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Molecular analysis and pathogenicity of the *Cladophialophora carrionii* complex, with the description of a novel species

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Abstract: *Cladophialophora carrionii* is one of the four major etiologic agents of human chromoblastomycosis in semi-arid climates. This species was studied using sequence data of the internal transcribed spacer region of rDNA, the partial β-tubulin gene and an intron in the translation elongation factor 1-alpha gene, in addition to morphology. With all genes a clear bipartition was observed, which corresponded with minute differences in conidiophore morphology. A new species, *C. yegresii*, was introduced, which appeared to be, in contrast to *C. carrionii*, associated with living cactus plants. All strains from humans, and a few isolates from dead cactus debris, belonged to *C. carrionii,* for which a lectotype was designated. Artificial inoculation of cactus plants grown from seeds in the greenhouse showed that both fungi are able to persist in cactus tissue. When reaching the spines they produce cells that morphologically resemble the muriform cells known as the "invasive form" in chromoblastomycosis. The tested clinical strain of *C. carrionii* proved to be more virulent in cactus than the environmental strain of *C. yegresii* that originated from the same species of cactus, *Stenocereus griseus*. The muriform cell expressed in cactus spines can be regarded as the extremotolerant survival phase, and is likely to play an essential role in the natural life cycle of these organisms.

Taxonomic novelty: *Cladophialophora yegresii* de Hoog, sp. nov.
Key words: Cactus, chromoblastomycosis, *Cladophialophora*, endophyte, extremotolerance, phylogeny, taxonomy.

INTRODUCTION

Cladophialophora carrionii (Trejos) de Hoog, Kwon-Chung & McGinnis is one of the most frequent etiologic agents of human chromoblastomycosis, a chronic cutaneous disease characterised by verrucose skin lesions eventually leading to emerging, cauliflower-like eruptions. The species is particularly observed in arid and semi-arid climates of e.g. South and Central America (Lavelle 1980) and Australia (Trejos 1954, Riddley 1957). The current hypothesis is that patients suffering from chromoblastomycosis are rural workers who acquire the infection after being pricked by cactus thorns or splinters (Rubin *et al*. 1991, Fernández-Zeppenfeldt *et al*. 1994). A classical case reported by O'Daly (1943) concerns traumatic inoculation with thorns of "guazábara" (*Opuntia caribaea*), a common xerophilic plant in semi-arid Venezuela. This hypothesised traumatic route of infection was later supported by Richard-Yegres & Yegres (Richard-Yegres & Yegres 1987; strain SR3 = CBS 863.96) and Fernández-Zeppenfeldt *et al*. (1994), who isolated strains from *Prosopis juliflora* litter. *Cladophialophora* Borelli has also been detected in association with spines of the common xerophyte *Aloe vera* and of the *Cactaceae*: *Opuntia caribaeae*, *O. caracasana*, *Stenocereus griseus* and *Cereus lanuginosus* (Borelli 1972, Yegres *et al*. 1996). Thorny American cacti are important components of the xerophyte flora of the arid climate of our study area in Falcon State, Venezuela (Richard-Yegres *et al*. 1992). Muriform cells are produced in human skin and represent the supposed pathogenic invasive form of fungi causing chromoblastomycosis (Mendoza *et al*. 1993). Early experiments involving the inoculation of several species of cold-blooded animals have shown the abundant production of the characteristic muriform cells *in vivo* (Trejos 1953).

A similar plant origin of chromoblastomycosis has been supposed for a related agent of chromoblastomycosis, *Fonsecaea pedrosoi* (Brumpt) Negroni. Marques *et al*. (2006) isolated this species from the shells of Babassu coconuts (*Orbignya phalerata*). The habit of local people to sit on these shells might explain the frequent occurrence of lesions on the buttocks (Silva *et al*. 1995). Salgado *et al*. (2004) found the species on the thorns of a *Mimosa pudica* plant which a patient could identify as the source of traumatic onset of his chromoblastomycosis.

Recently, with the development of molecular tools for species identification, doubt has arisen about the correctness of this supposed route of infection. The question whether environmental and clinical strains represent exactly the same species needs to be re-determined. In order to establish this for *C. carrionii*-associated chromoblastomycosis, reference strains from the CBS culture collection, supplemented with a large set of strains from semi-arid Venezuela, have been verified using molecular tools that are currently routinely employed to answer taxonomic questions in black yeasts and their filamentous relatives (de Hoog *et al*. 2003), particularly the internal transcribed spacer (ITS) region of rDNA, the partial β-tubulin gene (BT2), and an intron in the translation elongation factor 1-alpha (EF1). In addition, a series of three experiments has been conducted concerning inoculation into and superficial application onto germlings of *Stenocereus griseus* obtained by cultivation *in vitro*, mature plants of *S. griseus* from the wild, and in spines of *S. griseus* collected in the semi-arid area of study. Our aim is to reveal the role of the cactus *S. griseus* in the life cycle of its associated *Cladophialophora* spp., and to determine whether a link could be made to *C. carrionii* for obtaining a better understanding of human chromoblastomycosis.

MATERIALS AND METHODS

Fungal strains and morphology

Strains studied are listed in Table 1. This list comprises strains which have morphologically been identified as *C. carrionii*. Reference strains from the CBS culture collection, as well as fresh isolates from patients and the environment have been included. Strains were lyophilised and stored in liquid nitrogen soon after deposit at CBS. Stock cultures for transient working collections were grown 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 potato-dextrose agar (PDA) (de Hoog *et al*. 2000) and mounted in lactophenol cotton blue.

Table 1. Isolation data of *Cladophialophora* strains examined.

Name	CBS nr.	Other reference(s)[1]	GenBank ITS, BT2, EF1	mtDNA* (Kawasaki *et al.* 1993)	Source [human: duration, localization, sex, age (Pérez-Blanco *et al.* 2003)].	Geography
A / I / 1: *C. carrionii*						
	117904	UNEFM 0004-02 = dH 14480	EU137281, –, –		Chromoblastomycosis; 14 y; hip, thigh, leg; male 38	Falcon State, Venezuela
	117891	UNEFM 0002-00 = dH 14475	EU137278, –, EU137222		Chromoblastomycosis; 1 y; male 62	Falcon State, Venezuela
	117906	UNEFM 0014-96 = dH 14504	EU137288, EU137171, EU137231		Chromoblastomycosis; 0.5 y; hand; male 45	Falcon State, Venezuela
	117897	UNEFM 0011-03 = dH 14497	EU137314, –, EU137254		Chromoblastomycosis; 0.5 y; hand; male 42	Falcon State, Venezuela
	859,96	UNEFM 9617 = dH 10703	EU137295, EU137178, EU137237		Dry plant debris, arid zone	Falcon State, Venezuela
	117898	UNEFM 0010-98 = dH 14496	EU137308, –, EU137246		Chromoblastomycosis; 20 y; hand; female 59	Falcon State, Venezuela
	117889	UNEFM 0003-04 = dH 14478	–, EU137190, –		Chromoblastomycosis; 20 y; thigh, leg; female 78	Falcon State, Venezuela
	114392	UNEFM 82267 = dH 13261	EU137267, EU137150, EU137211		Chromoblastomycosis; leg; female	Falcon State, Venezuela
	114394	UNEFM 9803 = dH 13263	EU137307, –, EU137245		Chromoblastomycosis; hand; male 22	Falcon State, Venezuela
	114396	UNEFM 2001/1 = dH 13265	EU137269, EU137152, EU137213		Chromoblastomycosis; arm; male 35	Falcon State, Venezuela
	114399	UNEFM 2003/2 = dH 13268	EU137272, EU137155, EU137216		Chromoblastomycosis; arm; female 64	Falcon State, Venezuela
	114401	UNEFM 9901 = dH 13270	EU137274, EU137157, EU137218		Chromoblastomycosis; arm; female 40	Falcon State, Venezuela
	114402**	UNEFM 9902 = dH 13271	EU137275, EU137158, EU137219		Chromoblastomycosis; arm; female 40	Falcon State, Venezuela
	114403	UNEFM 95195 = dH 13272	EU137276, EU137159, EU137220		Chromoblastomycosis; arm; male	Falcon State, Venezuela
	117899	UNEFM 0010-04 = dH 14495	EU137301, EU137183, EU137241		Chromoblastomycosis; 2 y; hand; male 57	Falcon State, Venezuela
	117901	UNEFM 0009-03 = dH 14492	EU137312, EU137197, EU137252		Chromoblastomycosis; 8 y; arm; female 41	Falcon State, Venezuela
	114393	UNEFM 9801 = dH 13262	EU137268, EU137151, EU137212		Chromoblastomycosis; hand; male 72	Falcon State, Venezuela
	108.97**	UNEFM 9501 = dH 10704	EU137306, EU137188, EU137265		Chromoblastomycosis; skin	Falcon State, Venezuela
	114397	UNEFM 84020 = dH 13266	EU137270, EU137153, EU137214		Chromoblastomycosis; hand, arm; male 54	Falcon State, Venezuela
	114404	UNEFM 95656 = dH 13273	EU137311, EU137196, EU137251		Chromoblastomycosis; arm; male	Falcon State, Venezuela
	117902	UNEFM 0008-03 = dH 14489	EU137283, EU137166, EU137226		Chromoblastomycosis; 3 y; arm; male 42	Falcon State, Venezuela
	117893	UNEFM 0001-00 = dH 14470	EU137316, EU137200, –		Chromoblastomycosis; 2 y; knee; male 19	Falcon State, Venezuela
	117892	UNEFM 0001-02 = dH 14471	EU137277, EU137160, EU137221		Chromoblastomycosis; 8 y; knee; male 52	Falcon State, Venezuela
	117908	UNEFM 0013-04 = dH 14502	–, EU137191, –		Chromoblastomycosis; 6 y; back; male 13	Falcon State, Venezuela
	109.97**	UNEFM 9503 = dH 10706	–, –, –		Chromoblastomycosis; skin	Falcon State, Venezuela
	857.96	UNEFM 9408 = dH 10707	EU137294, EU137177, EU137236		Chromoblastomycosis; skin	Falcon State, Venezuela
	114398	UNEFM 2003/1 = dH 13267	EU137271, EU137154, EU137215		Chromoblastomycosis; arm; female 67	Falcon State, Venezuela
	114400	UNEFM 2003/3 = dH 13269	EU137273, EU137156, EU137217		Chromoblastomycosis; arm; male 50	Falcon State, Venezuela
	117909	UNEFM 0013-00 = dH 14501	EU137287, EU137170, EU137230		Chromoblastomycosis; arm; male	Falcon State, Venezuela
	114395	UNEFM 9802 = dH 13264	EU137299, EU137182, EU137240		Chromoblastomycosis; leg; female 22	Falcon State, Venezuela
	166.54	MUCL 10088	EU137290, EU137173, –		Skin lesion in human	Falcon State, Venezuela
	862.96	UNEFM 9603 = dH 10700	EU137315, EU137199, EU137255		Dry plant debris, semi-arid zone	Falcon State, Venezuela
	863.96**	IFM 41444 = UNEFM SR3 = dH 10699	AB109169 / EU137296, EU137179, EU137238		Dry spine (*Opuntia caribaea*) on soil, semi-arid zone	Falcon State, Venezuela
	861.96	UNEFM 9607 = dH 10701	EU137309, EU137194, EU137249		Dry plant debris, semi-arid zone	Falcon State, Venezuela
	117896	dH 14498	EU137285, –, EU137228		Hand lesion	Falcon State, Venezuela
	114397	UNEFM 84020 = dH 13266	EU137270, EU137153, EU137214		Chromoblastomycosis, hand and arm	Falcon State, Venezuela

