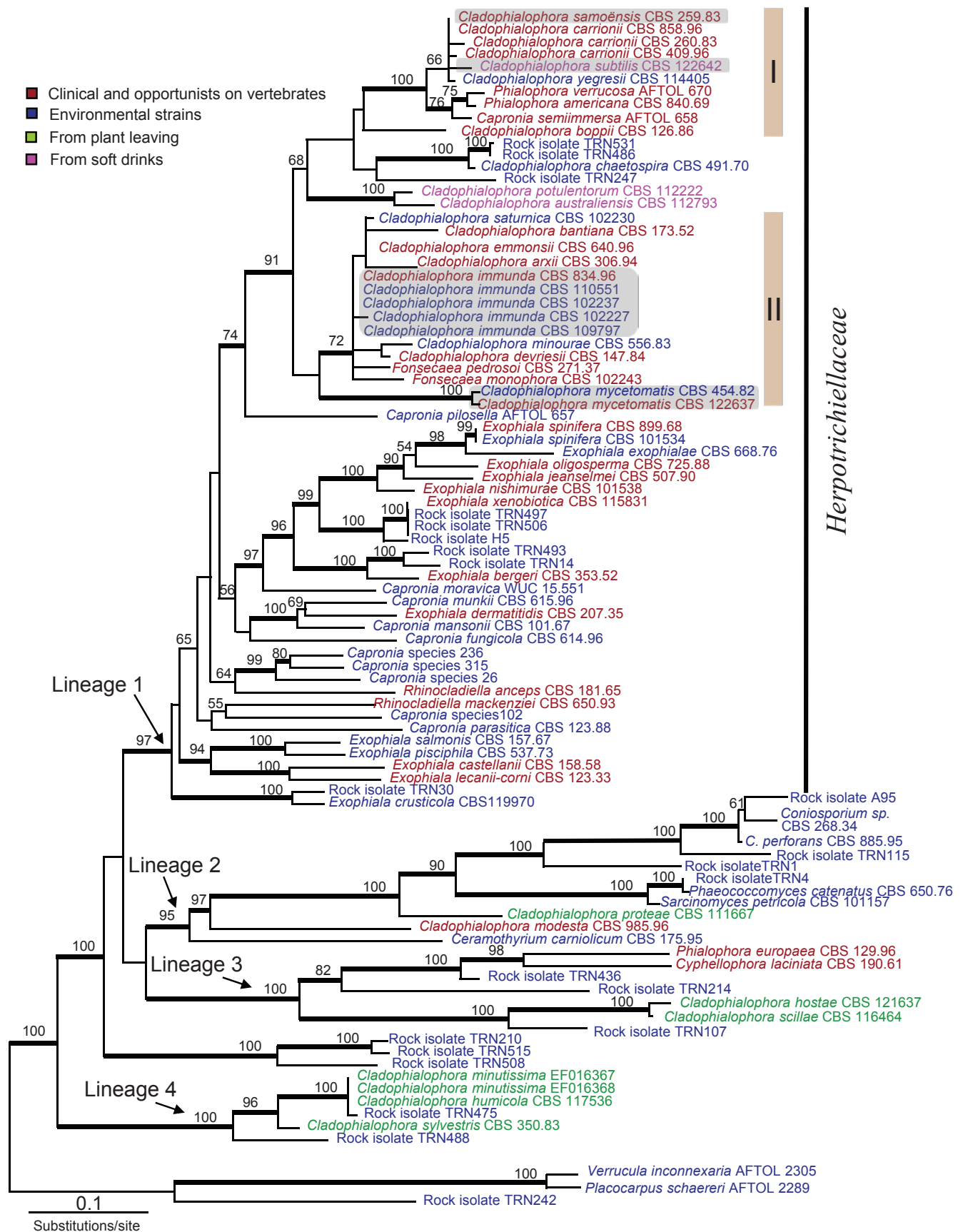
Fig.1. Phylogeny obtained from a ML analysis of three combined loci (SSU, LSU and *RPB*1) using RAxML. Bootstrap support values were estimated based on 500 replicates, and are shown above the branches (thick branch for values ≥ 70 %). The tree was rooted using *Verrucula inconnexaria*, *Placocarpus schaereri* and the rock isolate TRN242. New species are highlighted with grey boxes.
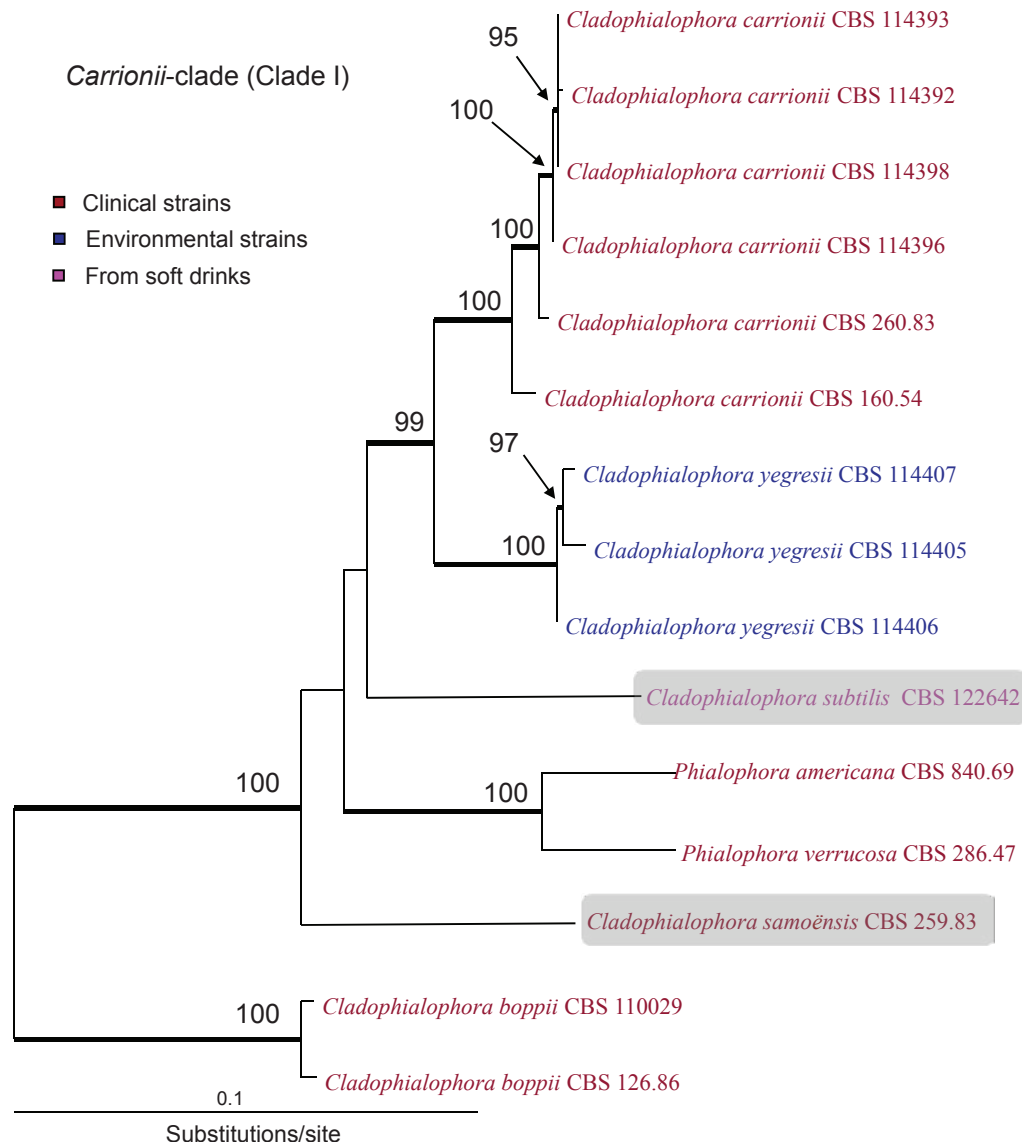
Fig. 2. Phylogeny of Clade I obtained from a ML analysis of three combined loci (ITS rDNA, *EF1-α* and *TUB*) using RAxML. Bootstrap support values were estimated based on 500 replicates, and are shown above the branches (thick branch for values ≥ 70 %). New species are highlighted using with grey boxes. *Cladophialophora boppii* (CBS 126.86 and CBS 110029) were taken as outgroup.

monophyletic clades including both clinical and environmental strains. For *C. immunda*, strains from different geographical areas and ecological preferences all cluster together. The environmental species *C. minourae* is sister to the pathogenic species *C. arxii*. The truly pathogenic species *C. bantiana* is sister to *C. emmonsii*, although with no support. Finally, the genus *Fonsecaea* forms a well-supported monophyletic group nested within this clade of *Cladophialophora*.