117905	dH 14505	EU137300, –, –		Chromoblastomycosis, hand, male	Falcon State, Venezuela
117900	dH 14493	EU137284, –, EU137227		Chromoblastomycosis, hand, male	Falcon State, Venezuela
114392	UNEFM 82267 = dH 13261	EU137267, EU137150, EU137211		Chromoblastomycosis, leg, female	Falcon State, Venezuela
–	FMC 248	AF397181, –, –		Chromoblastomycosis	Venezuela
–	IFM 41807	AB109175, –, –	Group mt-I	–	Venezuela
–	IFM 4812	AB109168, –, –	Group mt-I	–	Venezuela
–	IMTSP 690	AF397180, –, –		Chromoblastomycosis	Brazil
410.96	UAMH 4392 = NCMH 1010 = DUKE 2403	EU137310, EU137195, EU137250		Chromoblastomycosis	–
163.54		EU137304, EU137186, EU137243		Chromoblastomycosis	Australia
117903	dH 14482	EU137282, –, EU137225		Chromoblastomycosis, forearm, male	–
362.70	M.J. Campos 4555 = dH 15806	EU137302, EU137184, EU137242		Human	Mozambique
260.83	CDC B-1352 = FMC 282 = ATCC 44535 (ex-T of *C. ajelloi*)	EU137292, EU137175, EU137234	Group mt-I	Skin lesion in human	Uganda
986.96	UAMH 5717	EU137297, EU137180, –		Clinical material	–
–	IFM 4805	AB087204, –, –		–	–
–	IFM 4811	AB109178, –, –		–	–
–	IFM 41814	AB109176, –, –		–	–
B / II / 2: *C. carrionii*					
160.54	ATCC 16264 = CDC A-835 = MUCL 40053 = IFM 4808 (ex-LT of *C. carrionii*)	AB109177 / EU137266, EU137201, EU137210	Group mt-II	Chromoblastomycosis, human	Australia
–	Todd Pryce 200867 = dH 13218			Human	Australia
406.96	MRL 1114 = UAMH 4366 = dH 15847	EU137317, EU137202, EU137256		Human	Queensland, Australia
100434	ATCC 32279 = dH 10745 = IP 518 = RV 16499	EU137289, EU137172, EU137232		Human	Madagascar
–	IFM 4810	AB109170, –, –		–	–
–	IFM 41446 = DCU 606	AB109171, –, –		–	–
C: *C. carrionii*					
–	IFM 41651	AB109174, –, –	Group mt-I	–	China
–	IFM 41650	AB109173, –, –	Group mt-I	–	China
–	IFM 41641	AB109172, –, –	Group mt-I	–	China
–	IFM 4985	AB109179, –, –		–	–
–	IFM 4986	AB109180, –, –		–	–
D / III / 3: *C. yegresii*					
114406	UNEFM SgSR1 = dH 13275	EU137323, EU137208, EU137263		*Stenocereus griseus* asymptomatic plant	Falcon State, Venezuela
114407	UNEFM SgSR2 = dH 13276	EU137324, –, EU137264		*Stenocereus griseus* asymptomatic plant	Falcon State, Venezuela
114405**	UNEFM SgSR3 = dH 13274 (ex-T of *C. yegresii*)	EU137322, EU137209, EU137262		*Stenocereus griseus* asymptomatic plant	Falcon State, Venezuela

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[1]Abbreviations: ATCC = American Type Culture Collection, Manassas, U.S.A.; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CDC = Centers for Disease Control and Prevention, Atlanta, U.S.A.; DCU = Department of Dermatology, School of Medicine, Chiba, Japan; dH = G.S. de Hoog working collection; FMC = Faculdade de Medicina, Caracas, Venezuela; ITMSP = Instituto de Medicina Tropical de São Paulo, São Paulo, Brazil; IFM = Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan; IP = Institut Pasteur, Paris, France; MUCL = Mycotheque de l'Université de Louvain, Louvain-la-Neuve, Belgium; RV = Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium; UAMH = The University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada; UNEFM = Universidade Nacional Experimental Francisco de Miranda, Coro, Falcon, Venezuela.

Ex-T = Type strain; ex-LT = Lectotype strain.

*Type I and II groups based on mitochondrial DNA restriction fragment length polymorphism: H-1 and H-2 = restriction patterns 1 and 2, respectively, with *Hae*III enzyme; M-1 and M-2 = restriction patterns 1 and 2, respectively, with *Msp*I enzyme; S-1 and S-2 = restriction patterns 1 and 2, respectively, with *Sau*3AI enzyme.

** Used in plant and mouse inoculation experiments.

Table 2. Results from MrAic using corrected Akaike Information Criterion (AICc).

Fragment/Gene	*Model*	*df**	*lnL**	*AICc**	*wAICc**
rRNA ITS	TrNG	89	-21.840.556	4575.9992”	0.3080
EF-1α	HKYG	88	-19.392.355	41.147.171	0.5242
ß-Tubulin	SYMIG	90	-28.547.745	59.261.933	0.1913

**df* = degrees of freedom; *lnL* = log likelihood; *AICc* = corrected AIC; *wAICc* = weighted corrected AIC.

DNA extraction

Approximately 1 cm^2 mycelium of 30-d-old cultures was transferred to a 2 mL Eppendorf tube containing 300 µL TES-buffer (Tris 1.2 % w/v, Na-EDTA 0.38% w/v, SDS 2 % w/v, pH 8.0) and about 80 mg of a silica mixture (Silica gel H, Merck 7736, Darmstadt, Germany / Kieselguhr Celite 545, Machery, Düren, Germany, 2 : 1, w/w). Cells were disrupted mechanically in a tight-fitting sterile pestle for approximately 1 min. Subsequently 200 µL TES-buffer was added, the mixture was vortexed, 10 µL proteinase K was added and incubated for 10 min at 65 °C. After addition of 140 µL of 5 M NaCl and 1/10 vol CTAB 10 % (cetyltrimethylammoniumbromide) buffer, the material was incubated for 30 min at 65 °C. Subsequently 700 µL SEVAG (24 : 1, chloroform : isoamylalcohol) was mixed to solution, incubated during 30 min on ice water and centrifuged for 10 min at 14 000 rpm. The supernatant was transferred to a new tube with 225 µL 5 M NH_4-acetate, incubated on ice water and centrifuged again for 10 min at 14 000 rpm. The supernatant was transferred to another Eppendorf tube with 0.55 vol isopropanol and spun for 5 min at 14 000 rpm. Subsequently, the pellet was washed with ice cold 70 % ethanol. After drying at room temperature it was re-suspended in 48.5 µL TE buffer (Tris 0.12 % w/v, Na-EDTA 0.04 % w/v) plus 1.5 µL RNAse 20 U/mL and incubated for 15–30 min at 37 °C.

Sequencing and phylogenetic reconstruction

Three loci, namely the internal transcribed spacers (ITS), ß-tubulin (BT2) and translation elongation factor 1-α (EF1), were sequenced. For ITS sequencing, amplification was performed with V9G (5’-TTACGTCCCTGCCCTTTGTA-3’) and LS266 (5’-GCATTCCCAAACAACTCGACTC-3’). Sequencing reactions were conducted with ITS1 and ITS4 primers (White *et al*. 1990). For BT2 amplification and sequencing, primers Bt2a (5’-GGTAACCAAATCGGTGCTGCTTTC-3’) and Bt2b (5’-ACCCTCAGTGTAGTGACCCTTGGC-3’) were used (Glass & Donaldson 1995) and for EF1 amplification and sequencing, primers EF1-728F (5’CATCGAGAAGTTCGAGAAGG-3’) and EF1-986R (5’-TACTTGAAGGAACCCTTACC-3’) (Carbone & Kohn, 1999). Sequences were aligned in BioNumerics v. 4.5 (Applied Maths, Kortrijk, Belgium), exported and converted into Phylip interleaved format (Felsenstein 1993).

Calculation of ILD (incongruence length difference) was performed in PAUP v. 4.0b10 (Swofford 2003). A combined data set of ITS, EF1 and BT2 sequences was created. Optimality criterion was set to parsimony. The total number of characters was 1 263 with equal weight, while 677 characters were constant, and 396 parsimony-informative. Gaps were treated as missing, and tree-bisection-reconnection (TBR) was used as branch-swapping algorithm. Maximum number of trees was set to 100 and left unchanged.

Substitution model testing

The program MrAic (www.abc.se/~nylander/; Nylander 2004) was used to select a substitution model. MrAic is a Perl script for calculating the Akaike Information Criterion (AIC), corrected Akaike Information Criterion (AICc), Bayesian Information Criterion (BIC), and Akaike weights for nucleotide substitution models and model uncertainty. Using an ML algorithm, likelihood scores under different models were estimated using Phyml (http://atgc.lirmm.fr/phyml/). All 56 models implemented in Modeltest (Posada & Crandall 1998) were evaluated. These models were also combined with proportion of invariable sites (I) and/or gamma distribution shape parameter (G). A difference between Modeltest and MrAic is that the latter does not evaluate all models on the same, approximate topology as in PAUP (Swofford 1981). Instead, Phyml was used to try to find the maximum of the likelihood function under all models. This is necessary for finding AIC, AICc, or BIC for the models. The AICc calculation (Table 2) was used to select the right model for the ratio of parameters to characters (Nchar/Nparameters < 40; Burnham & Anderson 2002) for all loci. The substitution matrix of the models is printed next to the trees. Another advantage of using MrAic in combination with Phyml was the obtained accuracy of tree topology and the greater calculation speed (Guindon & Gascuel 2003).

Population genetic analyses

In order to confirm the intraspecific diversity shown in the MP trees, the number of populations in the *C. carrionii* complex was inferred with Structure v. 2.2 (Pritchard *et al.* 2000) using genotype data of the ITS regions of rRNA gene and of the partial EF1 and BT2 genes. Genotypes of these three loci of 43 isolates were sorted on the basis of sequence similarity. Structure is a model-based clustering method for using multilocus genotype data to infer population structure and assign individuals to populations. The parameters were as follows: the length of burn-in period was set to 10^6, number of MCMC repeats after burn-in 30 000; the ancestry model: admixture (individuals have mixed ancestry and is recommended as starting point for most analyses). Uniform prior for ALPHA was set to 1.0 (default) and all allele frequencies were taken as independent among populations with λ set to 1.0 (default). Probability of the data (for estimating K) was also computed (Falush *et al*. 2003). The burn-in period length and number of MCMC repetitions after burn-in were set as 10 000 and 100 000, and admixture model and allele frequencies correlated model were chosen for analysis. The number of populations (K) was assumed from two to four.

Association of multilocus genotypes was screened with the multilocus option in BioNumerics. To test for reproductive mode in each population, index of association (I_A, a measure of multilocus linkage disequilibrium) was calculated with Multilocus v. 1.2.2 (www.bio.ic.ac.uk/evolve/software/multilocus). The null hypothesis for this analysis was complete panmixia. The values of I_A were compared between observed and randomised data sets. The hypothesis would be rejected when $p < 0.05$. Population differentiation (index: theta, θ) was also detected using the same software and a null hypothesis for this analysis is no population differentiation. When observed θ is statistically significantly different from those of random datasets ($p < 0.05$), population differentiation should be considered.

A reticulogram was reconstructed using T-REX (Makarenkov 2001, Makarenkov & Legendre 2004) (www.labunix.uqam.ca/~makarenv/trex.html) on *C. carrionii / Cladophialophora* sp. The program first computed a classical additive tree using one of the five available tree reconstruction algorithms. Subsequently, at each step of the procedure, a reticulation (a new edge) was chosen that minimised the least-squares or the weighted least-squares loss function; it was added to the growing reticulogram. Two statistical criteria (Q1 and Q2) were proposed to measure the gain in fit when reticulations were added. The minimum of each of these criteria may suggest a stopping rule for addition of reticulations. With HGT (horizontal gene transfer) reticulogram reconstruction option (Makarenkov 2001) the program mapped the gene tree into the species tree using the least-squares method. Horizontal transfers of the considered gene were then shown in the species tree. The reticulate network was created in the ITS tree, which served as a species tree and compared with a gene tree, EF1. Degrees of recombination or horizontal gene transfer were also visualised using SPLITSTREE v. 4.8 software (Huson & Bryant 2006). Split decomposition (Bandelt & Dress 1992) was applied on three loci of the entire *C. carrionii* complex. Calculation was done with default settings of characters transformation using uncorrected P-values, equal angles and optimise box iterations set to 1. Star- or brush-like trees indicate clonal development, while reticulation indicates genetic exchange.

Isolation of fungi for inoculation experiments

Nine plants of *Stenocereus griseus*, located within 50 m radius of the house of a patient with chromoblastomycosis due to strain UNEFM 9902 = CBS 114402 (*C. carrionii*) in Sabaneta (Miranda, Falcón State, Venezuela), were analysed. Four fragments of approx. 2 × 3 × 1 cm were excised from each plant at brownish superficial lesions in upper branches. Sampled fragments were soaked in mineral oil for 15 min at 23 °C under agitation at 150 rpm (Fernández-Zeppenfeldt *et al*. 1994). Subsequently four cultivations were made per sample on agar slants. Strains with cultural characteristics and morphology similar to *C. carrionii* (de Hoog *et al*. 2000) were selected. Final identification was made by sequencing of the ITS region, by determining the ability of strains to grow at 35, 37, 38 and 40 °C, and whether they could break down 20 % gelatin (Richard-Yegres & Yegres 1987, Fernández-Zeppenfeldt *et al*. 1994). Environmental strain UNEFM-SgSR3 = CBS 114405 (*Cladophialophora* sp.) and clinical strain UNEFM 9902 = CBS 114402 (*C. carrionii*) were selected for the inoculation experiments.

Inoculum preparation

Approximately 1 cm^2 of a culture on Sabouraud's glucose agar (SGA) was transferred to 50 mL YPG medium (yeast extract 0.5 %, peptone 0.5 %, glucose 2 %) (de Hoog *et al*. 2000), shaken at 150 rpm and incubated for 3 d at 23 °C (Yegres *et al*. 1991). Five mL aliquots of the starter culture were transferred serially every 4 d to 500 mL flasks containing 100 mL synthetic medium (D-glucose 2 %, KH_2PO_4 0.2 %, NH_4SO_4 0.1 %, urea 0.03 %, $MgSO_4$ 0.03 %, $CaCl_2$ 0.003 %; pH 6.2) shaken at 150 rpm at 23 °C. After 4 d the suspensions, which were predominantly conidia, were filtered through sterile gauze, ground in 50 mL 0.85 % saline, centrifuged at 2 000 rpm, and repeatedly washed with saline until a clear supernatant was obtained. The suspensions were adjusted to 5 × 10^6 cells/mL (Yegres *et al*. 1991, Cermeño & Torres 1998). Inocula of 2 mL were checked for viability in lactritmel medium (de Hoog *et al*. 2000).

Experimental cactus germlings

Young cactus plants (*Stenocereus griseus*) were obtained in the laboratory (Clausnitzer 1978) by cultivation from seeds of a single cardon fruit collected near the house of the patient infected with CBS 114402 in the endemic area for chromoblastomycosis in Falcon State, Venezuela. The seeds were rinsed with sterile distilled water, the contents washed by agitation for 10 min at 120 rpm in 250 mL sodium hypochlorite 4 % (v/v), and subsequently with sterile distilled water at 120 rpm for 5 min. The supernatant was decanted, 250 mL HCl 20 % was added, the seeds were incubated for 3 h, decanted and washed repeatedly with sterile distilled water. Seeds were then dried for 24 h on filter paper at 37 °C. Onset of germination was obtained by incubation of the seeds in a moist chamber on filter paper for 15 d under alternately 8 h of continuous white light (26 W) and 16 h of darkness; bud emergence was observed daily. Germlings of 1 cm in length, with green colour and having two leaves were transplanted to 128-container germinators until roots developed. The sterile substrate contained 5 parts Sogemix® and 1 part river soil from the region where the fruit was collected. The daily light regime was as above; plants were watered every 10 d with 5 mL sterile tap water for 1 yr.

Inoculation of *S. griseus* germlings

Fungal suspensions (0.1 mL) were either injected using a syringe (13 × 0.4 mm) at a depth of approximately 5 mm into cortical tissue (Fig. 1A), or superficially applied onto (Fig. 1B) 96 randomly selected 1-yr-old plants: 50 % using clinical strain CBS 114402 (*C. carrionii*) and 50 % using environmental strain CBS 114405 (*Cladophialophora* sp.). The controls were 64 plants which were treated similarly, but using sterile saline (0.85 %). The growth chambers with inoculated plants stayed in the laboratory under the conditions specified above. From day 15 post inoculation onwards every 15th day, six plants of each treatment were sectioned longitudinally from the apex and transversely by means of a hand-held microtome, examined directly in glycerin water (25 %), and cultured in lactritmel medium (Fernández-Zeppenfeldt *et al*. 1994).