Physiology

The cardinal growth temperature test showed that all cultures obtained in this study had their optimal development at 27-30 °C, with growth abilities ranging between 9-37 °C. No growth was observed at 40 °C. For *C. samoënsis*, *C. immunda* and *C. mycetomatis,* the optimum growth temperature on MEA and PDA was 27 °C, with minimum and maximum of 15 and 37 °C, respectively. For all the other species, growth temperatures were identical except for the minimum temperature, which was 12 °C in *C. subtilis*. However, neither plant associated species nor strains isolated from sport drink nor apple juice (*C. australiensis* and *C. potulentorum*) had

the ability to grow at 37 and 40 ° C (Fig. 4). Growth characteristics were studied at low pH after addition of 0.1 mM $CaCl_2$ to the basal medium, inducing conversion of hyphae of *Cladophialophora* species into muriform cells when incubated at 25−37 °C (Table 3). *Cladophialophora subtilis* developed extensive mycelia and produced muriform cells at 25 °C after one wk incubation (Fig. 5). Hyphae were generally attached to these muriform cells in either terminal or intercalary positions (Fig. 5). However, no muriform cells were observed under the same conditions at 25 °C in other species (*C. immunda, C. mycetomatis* and *C. samoënsis*). Hyphae of *C. subtilis, C. immunda* and *C. samoënsis* converted to large numbers of muriform cells when incubated in the same conditions at 37 °C. Moreover, muriform cells were not observed for plant-associated species of *Cladophialophora* and for *C. mycetomatis* and *C. yegresii* neither at 25 nor at 37 °C (Table 3).

Taxonomy of *Cladophialophora*

Cladophialophora carionii (Trejos) de Hoog, Kwon-Chung & McGinnis, J. Med. Vet. Mycol. 33: 345 (1995).

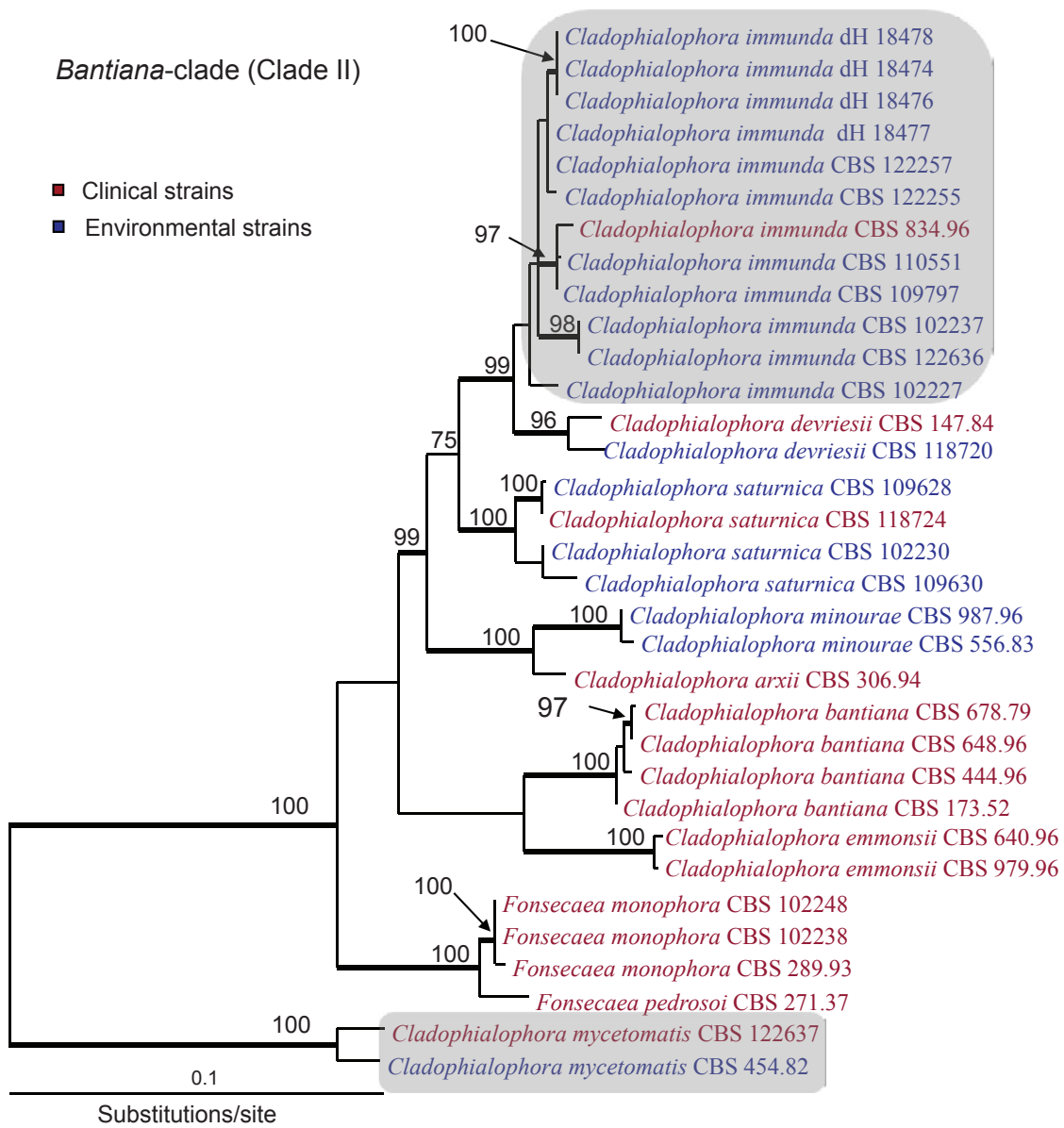≡ *Cladosporium carrionii* Trejos, *Revista de Biologia Tropicale*, Valparaiso 2:

Fig. 3. Phylogeny of Clade II obtained from a ML analysis of three combined loci (ITS rDNA, *EF1-α* and *TUB*) using RAxML. Bootstrap support values were estimated based on 500 replicates, and are shown above the branches (thick branch for values ≥ 70 %). New species are highlighted using with grey boxes. The tree was rooted with two strains of *Cladophialophora mycetomatis* (CBS 454.82 and CBS 122637).
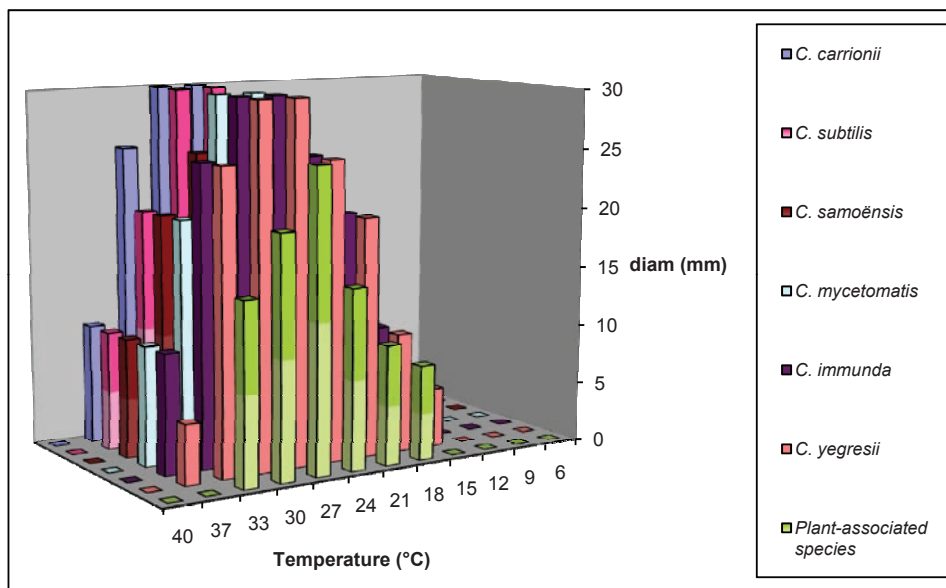

Fig. 4. Colony diameters of novel *Cladophialophora* species at different temperatures ranging from 6 to 40 °C, measured after two wks on 2 % MEA.
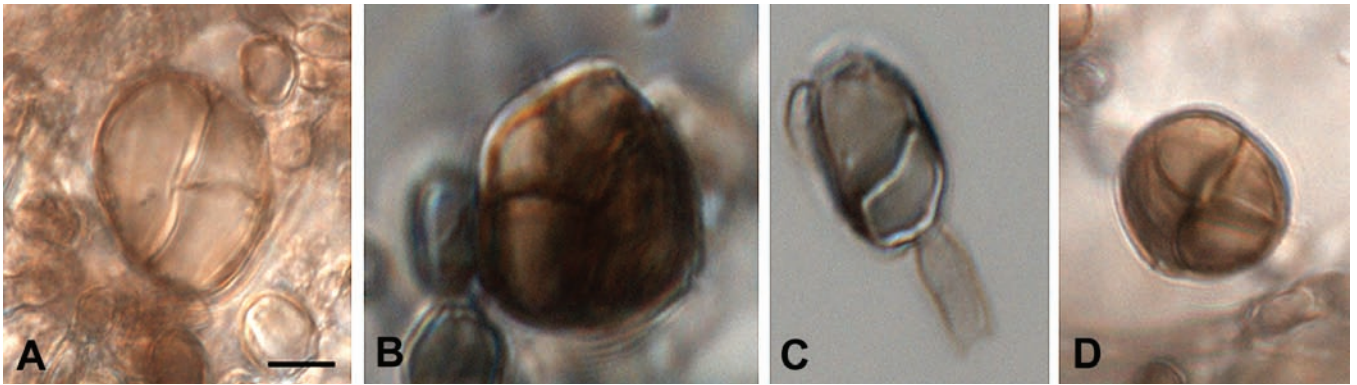
Fig. 5. Morphology of muriform cells in three species of *Cladophialophora*. A–B. *Cladophialophora subtilis*. C. *Cladophialophora immunda*. D. *Cladophialophora carrionii*. Scale bar = 10 µm.

Table. 3. Effect of calcium and temperature on production of muriform cells under the acidic conditions in basal medium for 13 species of *Cladophialophora*.

Cladophialophora species	Muriform Cells at 25 °C, 0.1 mM Ca²⁺ and pH 2.5	Muriform Cells at 37 °C, 0.1 mM Ca²⁺ and pH 2.5
C. carrionii	+	++
C. samoënsis	–	+
C. subtilis	+	+++
C. mycetomatis	–	–
C. immunda	–	+
C. australiensis	–	–
C. potulentorum	–	–
C. yegresii	–	–
C. sylvestris	–	–
C. humicola	–	–
C. hostae	–	–
C. proteae	–	–
C. scillae	–	–

No production of muriform cells is indicated by –, low production of small muriform cells by +, moderate production of large muriform cells by ++, and large production of large muriform cells by +++.

106 (1954)
= *Cladophialophora ajelloi* Borelli, *Proceedings of the 5th International Conference on Mycoses*: 335 (1980)

Type: Trejos 27 (CBS H-18465, lectotype designated here; CBS 160.54 = ATCC 16264 = CDC A-835 = MUCL 40053, ex-type).

Trejos (1954) introduced *Cladophialophora carrionii* but he did not indicate a holotype. For this reason, the isolate Trejos 27 = Emmons 8619 = CBS 160.54, the first strain mentioned by Trejos (1954), is selected here as **lectotype** for *C. carrionii*. The ex-type strain of *Cladophialophora ajelloi*, CBS 260.83, proved to be indistinguishable from *C. carrionii* based on both morphology and molecular data. This former species was also known to be able to produce phialides in addition to catenate conidia (Honbo *et al.* 1984). *Cladophialophora ajelloi* is here proposed as a taxonomic synonym of *C. carrionii*.

Cladophialophora samoënsis Badali, de Hoog & Padhye, **sp. nov**. MycoBank MB511809. Fig. 6–7.

Etymology: Named after Samoa, the Pacific Island where the species was encountered in a human patient.

Coloniae fere lente crescentes, ad 30 mm diam post 14 dies, olivaceo-virides vel griseae, reversum olivaceo-nigrum. Cellulae gemmantes absentes. Hyphae leves, hyalinae vel pallide brunneae, 2–3 µm latae. Conidiophora semi-macronemata, septata, lateralia vel terminalia; stipites et ramoconidia denticulata. Conidia holoblastica, dilute brunnea, late fusiformia, unicellularia, levia, catenas longas ramosas cohaerentes formantia, cicatricibus dilute brunneis, 3–4 × 2–3 µm. Synanamorphe not visa. Chlamydosporae absentes. Teleomorphe ignota.

Description based on CBS 259.83 at 27 °C on MEA after 2 wks in darkness.

Cultural characteristics: Colonies growing moderately slowly, reaching up to 30 mm diam, olivaceous-green to grey, with a thin, dark, well-defined margin, dry, velvety, darker after 4 wks; reverse olivaceous-black. Cardinal temperatures: minimum 15 °C, optimum 27−30 °C, maximum 37 °C. No growth at 40 °C.

Microscopy: Budding cells absent. Hyphae smooth, hyaline to pale brown, branched, 2−3 µm wide, locally forming hyphal
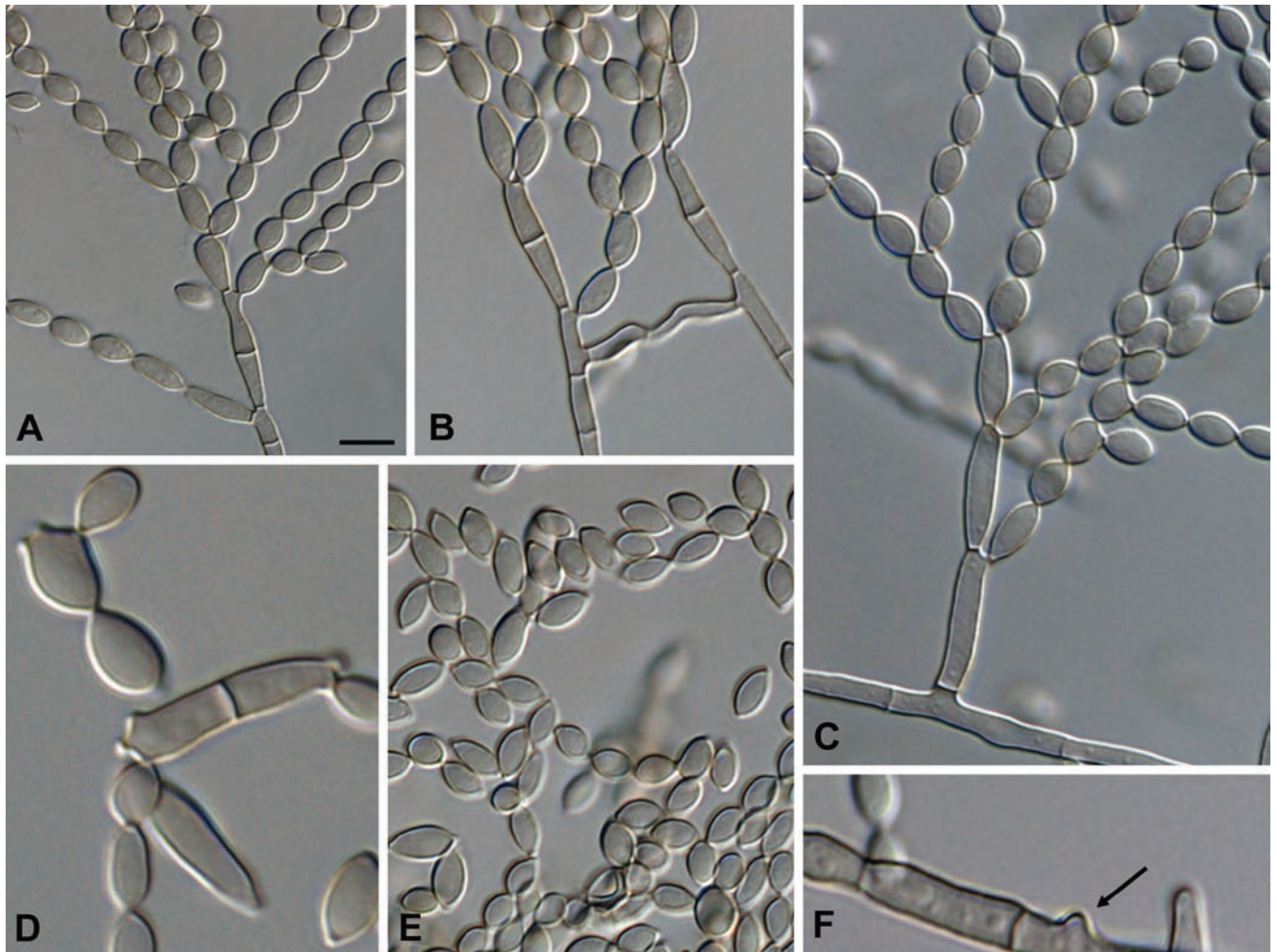
Fig. 6. *Cladophialophora samoënsis* (CBS 259.83). A. Conidiophore. B–E. Conidial chains with ramoconidia and conidia. F. Conidiogenous loci (arrows). Scale bar = 10 µm.

strands and coils. Conidiophores semi-macronematous, septate, lateral or terminal, with denticles on the stipe and on 0–1-septate ramoconidia. Conidia holoblastic pigmented, broadly fusiform, one-celled, pale brown, smooth-walled, forming long, cohering, branched acropetal chains of conidia; conidial scars pale. Conidia 3–4 × 2–3 µm. Synanamorph not seen. Chlamydospores absent. Teleomorph unknown.

Specimen examined: **U.S.A.**, Samoa (Pacific)*,* isolated from human patient with chromoblastomycosis, November 1979 (CBS H-20113, **holotypus**; CDC B-3253 = CBS 259.83, ex-type).