Experimental cactus plants

A total of 150 whole *S. griseus* plants ≤ 15 cm tall and without macroscopically visible lesions, were dug from an area within a 50 m radius of the house of the patient with chromoblastomycosis as specified above. Plants were transported to the laboratory and transplanted individually into polyethylene bags with a capacity of 1 kg, using as substrate river soil from the same area. Plants were maintained outside, directly adjacent to the laboratory to adjust at average temperatures of 32 °C and with natural daylight. They were watered with tap water every 15th d for a period of 6 mo.

Scar formation in mature *S. griseus* plants

For inoculation purposes, 150 sharp, wooden toothpicks 4 × 0.3 × 0.2 cm were washed and boiled for 3 min in tap water to eliminate resins (Yegres & Richard-Yegres 2002). This procedure was repeated three times. Three batches of 50 toothpicks each were kept separate in Petri dishes. Plates were incubated for 15 d at 23 °C after inoculating each batch with 1 mL fungal suspension (5 × 10^6 cells/mL) of either strain CBS 114405 or CBS 114402, with sterile water as control. A total of 50 randomly selected plants were inoculated (Fig. 1C) halfway up the shaft with a toothpick colonised with CBS 114402 (*C. carrionii*), CBS 114405 (*Cladophialophora* sp.) or the control (Yegres & Richard-Yegres 2002).

Starting from 2 wk post inoculation, 10 plants were randomly chosen every 15 d, and tissue samples taken at the point of

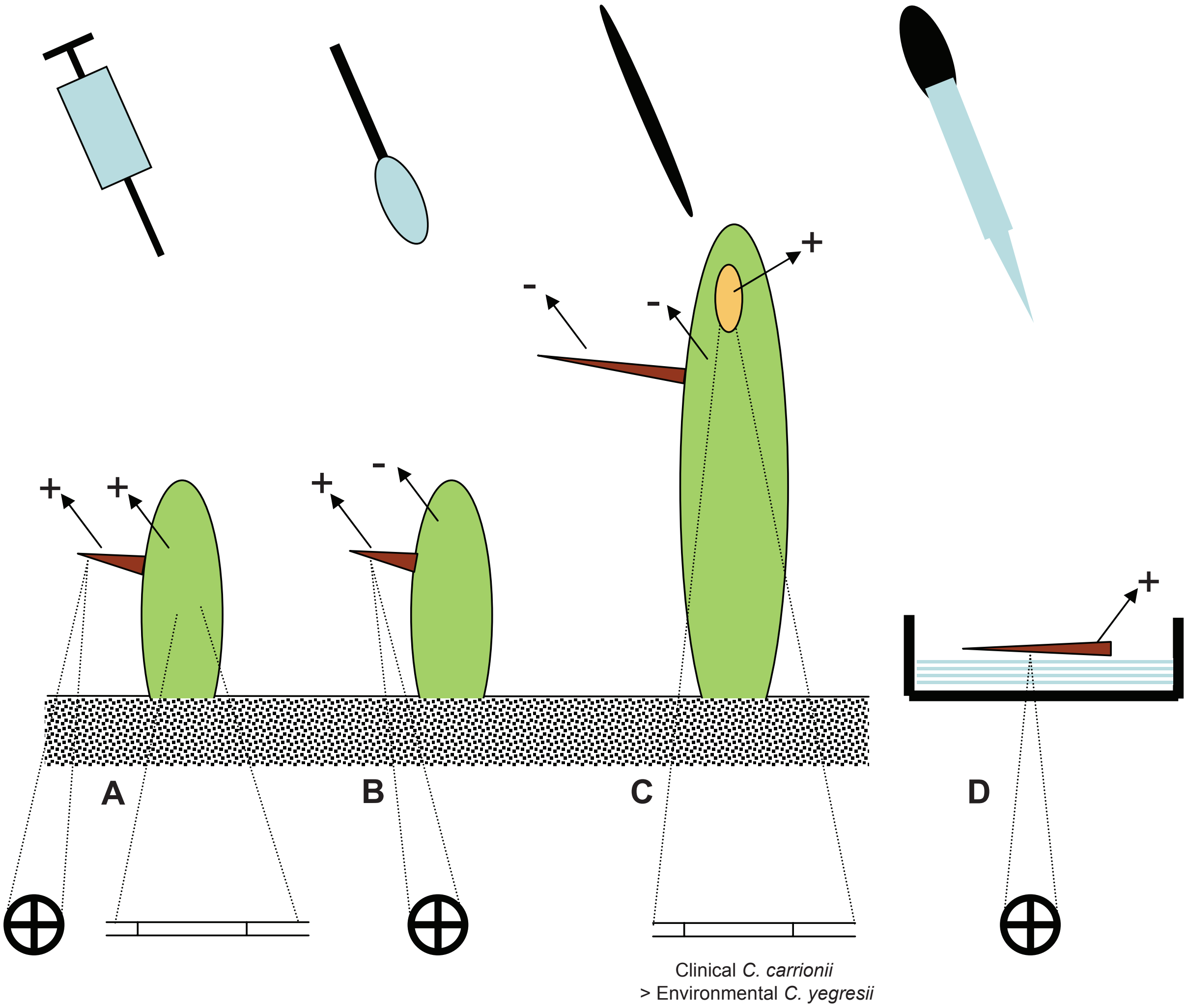

Fig. 1. Diagram of inoculation experiments with results. A. Inoculation of young cactus; B. Superficial application of young cactus; C. Traumatic application of mature cactus, with brown resulting scar; D. Superficial application of mature spines. Indications +/- refer to positive resp. negative results of re-isolated strains. Lower line: circles represent production of muriform cells, filaments represent hyphal growth.

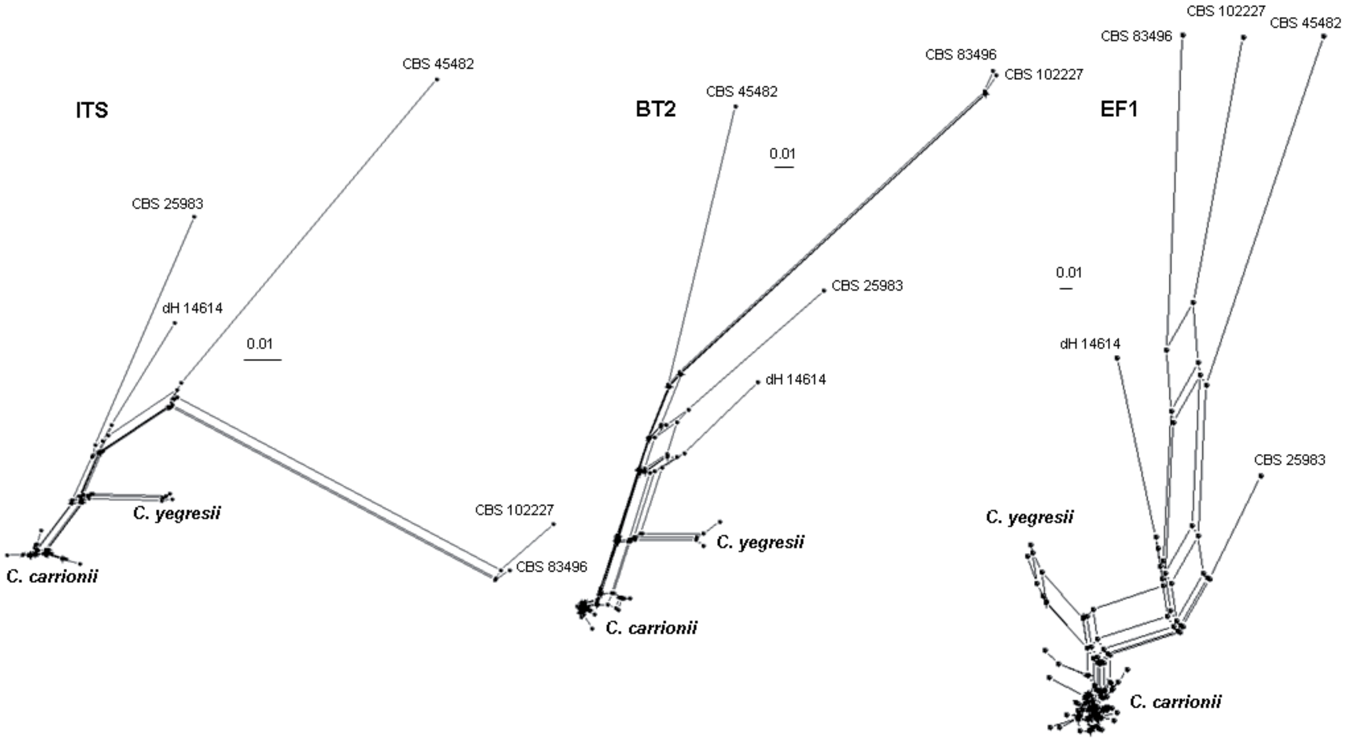

Fig. 2. Split decomposition of the *C. carrionii* complex using SplitsTree with uncorrected (P-value) distances. Nodes are shown only with different genotypes; hence EF1 shows the largest number of nodes. Extensive reticulation is noted in all loci.