Notes: This strain (CBS 259.83, from Samoa) was previously identified as *C. ajelloi* (Goh *et al*. 1982). In our multigene analysis, it clustered within the *carrionii* complex (Clade I; Fig. 2), but proved to be consistently different from all described species.
Case Report: According to the description by Goh *et al*. (1982), a healthy, 43-yr-old male patient had a 5 × 3 cm erythematous, scaling lesion on his arm which was first observed about 3 yr earlier. The patient did not remember the circumstances under which the infection had been acquired. Muriform cells were revealed in superficial dermis and stratum corneum after skin scrapings. Histological examination showed segments of well-differentiated stratified squamous epithelium with moderate keratin production and underlying coarsely fibrillar dermal connective tissue. In this tissue, dense aggregates of chronic inflammatory cells, including lymphocytes, plasma cells and multinucleated foreign bodies (giant cells), were observed. Hematoxylin and eosin (H&E) and periodic

acid-Schiff (PAS) stained sections characterized dark brown, thick-walled, multiseptate muriform cells, measuring 6−12 µm in diameter, and dividing by fission. The histopathological observations led to the diagnosis of chromoblastomycosis, and the strain was identified as *C. ajelloi* (Goh *et al*. 1982). The taxon name *C. ajelloi* is not available for this taxon, as it was shown here to be a synonym of *C. carrionii*. Hence *Cladophialophora samoënsis* is described as a novel agent of chromoblastomycosis. Morphological observation showed branched chains of holoblastic conidia identical to those of *Cladophialophora carrionii*.

Cladophialophora subtilis Badali & de Hoog, **sp. nov**. MycoBank MB511842. Figs 8, 11.

Etymology: Named after thin-walled, conidial structures.

Coloniae fere lente crescentes, ad 30 mm diam post 14 dies, velutinae, olivaceo-virides vel griseae, reversum olivaceo-nigrum. Cellulae gemmantes absentes. Hyphae leves, hyalinae vel pallide brunneae, 2−3 µm latae. Conidiophora micronemata, septata, lateralia vel terminalia; stipites et ramoconidia denticulata. Conidia holoblastica, dilute brunnea, late fusiformia, unicellularia, levia, catenas longas ramosas cohaerentes formantia, cicatricibus dilute brunneis, 5−6 × 2−3 µm. Synanamorphe not visa. Chlamydosporae absentes. Teleomorphe ignota.

Description based on CBS 122642 at 27 °C on MEA after 2 wks in darkness.

Cultural characteristics: Colonies growing slowly, reaching 30 mm
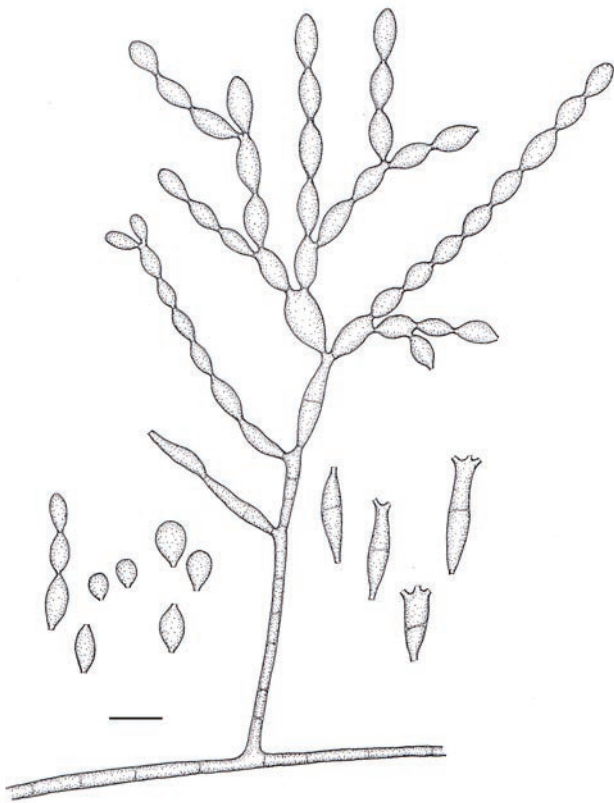
Fig. 7. Microscopic morphology of *C. samoënsis* (CBS 259.83). Branched conidial chains with ramoconidia and conidia. Conidiophores septate, lateral or terminal, with denticles on the stipe and on 0–1-septate ramoconidia. Scale bar = 10 μm.
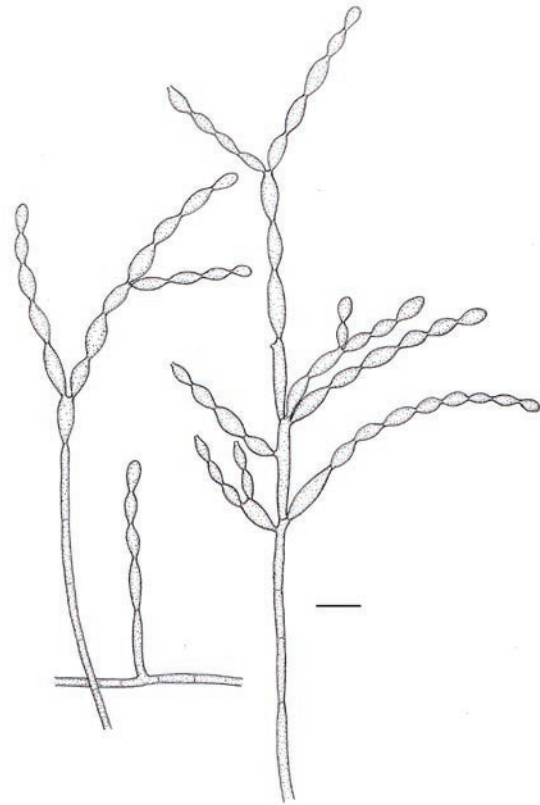

Fig. 8. Microscopic morphology of *C. subtilis* (CBS 122642). Fertile hyphae septate, ascending to erect. Conidiophores apically branched, cylindrical to sub-cylindrical Branched conidial chains with ramoconidia and conidia. Scale bar = 10 μm.
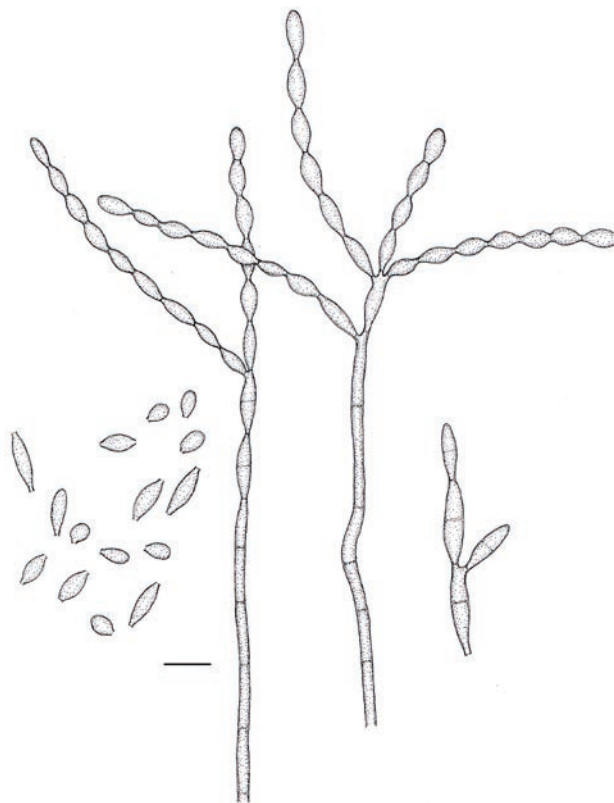

Fig. 9. Microscopic morphology of *C. mycetomatis* (CBS 122637 and CBS 454.82). Septate hyphae creeping, ascending to sub-erect. Conidiophores solitary, micronematous, cylindrical, apically branched. Conidia holoblastic, fusiform produced in long chains. Scale bar = 10 μm.
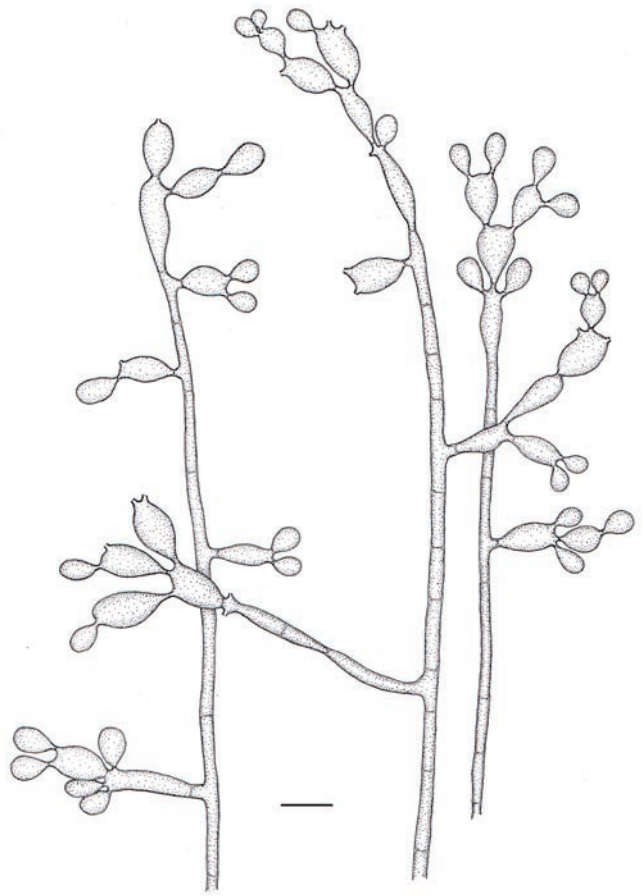

Fig. 10. Microscopic morphology of *C. immunda* (CBS 834.96, CBS 109797, CBS 110551, CBS 102227, CBS 102237). Hyphae branched, septate, straight, ascending to erect, Hyphae giving rise to conidiophores. Lemon-shaped to pyriform to guttuliform, narrowed towards one or both ends, coherent or deciduous. Scale bar = 10 μm.
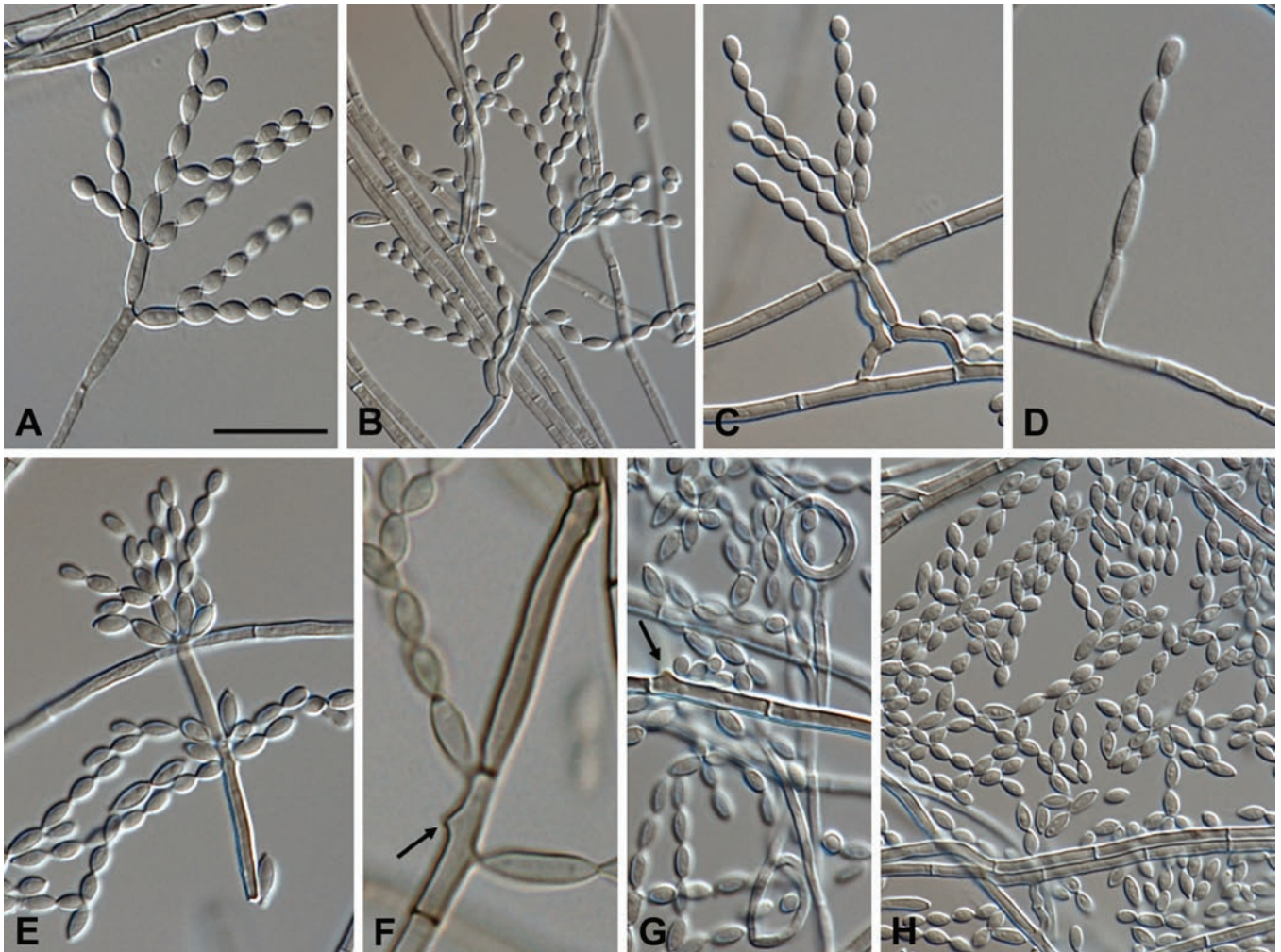
Fig. 11. *Cladophialophora subtilis* (CBS 122642). A–D. Conidiophores with branched conidial chains and ramoconidia. E. Sympodial conidiogenesis. F–G. Conidiogenous loci (arrows). H. Conidia. Scale bars = 10 μm.

diam (10 mm at 37 °C on PDA). Colonies velvety, olivaceous-black, with a wide well defined margin darker than the colony centre, and a compact suede-like to downy surface; reverse olivaceous-black. Cardinal temperatures: minimum 12 °C, optimum 27−30 °C, maximum 37 °C. No growth at 40 °C.

Microscopy: Fertile hyphae septate, ascending to erect, smooth, thin-walled, hyaline to pale olivaceous, guttulate, branched, 2−3 μm wide, forming hyphal strands and hyphal coils. Conidiophores either micronematous, erect, sub-cylindrical, often reduced to a conidiogenous cell, or semi-macronematous, with stalks 60−80 μm long, guttulate, hyaline to pale olivaceous, cylindrical to sub-cylindrical, apically branched, 2.5−3 μm wide. Conidiogenous cells pale brown, slightly darker than conidia, sub-cylindrical to fusiform, with pigmented scars, smooth-walled, proliferating sympodially with 1−3, denticle-like extensions and guttulate. Ramoconidia present. Conidia one-celled, produced in long coherent chains, subhyaline to hyaline, smooth, thin-walled, guttulate; conidia and ramoconidia ellipsoidal to ovoid, 5−6 × 2−3 μm, non-septate. Chlamydospores absent. Teleomorph unknown.

Specimen examined: **The Netherlands**, Utrecht, isolated from ice tea, December 2004, (CBS H-20114, **holotypus**; CBS 122642 = dH 14614, ex-type).

Notes: The species is morphologically similar to *C. carrionii*, an agent of chromoblastomycosis. However, *C. subtilis* has distinct erect conidiophores which arise at right angles from fertile hyphae; conidiogenous cells are pale brown, slightly darker than conidia, sub-

cylindrical to fusiform, with pigmented scars, proliferating sympodially with 1−3, denticle-like extensions.

The species is known from a single strain originating from commercial ice tea. Several *Cladophialophora* species have been isolated from sugared drinks (*C. potulentorum, C. australiensis*), while pathogenic *Exophiala* species also have a preference for sugar-rich surfaces of fruits (Sudhadham *et al.* 2008). The association of Chaetothyrialean anamorphs with drinks is thus not surprising. The group is also associated with human disorders (de Hoog *et al.* 2000, Levin *et al.* 2004). Our species has the ability to grow at 37 °C and produces muriform cells when incubated at 25 and 37 °C at low pH (pH = 2.5). Further studies are required to evaluate its pathogenic ability.

Cladophialophora mycetomatis Badali, de Hoog & Bonifaz, **sp. nov.** MycoBank MB511843. Figs 9, 12.

Etymology: Named after the clinical picture mycetoma caused by one of the strains.