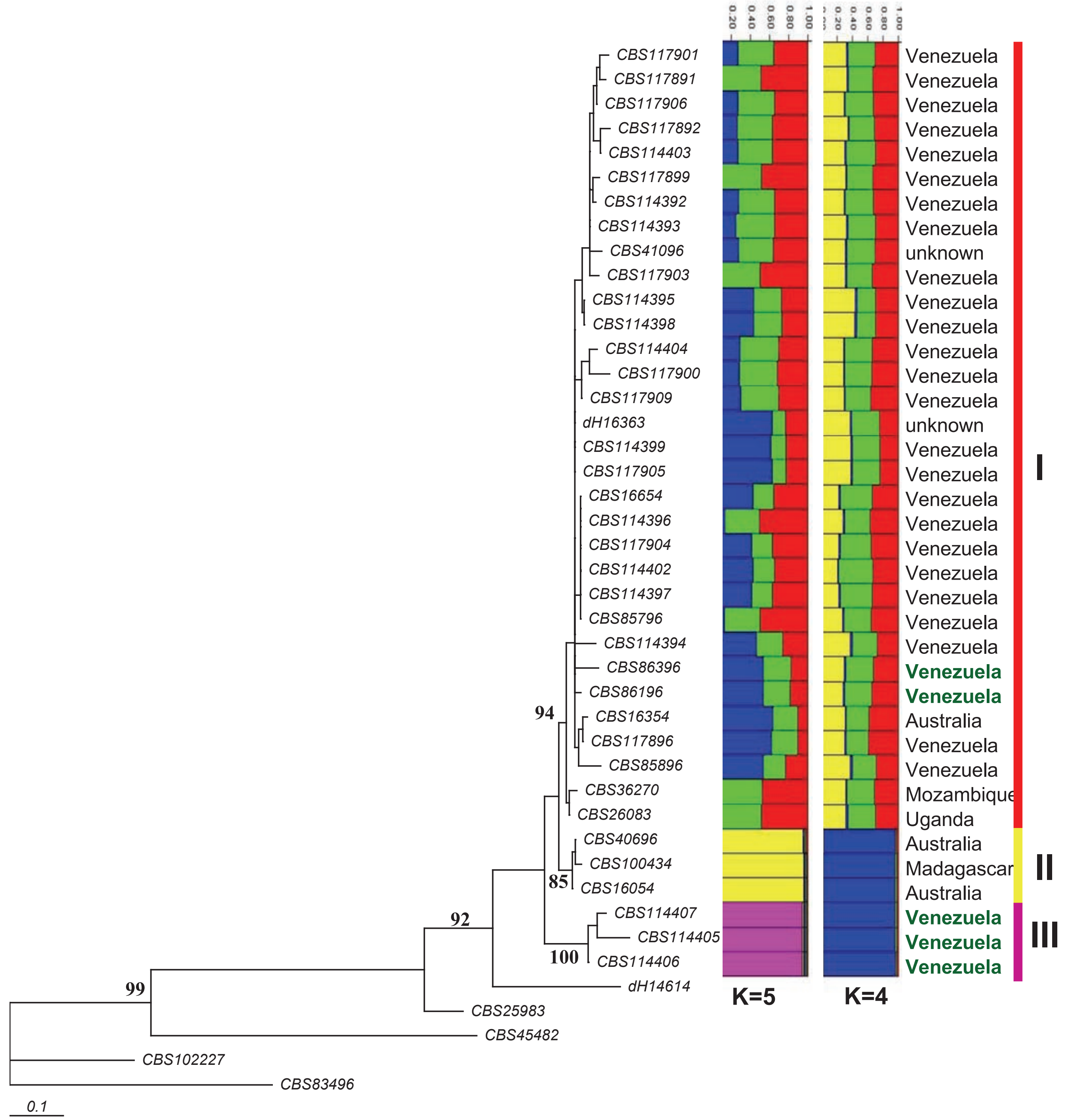

Fig. 3. Phylogenetic tree (Neighbour-joining) of the *C. carrionii* complex based on EF1 (with grouping I–III) using the same strains of Fig. 1, generated using the HKY+G model. The model was calculated using ML in MrAic software. Bootstrap cut-off = 80 %. CBS 834.96 was taken as outgroup. Columns were generated with Structure software hypothesising K = 4 and K = 5, and alleles independent. Geographical origins in black refer to isolates from humans (chromoblastomycosis); origins in green refer to isolates from plant material.

inoculation, and from the thorns directly adjacent to this area. Samples were rinsed with 4 % sodium hypochlorite for 3 min, and subsequently washed in sterile distilled water for re-isolations, and for histological study by means of light microscopy (Fernández-Zeppenfeldt *et al*. 1994).

Experiments with spines of *S. griseus*

Ninety cactus spines of 2.5 cm av. length were collected from a single *S. griseus* plant located near the home of the patient infected with CBS 114402, at approx. 2.5 m height, superficially sterilised, and divided into three groups, of which 30 spines were inoculated with CBS 114402 (*C. carrionii*), 30 with CBS 114405 (*Cladophialophora* sp.) and 30 to be used as control, inoculated with a saline solution (Fig. 1D). A similar series composed of 90 spines of 1.5 cm average length was collected at approx. 1 m height. All spines were incubated in sterile Petri dishes with filter paper (Whatman #1) with 2 mL saline solution; subsequently 0.1 mL fungal suspension was applied. Twenty spines were analysed weekly by means of longitudinal sectioning with a hand-held microtome, cultured as above and observed microscopically until day 75 post incubation.

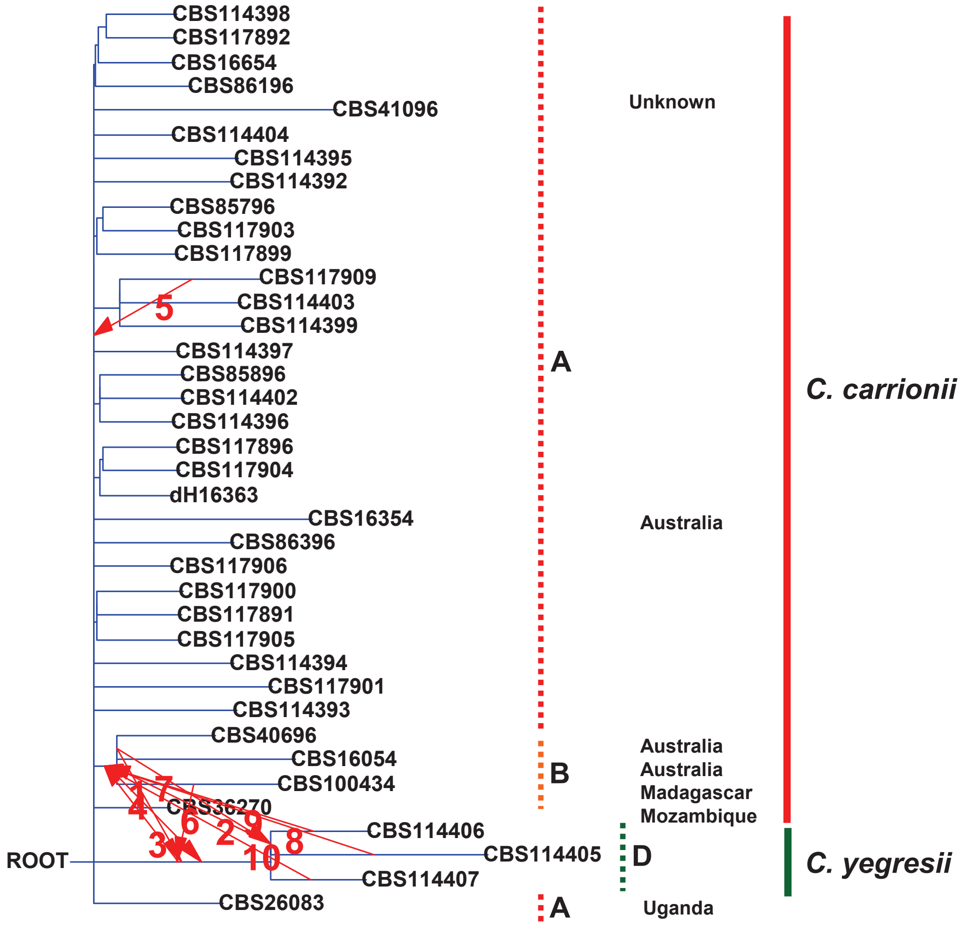

Fig. 4. Reticulogram of South American strains of *Cladophialophora* species and strains from other continents (mentioned) constructed with T-rex software. ITS rDNA (with grouping A, B, D) was used as species tree and compared with the ß-tubulin gene tree. First 10 reticulations are shown with numbers.

Statistics

Survival of the cactus seedlings and collected plants following inoculation were evaluated using the X^2-test (P = 0.05 was considered significant). Stem lesions resulting from inoculations were analysed with Student's T-test (P = 0.01 was considered significant).

RESULTS

The rDNA ITS region was sequenced for 43 strains identified as *C. carrionii* based on morphology. Sequences of 16 additional strains were downloaded from GenBank. Five distantly related, unidentified cladophialophora-like species were added, with CBS 834.96 as outgroup. In *C. carrionii*, 203 positions were compared in ITS1, 158 in the 5.8S rRNA gene and 182 in ITS2 (Table 3). The sequences could be aligned with confidence over their entire lengths. Over the data set, 35 positions were polymorphic, of which 33 were phylogenetically informative (Table 3), the two remaining being variable T-repeats near the ends of ITS1 and 2.