Coloniae fere lente crescentes, ad 30 mm diam post 14 dies, velutinae, olivaceo-griseae, reversum olivaceo-nigrum. Cellulae gemmantes absentes. Hyphae leves, hyalinae vel pallide brunneae, 2.5−3 μm latae. Conidiophora solitaria micronemata, dilute brunnea, cylindrica, sursum ramosa, 3.0−3.5 μm lata. Cellulae conidiogenae dilute brunneae, leves, sympodialiter proliferentes. Ramoconidia 0−(1)-septata, cylindrica vel fusiformia, 2.5−4 × 2.5−3 μm. Conidia holoblastica, dilute brunnea, late fusiformia , unicellularia, levia in catenis longis ramosis cohaerentia, cicatricibus dilute brunneis, 2.5−3 × 2−3. Synanamorphe not visa. Chlamydosporae absentes. Teleomorphe ignota.
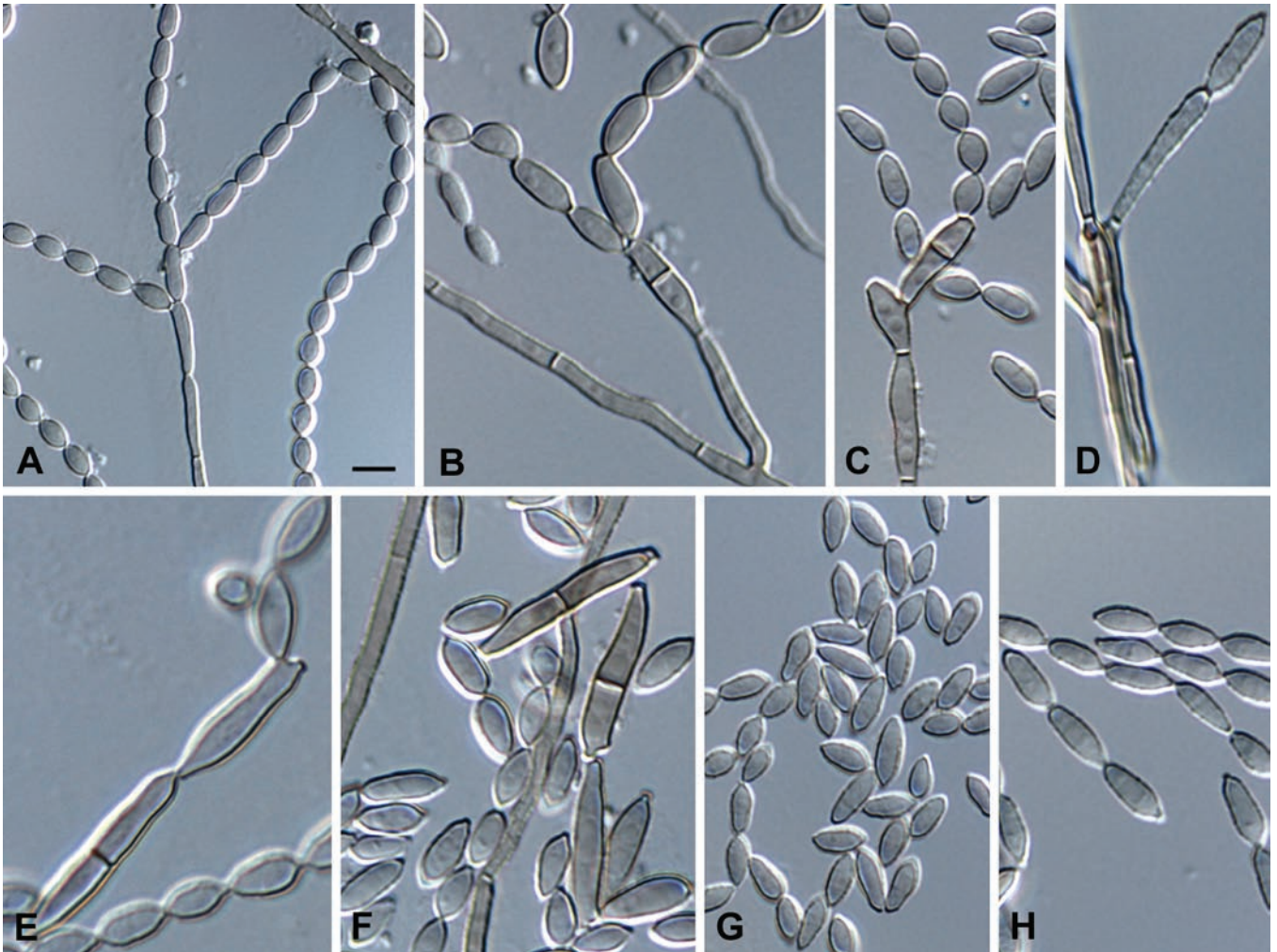
Fig. 12. *Cladophialophora mycetomatis* (CBS 122637 and CBS 454.82). A–B. Conidiophores and conidial chains with ramoconidia and conidia. C–D. Cylindrical, septate conidiophores. G. Ellipsoidal to fusiform conidia. Scale bars = 10 μm.

Description based on CBS 122637 at 27 °C on MEA after 2 wks in darkness.

Cultural characteristics: Colonies moderately expanding, reaching up to 30 mm diam, olivaceous-grey, velvety; reverse olivaceous-black. Cardinal temperatures: minimum 15 °C, optimum 27−30 °C, maximum 37 °C [maximum growth temperature 33 °C; CBS 454.82].

Microscopy: Budding cells absent. Septate hyphae creeping, ascending to sub-erect, smooth-walled, hyaline to pale olivaceous, guttulate, branched, 2.5−3.0 μm wide. Conidiophores solitary, micronematous, pale brown, cylindrical, apically branched, 3.0−3.5 μm wide. Conidiogenous cells pale brown, smooth-walled, sympodially proliferating. Ramoconidia cylindrical to fusiform, 2.5–4.0 × 2.5−3.0 μm. Conidia holoblastic, fusiform produced in long chains; subhyaline to pale olivaceous, guttulate. Chlamydospores absent. Teleomorph unknown.

Specimen examined: **Mexico**, Jicaltepec, isolated from human patient with mycetoma, 2006 (CBS H-20116, **holotypus**; CBS 122637, ex-type).

It is remarkable that an environmental strain of *C. mycetomatis* (CBS 454.82) was isolated by W. Gams as a culture contaminant in the strain of *Scytalidium lignicola* Pesante (CBS 204.71) from the Netherlands. It was morphologically very similar to *C. carrionii* but was methyl-α-D-glucoside and melibiose negative and assimilated

D-glucosamine and galactitol (de Hoog *et al.* 1995).

Case Report: A 49-yr-old male farmer mainly growing corn and resident of Jicaltepec, in the semi-arid zone Pinotepa Nacional Oaxaca, approximately 450 km south of Mexico City presented with a dermatosis localised to the left leg at the dorsum of the foot, affecting the third toe (Fig. 13A). The lesion consisted of a tumorous area, with deformation, and nodules with draining sinuses releasing thread-like material including black granules. The dermatosis had begun one and a half yr before, after a trauma with a thorn of cactaceous plant called nopal (*Opuntia* sp.). These led to progressive swelling of the region and occasional pain (Fig. 13A). Initial treatment included penicillin and sulfametoxazole-trimetroprim. The presumptive clinical diagnosis was that of mycetoma. Direct examination with KOH (10 %) showed some black granules approximately 500 μm in size. The granules were composed of branched, septate, dematiaceous hyphae (Fig. 13B). Clinical specimens cultured on Sabouraud Glucose Agar (SGA) with or without antibiotics (Mycosel) resulted in the growth of a dematiaceous fungus morphologically identified as *Cladophialophora* sp. Once the diagnosis of eumycetoma had been made, laboratory tests consisted of a complete blood count, blood chemistry and liver function tests; all of these were within normal limits. Foot radiographs (lateral and PA) showed no involvement of bones. Treatment with itraconazole, 200 mg/d was instituted, with significant clinical improvement at 8 mos. Liver and hematological function tests were monitored throughout the treatment period at
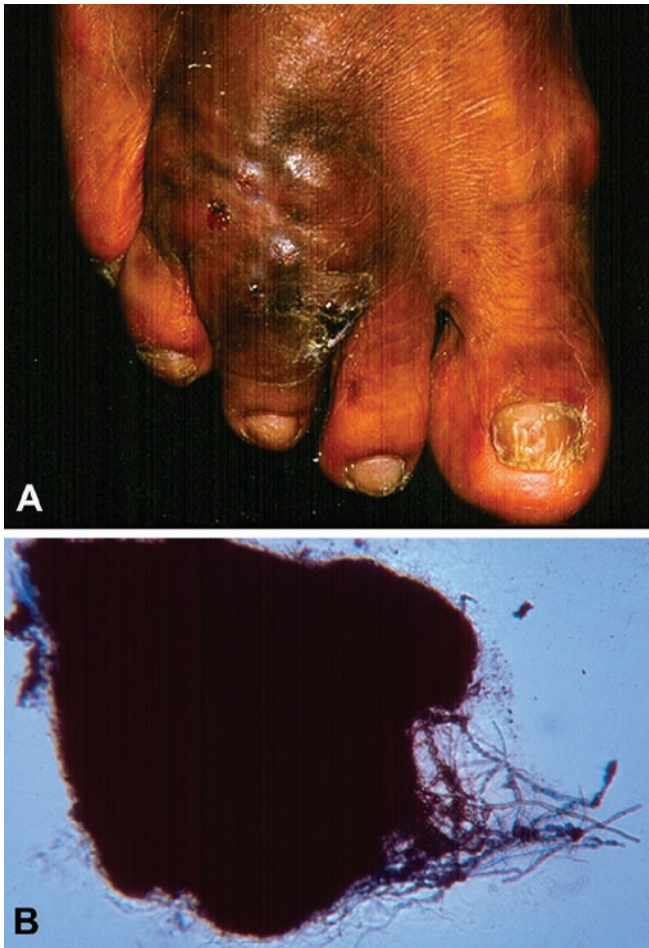
Fig.13. Clinical manifestations of Eumycetoma caused by *Cladophialophora mycetomatis*. A. Deformed tumorous area of the foot, with nodules, draining sinuses and discharging purulent fluid. B. Branched black granule, approximately 500 µm in size (in 10 % KOH), with septate hyphae.

4-mo intervals, with no alterations.

The province where the patient lived in the South of Mexico is a very poor region in a semi-arid area climate zone. The majority of inhabitants are farmers growing corn and other vegetables. Cactaceous plants are very common. The trauma with a thorn of a nopal cactus and the micromorphology of the fungus reminded of *Cladophialophora carrionii*, which is abundant under similar climatic conditions in Venezuela (de Hoog *et al.* 2007), but according to molecular data the fungus proved to be clearly separate. In addition to *Cladophialophora*, *Exophiala* species (particularly *E. jeanselmei*) are also known to cause mycetoma (de Hoog *et al.* 2003). Otherwise, subcutaneous infections by members of *Chaetothyriales* mostly lead to chromoblastomycosis-like infections which are characterised by muriform cells rather than granules, and do not lead to necrosis and draining.

Cladophialophora immunda Badali, Satow, Prenafeta-Boldú & de Hoog, **sp. nov**. MycoBank MB511844. Figs 10, 14.