For ITS sequences the AICc selected the TrN+G model (TrNG; Tamura & Nei 1993). The base frequency of ITS: T = 0.2467, C = 0.2897, A = 0.2247, G = 0.2390, TC = 0.5364, AG = 0.4636. The EF1 tree was built with substitution model HKY+G; the base frequency of EF1: T = 0.2990, C = 0.2665, A = 0.2123, G = 0.2221, TC = 0.5655, AG = 0.4345. The best model for BT2 sequences was the SYM+I+G (symmetrical model). The base frequency for BT2: T = 0.2255, C = 0.2953, A = 0.2463, G = 0.2328, TC = 0.5208, AG = 0.4792. Bootstrap values of the EF1 tree were calculated with PAUP using parsimony and with maxtrees set to 500 and 500 replicates (data not shown). Total number of characters was 191 of which 101 were parsimony-informative. Tree length was 365 and had the following indices: Consistency Index = 0.685, Retention Index = 0.542 and Homoplasy Index = 0.315.

The original tree length, L_o was 1 055, the tree length of the combined data, L_c was 1 062. The resulting incongruence length difference $L = (L_c – L_o)$ was 7 (P = 0.24). The observed ILD was not significantly greater than expected by chance and it was concluded that the sequences were congruent and could be used together in a combined analyses.

Split decomposition based on the same alignment generated extensive recombination. The structure found with three loci was robust, with the exception of separation of CBS 834.96 and CBS 102227 with EF1 (Fig. 2).

The core of the network, comprising the strains listed in Table 1, was analysed in more detail. With ITS, four groups were recognised (A–D; Table 1). (A) was the main group with 36 strains / sequences; FMC 248 differed only by a small T-repeat and was regarded as a member of (A). The remaining groups were smaller, differing from group (A) maximally by two consistent positions (Table 3). Group (C) mainly comprised sequences from GenBank and all originated from Abliz *et al*. (2004). One of the strains of group (C), IFM 4808, concerned a subculture of CBS 160.54, which is an original isolate of Trejos (1954) representing *C. carrionii*. Re-sequencing indicated that it was a member of group (B). Analysis of our electropherograms of this isolate was not suggestive of heterothallism. None of the positions characterising groups (A)–(C) were also found to differ in group (D), which deviated in 16 mutations in ITS1 and 8 in ITS2; 17 of the mutations were transitions, 7 were transversions and 7 indels. Group (D) was clearly distinct from the complex of (A)–(C),

Table 3. Nucleotide variability of ITS1-2 ribosomal DNA regions of *Cladophialophora carrionii* (A – C) and *C. yegresii* (D).

rDNA domains (length), with variable nucleotide positions.

ITS1 (201-203)	***A***	***B***	***C***	***D***
16	C	C	C	T
17	T	C	T	T
19	T	T	T	C
51	A	A	A	G
57	A	A	A	T
90-92	TG-	TG-	TG-	CGT
101	T	T	T	C
103	C	C	C	T
104	G	A	G	G
106	A	A	A	G
114	T	T	T	C
122	T	T	T	C
132	C	C	C	T
137	A	A	A	C
141	C	C	C	T
145	-	-	-	A
163-170	6-10T	6-10T	6-10T	TTGTATCT
180	-	-	-	A
183	G	G	G	A
190	T/A	A	A	A
5.8S (158)	***Monomorphic***			
ITS2 (178-182)	***A***	***B***	***C***	***D***
36	C	T	T	T
48	T	T	G	T
49	T	T	T	C
51	-	-	-	C
114	C	C	C	G
140	A	A	A	G
155	-	-	-	T
178-179	--	--	--	CT

with a total of 27 mutations.

For multilocus analysis with ITS, EF1 and BT2 a smaller set of strains was compared. Sequences of the 205 bp long element of EF1 contained 32 phylogenetically informative mutations. Three entities were distinguished (I–III; Fig. 3). With BT2, three groups with the same composition were recognised. Strains of ITS group (C) were not available for study.

On the basis of multilocus screening in BIONUMERICS, concordant groups (A)–(D) were tested with the STRUCTURE programme. When K was set at 4 or 5, consistent groupings were noted, indicated as I, II and III (Fig. 3), corresponding with ITS groups (A), (B) and (D), respectively in Table 1.

The possibility that group (D) / (III) included a member of another, morphologically similar but phylogenetically unrelated group of fungi was excluded by SSU sequencing. Genera morphologically similar to *Cladophialophora*, such as *Cladosporium* Link, *Devriesia* Seifert & N.L. Nick., *Phaeoramularia* Munt.-Cvetk., *Pseudocladosporium* U. Braun and *Stenella* Syd. proved to be remote (data not shown).

With T-REX, interaction between groups (B) and (D) was noted, rather than between groups (B) and (A), despite the high sequence similarity of (A) and (B) (Fig. 4).

Morphological observation revealed that representatives of ITS groups (A)–(C) generally had conidiophores that arise at right angles from creeping hyphae (Fig. 5), while those of (D) tend to be ascending, hyphae gradually becoming conidiophore-like. Since slight correspondence was found in independent markers and phenetic criteria, we considered group (D) to represent a separate species, which is described as follows.

Cladophialophora yegresii de Hoog, **sp. nov.** MycoBank MB500208. Figs 6, 7D–F.

Etymology: Named after Francisco Yegres, Venezuelean mycologist.

Coloniae in agaro PDA dicto 22 °C planae, olivaceo-virides, pulverulentae vel velutinae, margine integra; reversum olivaceo-atrum. Hyphae fertiles dilute olivaceo-virides, ascendentes, paulatim in catenas conidiorum concolorium vertentes. Conidiorum catenae ramosae, conidia dilute olivaceo-viridia, levia et tenuitunicata, 4.5–6 × 2.5 µm, faciliter liberata, cicatricibus modice pigmentatis. Chlamydosporae et cellulae zymosae absentes. Synanamorphe phialidica non visa. Teleomorphe ignota.

Holotypus cultura sicca CBS H-18464 in herbarium CBS praeservatur.

Colonies on PDA at 22 °C evenly olivaceous green, powdery to velvety, with entire margin; reverse olivaceous black. Fertile *hyphae* pale olivaceous green, ascending, gradually changing over into concolorous chains of conidia. *Conidial system* profusely branched. *Conidia* pale olivaceous green, smooth- and thin-walled, 4.5–6 × 2.5 µm, detached rather easily, with slightly pigmented scars. *Chlamydospores* and yeast cells absent. Phialidic synanamorph not observed. Teleomorph unknown.

Specimen examined: **Venezuela**, Falcon state, from asymptomatic *Stenocereus griseus* cactus, G. Fernández-Zeppenfeldt, CBS H-18464 **holotype**, culture ex-type CBS 114405 = UNEFM SgSR3.

Notes: Of the 48 dematiaceous isolates obtained from 36 fragments of the cactus *Stenocereus griseus*, four strains originating from four different plants of *S. griseus* presented morphological and physiological key characteristics of *Cladophialophora carrionii* or *C. yegresii* (de Hoog *et al*. 2000, 2006). Gelatin liquefaction was negative in all strains and the maximum growth temperature was 37 °C. After identification to species level using sequence data (de Hoog *et al*. 2006), both *C. carrionii* and *C. yegresii* appeared to be among the strains isolated.

A total of 256 plants obtained at the end of 1 yr from germlings, had ribs, spines, and an average height of 15 cm. The 96 germlings inoculated with fungal suspensions of the test strains CBS 114402 (*C. carrionii*, clinical) and CBS 114405 (*C. yegresii*, environmental) remained without visible external lesions during the year of experimentation. Histological sections of the 96 inoculated plants consistently revealed internal growth of the fungi in their filamentous form. Muriform cells were not observed, neither on the epidermis, nor in the internal tissue, spines or roots. The re-isolated cultures demonstrated the viability of the fungi during the entire experimental process: CBS 114402 (*C. carrionii*) was grown from 26 (54.16 %) of the plants and CBS 114405 (*C. yegresii*) in 23 (47.90 %) of the plants. The X^2 test did not reveal significant differences between the isolates ($X^2c = 0.0729 < X^2t = 3.84$). The 32 control plants remained without external lesions, and in the

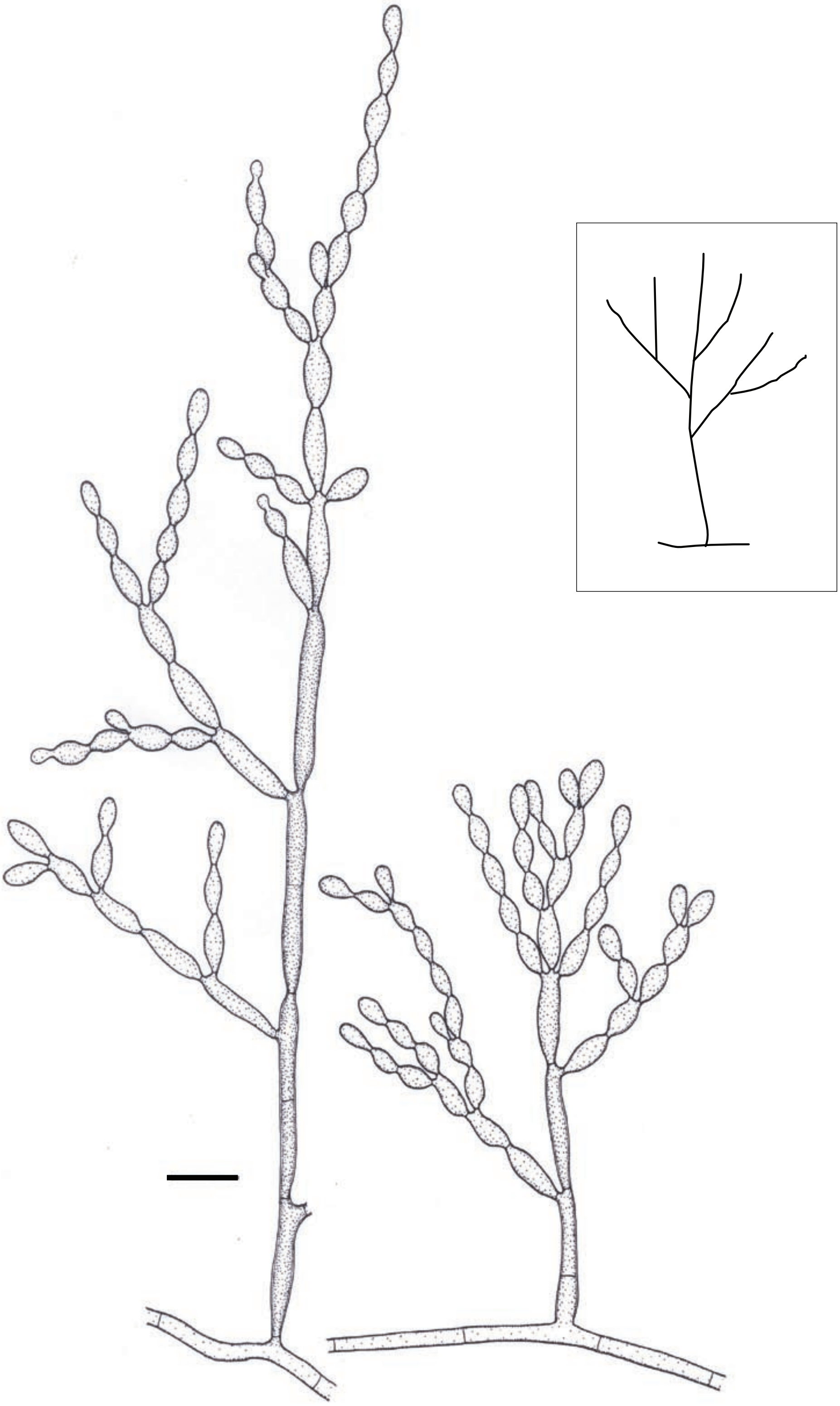

Fig. 5. Microscopic morphology of *C. carrionii*, strain CBS 160.54. Conidiophore erect, i.e. mostly arising at 90° from creeping hypha (sketch). Scale bar = 10 µm.

histological sections no internal or external fungal elements were observed. None of the fungi isolated from the control plants proved to be a species of *Cladophialophora.*

With 96 plants with superficial application of spore suspension (48 plants for each isolate, either clinical or environmental) neither internal nor external lesions were observed. Histological sectioning did not reveal fungal elements in or on plant tissue. Short hyphal elements and meristematic cells were occasionally seen around and inside the outer layers of the spines. The re-isolated strains proved that the fungi survived during the entire experimental procedure: CBS 114402 (*C. carrionii*) was isolated from 32 (66.67 %) plants and CBS 114405 (*C. yegresii*) from 33 (68.75 %). The X^2 test did not detect significant differences in survival rates among the isolates ($X^2c = 0.4375 < X^2t = 3.84$).

Mature plants inoculated using colonised toothpicks showed average scarring of 1.88 cm diam with *C. carrionii* and 1.33 cm diam with *C. yegresii*, around the point of inoculation. In histological sections of 100 plants, dark, septate hyphae with inflated elements were observed at the points of inoculation. Muriform cells were not observed. Re-isolated strains were evidence of isolate viability:

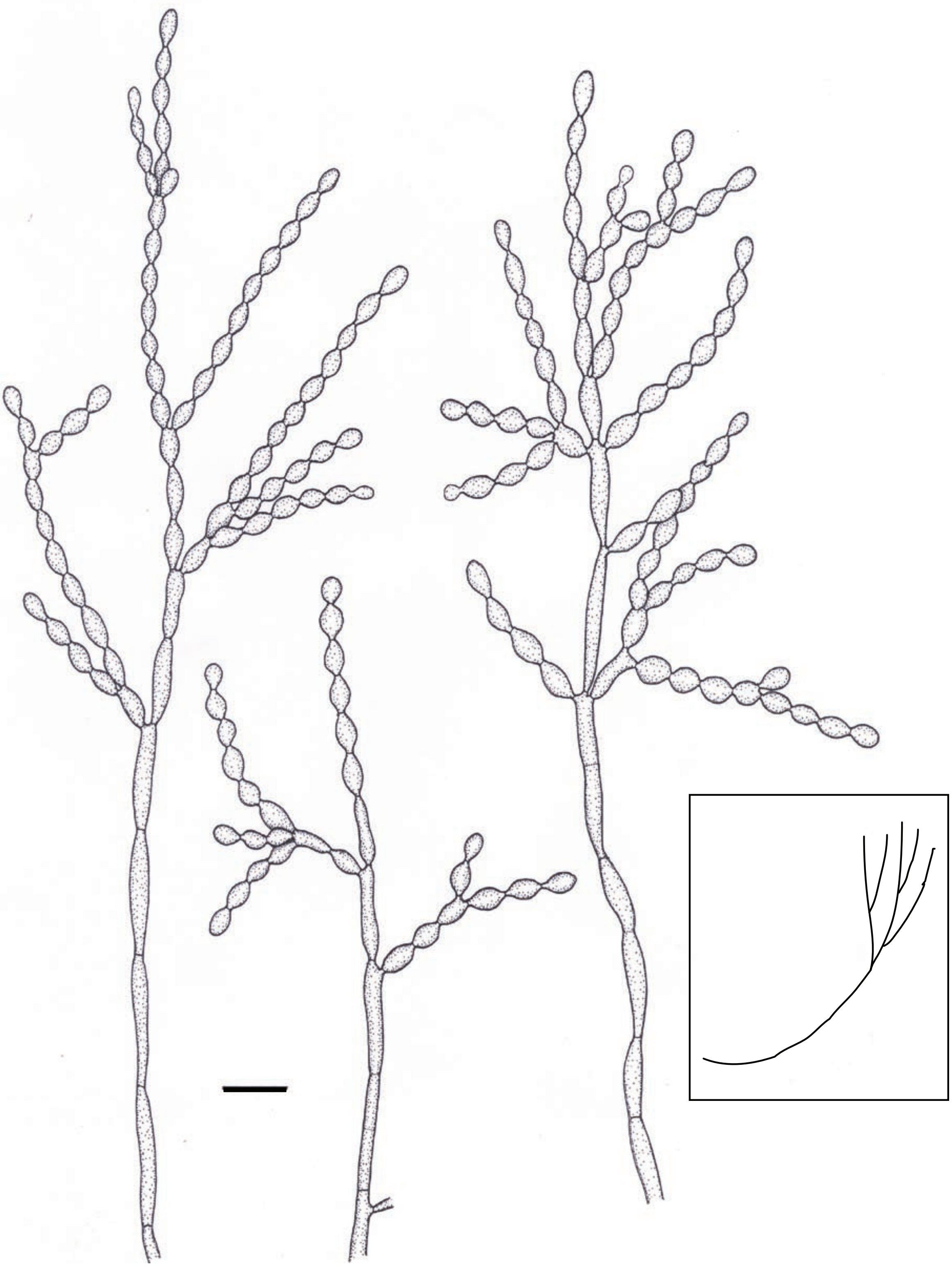

Fig. 6. Microscopic morphology of *C. yegresii*, strain CBS 114405. Conidiophore ascending, i.e. mostly emerging from hyphal end that is gradually growing upwards to become a conidiophore (sketch). Scale bar = 10 µm.

CBS 114402 (*C. carrionii*) was grown from 36 (72 %) plants and CBS 114405 (*C. yegresii*) from 30 (60 %). The fungi could not be isolated from spines. The 50 plants used as controls showed scarring of 1.06 cm diam on average around the point of inoculation. No fungal elements were seen in direct examinations and histological sections of these plants. The retro-cultures were negative. The scarring responses of the plants to the clinical strain, environmental strain and control proved to be highly significant:

Clinical CBS 114402 *vs.* environmental CBS 114405: þ = 0.000832, P = 0.01;

Clinical CBS 114402 *vs.* control: þ = 0.00003128, P = 0.01;

Environmental CBS 114405 *vs*. control: þ = 0.005343, P = 0.01.

Spines 2.5 cm av. in length, seeded with suspensions of CBS 114402 (*C. carrionii*) and CBS 114405 (*C. yegresii*), developed toruloid hyphal elements with some dark, swollen cells similar to muriform cells known in human tissue. The re-isolated strains proved the species to survive during the experimental procedure (< 75 d). Similar results were obtained with the spines 1.5 cm av. in length. No cladophialophora-like fungi were isolated from the controls.