Etymology: Named after its preference for polluted environments.

Coloniae fere lente crescentes, ad 30 mm diam post 14 dies 27–30 °C, velutinae, olivaceo-griseae vel olivaceo-virides, reversum olivaceo-nigrum. Cellulae gemmantes absentes. Hyphae leves, hyalinae vel pallide brunneae, adscendentes vel erectae, 2.5–4 µm latae, saepe circiter aggregatae. Conidiophora micronemata, septata. Conidia holoblastica, dilute brunnea, late fusiformia, unicellularia, levia, catenas cohaerentes formantia vel dilabentia, cicatricibus dilute brunneis, 3.0−4.5×2.5−4.0 µm. Synanamorphe non visa. Chlamydosporae vulgo absentes,

Teleomorphe ignota.

Description based on CBS 834.96 at 27 °C on MEA after 2 wks in darkness.

Cultural characteristics: Colonies growing moderately slowly, attaining up to 30 mm diam at 27−30 °C and 10 mm [5 mm in CBS 102237; 20 mm in CBS 110551] at 37 °C. Colonies dark olivaceous-grey [greyish green to olivaceous-green in CBS 102237] with a thin, dark, well-defined margin [wide grey margin in CBS 109797], spreading, downy, velvety; reverse olivaceous-black. Cardinal temperatures: minimum 15 °C, optimum 27−30 °C, maximum 37 °C. No growth at 40 °C.

Microscopy: Budding cells absent. septate hyphae , straight, ascending to erect, smooth, thin-walled, hyaline to pale brown, branched, 2−3 µm wide, frequently forming hyphal strands and coils [no hyphal coils in CBS 102237, CBS 110551]. Hyphae giving rise to conidiophores which are pale brown, erect, mostly straight, branched or unbranched, long, sub-cylindrical and cylindrical to fusiform [fusiform to ellipsoidal in CBS 110551, CBS 102227], 2.5−4 µm wide [with T-shaped foot cell in CBS 102227], with up to 6−8 septa. Conidiogenous cells branched, conspicuously denticulate, smooth-walled, guttulate, with pigmented scars. Conidia one-celled, acropetal, catenulate, sub-hyaline to pale brown [olivaceous brown in CBS 102227, CBS 102237], smooth [slightly verrucose in CBS 109797, CBS 102227], thin-walled, lemon-shaped to pyriform to guttuliform, narrowed towards one or both ends, with pale pigmented scars. Conidia 3.0−4.5 × 2.5−4.0 µm [3−4 × 2−3 µm in CBS 102227], coherent or deciduous. Phialidic synanamorph not seen. Chlamydospores absent [present in CBS 109797]. Teleomorph unknown.

Specimens examined: **U.S.A.**, Georgia, Atlanta, isolated from a sub-cutaneous ulcer on a 68-yr-old female treated with long-term immunosuppressive therapy, (CBS H-20115, **holotypus**; CDC B-6580 = CBS 834.96, ex-type). **Brazil,** Paraná, Colombo, isolated from *Syagrum romanzoffianum* stem, CBS 102227. **Brazil,** Paraná, isolated from decaying cover vegetable, CBS 102237. **Germany,** Kaiserslautern, isolated from biofilter inoculated with soil, CBS 109797. **The Netherlands**, isolated from hydrocarbon-polluted soil, CBS 110551 (Prenafeta-Boldú *et al.* 2006). **Brazil** isolated from hydrocarbon-polluted soil, CBS 122636, CBS 122255, CBS 122257, dH 18477, dH 18476, dH 18476, dH 18478 (Satow *et al.* 2008).

Case Report: According to the description by Padhye *et al.* (1999), a 68 yr-old-female who underwent long-term immunosuppressive therapy in view of a history of recurrent sinusitis, pneumonia, genital herpes, hysterectomy, chronic hypergammaglobulinemia, low grade lymphoma, Sjogren's disease, rheumatois arthritis. She had not any history of predisposing factor such as truma. The pretibial lesion was non-responsive to cephalexin or ofloxacin. Due to the clinical manifestation of the lesion, a biopsy was performed which consisted of dermis and subcutaneous tissue. Biopsy tissue section was stained by PAS (Fig. 15A) and Gomori's methanamine-silver (GMS, Fig. 15B) stains, showing septate hyphae, moniliform hyphae of different lengths, and thick-walled cells. The melanized fungal elements were within intense infiltrates of neutrophils and necrotizing granulomas with many giant cells. The histopathological observations led to the diagnosis of a subcutaneous phaeohyphomycosis infection. The strain was identified as *Cladophialophora* species (Padhye *et al.* 1999) resembling *C. devriesii* and *C. arxii*. It formed dry conidia in branched acropetal chains inserted on pronounced denticles, fusiform to lemon-shaped conidia, and being involved in a human infection. Unlike *C. arxii*,
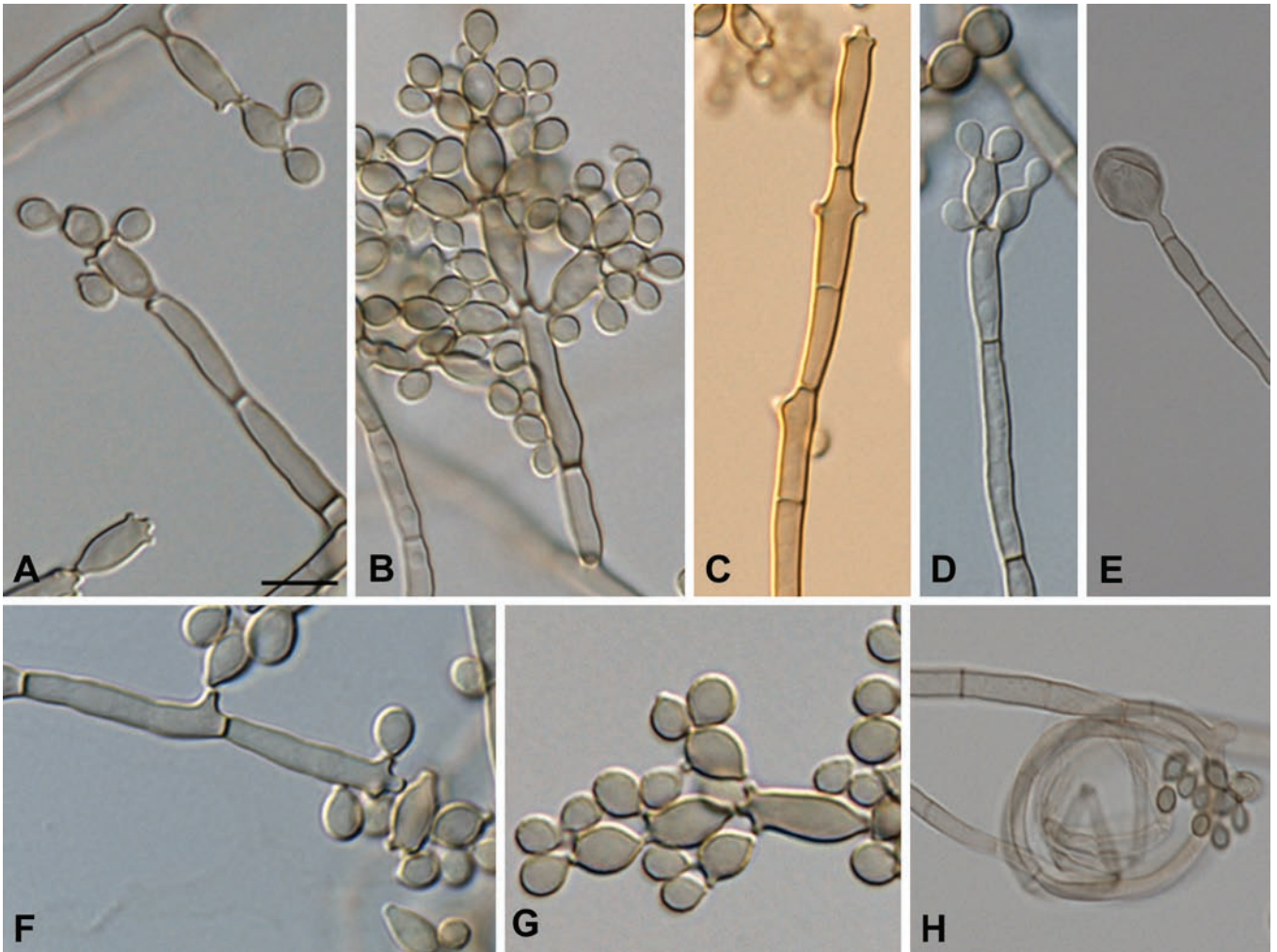
Fig. 14. *Cladophialophora immunda* (CBS 834.96, CBS 109797, CBS 110551, CBS 102227, CBS 102237). A–D. Conidiophores and conidial apparatus with T-shaped foot cell and cylindrical, septate, denticulate conidiogenesis. E. Chlamydospores. F–G. Thin-walled, lemon-shaped to pyriform to guttuliform conidia. H. Hyphal coil. Scale bar = 10 µm.
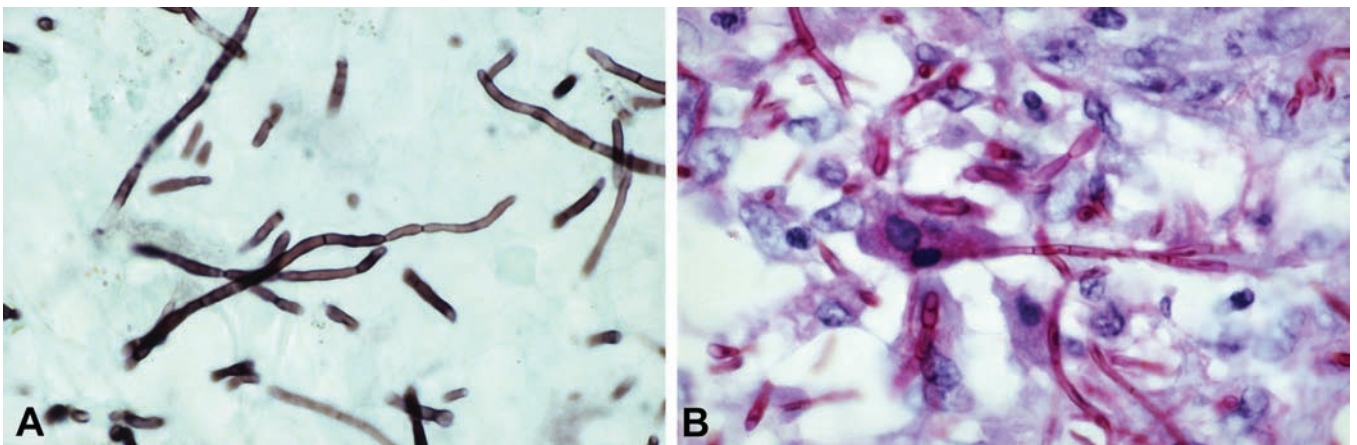

Fig. 15. *Cladophialophora immunda* (CBS 834.96). A. Gomori Methenamine-silver and B. Periodic acid-Schiff stained sections revealed septate hyphae, moniliform hyphae of different lengths, and thick-walled cells. (GMS & PAS × 360)

the species did not grow above 37 °C. We identified the strain with several environmental isolates by multilocus sequencing (Figs 1, 3 and Table 1). For the environmental strains, a striking association with aromatic hydrocarbons was observed: eight out of twelve strains of *C. immunda* originated from hydrocarbon-polluted soils. An association with assimilation of toxic monoaromatic compounds and infective potentials have been hypothesised (Prenafeta-Boldú *et al.* 2006). Black yeasts and their filamentous relatives in the ascomycete order *Chaetothyriales* are potent degraders of monoaromatic compounds and eventually tend to accumulate in industrial biofilters (Cox *et al.* 1997, Prenafeta-Boldú *et al.* 2001, de Hoog *et al.* 2006).

DISCUSSION

Cladophialophora-like anamorphs are polyphyletic

The genus *Cladophialophora* is morphologically characterized by poorly or profusely branched chains of dry, rather strongly coherent, moderately melanized conidia. Our results show that this *Cladophialophora* anamorphic type is convergent, as the genus *Cladophialophora* is polyphyletic. In our combined phylogeny of the *Chaetothyriales* (Fig. 1), the plant-associated species (*C. hostae, C. scillae, C. proteae, C. sylvestris, C. minutissima,* and *C. humicola*) appeared to be closer to *Ceramothyrium carniolicum* (a putative member of the family of *Chaetothyriaceae*), and only distantly related to most remaining *Cladophialophora* species. It is interesting to note that *Cladophialophora* species that are consistently associated with pathology to humans mostly belong to the *Herpotrichiellaceae*, a family particularly diverse in human opportunists (*e.g.*, *Exophiala*, *Fonsecaea*, *Rhinocladiella*, and *Veronaea*). The lineages to which the plant-associated species of *Cladophialophora* (lineages 2, 3 and 4) belong contain comparatively few human opportunists, all of them causing only mild cutaneous infections (*e.g.*, *Phialophora europaea*, and *Cyphellophora laciniata*), or traumatic infection (*Cladophialophora modesta*).

Similar Cladophialophora-like morphologies have also been observed in several unrelated environmental fungi, particularly in *Cladosporium*, *Pseudocladosporium* and *Devriesia* (Braun *et al.* 2003, Crous *et al.* 2007, Seifert *et al.* 2004). These genera are assigned to different families within the *Dothideales* and the *Capnodiales*, two ascomycete orders for which species are only exceptionally encountered in a clinical setting. Moreover, the conidial scars have a pale pigmentation in *Cladophialophora*, which is in contrast to members of the saprobic genera *Cladosporium*, *Devriesia* and *Pseudocladosporium*, where pronounced darker conidial scars are present. Furthermore, conidiophores in most *Cladophialophora* species are poorly differentiated, while those of *Cladosporium* species are usually erect and significantly darker than the rest of the mycelium. Finally, conidial chains of *Cladophialophora* species are coherent, while those of *Cladosporium* detach very easily.

Evolution of pathogenicity in *Cladophialophora*

Melanin pigments are common to all Chaetothyriales, although little is known concerning the pathogenic mechanisms by which these fungi cause disease, particularly in immunocompetent individuals. However, the production of melanin was shown to be involved in pathogenicity. Melanins are pigments of high molecular weight formed by oxidative polymerization of phenolic compounds and usually are dark brown or black; in case of fungal pathogens melanin appears to function in virulence by protecting fungal cells against fungicidal oxidants, by impairing the development of cell-mediated responses, interfere with complement activation and reduce the susceptibility of pigmented cells to antifungal agents. In the environment, they protect organisms against environmental factors (Butler *et al.* 1998, Jacobson 2000).

Another putative virulence factor is thermotolerance. According to de Hoog *et al.* (2000), species of *Cladophialophora* show a differential maximum growth at temperatures more or less coinciding with clinical predilections, species causing systemic infections being able to grow at 40 °C. The agents of chromoblastomycosis have a growth maximum at 37 °C, while a mildly cutaneous species, such as *C. boppii*, is not able to grow at this temperature (de Hoog *et al.* 2000). In the present study, all studied strains of *Cladophialophora* had an optimum growth around 27 °C, and were still able to grow at 37 °C, but not at 40 °C (Fig. 4). This observation agrees with the prevalent nature of these species as environmental saprobes, and their potential to cause superficial infections in humans, similarly to other opportunistic species. Tolerance of human body temperature is an essential requirement for pathogenicity, but this trait may have incidentally been acquired via adaptation to warm environmental habitants, such as hot surfaces in semiarid climates.

Early experiments involving the inoculation of *Cladophialophora* in several species of cold-blooded animals have shown the abundant production of characteristic muriform cells *in vivo* (Trejos 1953). For *C. yegresii* and *C. carrionii*, the muriform propagules are also present in cactus spines (de Hoog *et al.* 2007); in this plant host, they can be regarded as an extremotolerant survival phase, and are likely to play an essential role in the natural life cycle of these organisms. The capacity of some *Herpotrichiellaceae* to grow in a meristematic form both in human hosts and in extreme environmental conditions supports the suggestion that the muriform cells may indeed be a main virulence factor in the development of the disease, representing an adaptation to the conditions prevailing in host tissues. The conversion of hyphae to muriform cells can be induced *in vitro* under acidic conditions (pH = 2.5) and low concentration of calcium. In the present study, we observed different morphogenetic responses between environmental and clinical species of *Cladophialophora*. *C. subtilis* isolated from ice tea, under the above circumstances formed structures resembling muriform cells at 27 °C which became larger when incubated at 37 °C. The pathogenic *C. samoënsis* and *C. immunda* also produced muriform cells at 37 °C. Muriform cells were not observed, neither on the plant-associated species (plant leaving) of *Cladophialophora*, nor in the *C. mycetomatis* isolated from both environment and a patient.

CONCLUSIONS

Some members of *Herpotrichiellaceae* grow as ordinary filamentous moulds in pure culture, but can also be induced to form strongly melanized, isodiametrically expanding meristematic cells *in vitro*. This type of cells, dividing by sclerotic fission, is characteristically found in chromoblastomycosis. However, they can also be observed when these fungi grow in natural niches, in particular the ones characterized as extreme (*e.g.*, *F. pedrosoi* within dried cactus thorns; Sterflinger *et al.* 1999) and Vicente *et al.* (2008) have shown that the hydrocarbon-polluted environments yielded yet another spectrum of chaetothyrialean fungi. A generalized suite of adaptations to extreme environments, including melanin production and meristematic growth, as well as thermotolerance, is suggested to contribute to pathogenic potential (Gueidan *et al.* 2008). Many of the natural and artificial habitats that are associated with growth of *Herpotrichiellaceae*, such as decaying tree bark and creosoted poles and ties, are likely to favor fungi that, in addition to being able to break down the aromatic compounds occurring in these substrata, have a generalized set of adaptations to conditions that at least occasionally become highly stressful (extreme temperatures, pH, low availability of nutrients and growth factors, etc.). Many of these adaptations may incidentally predispose fungi towards human opportunistic pathogenesis.

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