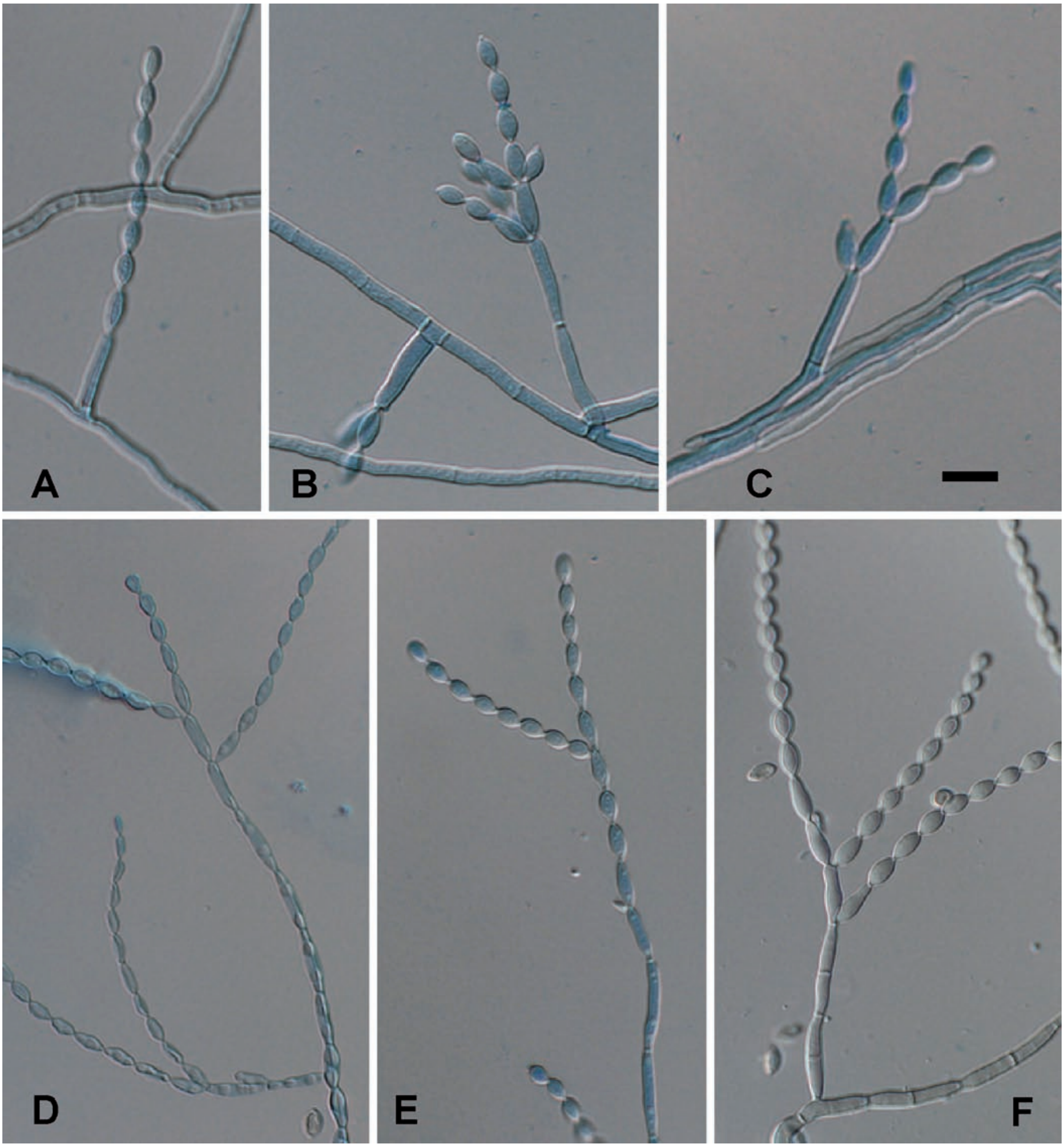

Fig. 7. Conidial morphology in selected branches of (upper row: A–C) *C. carrionii*, strain CBS 260.83; (lower row: D–F) *C. yegresii*, strain CBS 114405. In this respect the two species are identical. Scale bar = 10 µm.

DISCUSSION

Taxonomy of *Cladophialophora*

Judging from SSU rDNA phylogeny data, all *Cladophialophora* species that are consistently associated with pathology to humans belong to the *Herpotrichiellaceae* in the order *Chaetothyriales* (Haase *et al*. 1999). Within this order, the genus *Cladophialophora* is polyphyletic. Conidia of all species are produced in branched chains on poorly differentiated hyphae. This very simply structured conidial system may lead to confusion with morphologically similar but unrelated fungi that are encountered as contaminants in the hospital environment. The genus *Cladosporium* comprises ubiquitous airborne fungi which mostly have erect, more or less differentiated conidiophores, and dark conidial scars. They are associated with *Davidiella* Crous & U. Braun teleomorphs and belong to the *Dothideomycetes*, family *Davidiellaceae* (Braun *et al*. 2003, Schoch *et al*. 2006). *Pseudocladosporium* was introduced by Braun (1998) with three species differing from *Cladophialophora* mainly by intercalary hyphal cells with lateral extensions that bear conidial chains, having *Caproventuria* U. Braun teleomorphs (see Crous *et al*. 2007 – this volume). The group is classified in the *Venturiaceae* and *Mycosphaerellaceae* in the *Dothideales* (Braun *et al*. 2003). The anamorph genus *Devriesia* comprises thermophilic saprobes with a cladophialophora-like appearance and producing dark, multi-celled chlamydospores alongside the hyphae. Phylogenetically this genus is related to the *Mycosphaerellaceae,* in the *Dothideomycetes* (Seifert *et al*. 2004).

Cladophialophora carrionii was originally introduced by Trejos (1954) on the basis of 46 strains from Venezuela, Australia and South Africa. He did not indicate a holotype. For this reason isolate Trejos 27 = Emmons 8619 = CBS 160.54, the first strain mentioned by Trejos (1954), is selected here as representative for *C. carrionii*. A dried specimen of this strain has been deposited as **lectotype** in the Herbarium of the Centraalbureau voor Schimmelcultures as CBS H-18465.

The ex-type strain of *Cladophialophora ajelloi* Borelli, CBS 260.83, proved to be indistinguishable from *C. carrionii*, which was also known to be able to produce phialides in addition to catenate conidia (Honbo *et al*. 1984). Remarkably, a strain identified as *C. ajelloi* from Samoa (CBS 259.83; Goh *et al*. 1982) proved to be related to but consistently different from all strains of the *C. carrionii* complex. The 43-year-old male patient in otherwise good health carrying this fungus had a 5 × 3 cm erythematous, scaling lesion on his arm. Muriform cells were present in superficial dermis and stratum corneum. This clearly represents yet another agent of human chromoblastomycosis. The name *C. ajelloi* is not available for this taxon, as this is a synonym of *C. carrionii*. The taxon will be formally described in a forthcoming paper.

Members of ITS groups (A)–(D) were shown to be close to each other in SSU phylogeny (data not shown) underlining that all analysed species were correctly assigned to *Cladophialophora*. This genus was defined by melanised acropetal chains of conidia, near absence of conidiophores, and phylogenetic affinity to the order *Chaetothyriales*. Strains (A)–(D) clustered in a clade which contained a mixture of species of *Cladophialophora, Fonsecaea* Negroni and *Phialophora* Medlar. From a point of view of human disease, the species of the clade were known as agents of brain infection [*C. bantiana* (Sacc.) de Hoog *et al., F. monophora* (M. Moore & F.P. Almeida) de Hoog *et al.*], disseminated disease [*C. devriesii* (A.A. Padhye & Ajello) de Hoog *et al.*], cutaneous disease [*C. boppii* (Borelli) de Hoog *et al.*] and particularly chromoblastomycosis (*C. carrionii, Fonsecaea, Phialophora*).

Diversity of *Cladophialophora carrionii / C. yegresii*

Infraspecific variability was observed within *C. carrionii.* The groups (A)–(C) were separated on the basis of five mutations in the ITS region, which were supported by mutations in EF1 and BT2, as confirmed by analysis in Structure, where the same separation (K = 5) of entities was observed. Furthermore, K = 4 unites groups (B/II) and (D/III), despite the fact that the sequence of (B) is more close to those of (A). With T-rex software a similar relationship between [(B), *C. carrionii*] and [(D), *C. yegresii*] was noted, suggesting horizontal gene flow between these entities. This is remarkable, since (C) strains predominantly inhabit remote deserts in Madagascar and Australia, while (D) is found in equally remote localities in Venezuela. Extensive reticulation was observed in all genes with SplitsTree. With ITS and BT2, CBS 834.96 and CBS 102227 cluster closely together, while in the more variable EF1 data these are all widely apart, suggesting that in *Cladophialophora* other mechanisms than recombination may occur.

Group (C) contained ITS sequences taken from the public domain, originating from a single study (Abliz *et al*. 2004). Remarkably, strain IFM 4808 found in group (C) on the basis of data from Abliz *et al*. (2004), was the same isolate as CBS 160.54, which was found repeatedly in group (B) in our data set (Table 1). A similar phenomenon was observed with strain IFM 41444 = CBS 863.96, of which GenBank deposition AB109169 consistently deviated from our data in a frequently observed mutation. A possible explanation of these consistent sequence conflicts is heterozygosity. Although most chaetothyrialean fungi are supposed to be haploid (Szaniszlo 2002; Zeng *et al.* 2007), some strains have a double DNA content in yeast cells (Ohkusu *et al*. 1999). Teleomorphs are not known in *Cladophialophora* and related black yeasts, but many species are known to form profuse hyphal anastomoses (de Hoog *et al*. 2006), allowing parasexual processes and mitotic recombination. However, all electropherograms including those from the study of Abliz *et al*. (2004), which were kindly sent by K. Fukushima (Chiba, Japan), were unambiguous, without double peaks. This matched with the observation of preponderant clonality despite frequent anastomoses in *Exophiala* J.W. Carmich. (Zeng *et al*. 2007). An alternative explanation might be the occurrence of paralogous ITS repeats, as reported earlier in *Fusarium* Link (O'Donnell & Cigelnik 1997).

The remaining diversity within *C. carrionii* as confirmed by Structure shows some geographical structuring of populations, in that group (A) does not occur in Asia, group (B) is limited to Australia and Africa, and group (C) has thus far only been reported from Asia. The wide distribution of most genotypes suggests, however, that worldwide occurrence is likely to become apparent when more strains have been analysed. All climate zones where *C. carrionii* was isolated were semi-arid to arid, desert-like. Genotypes were not limited to the endemic semi-arid areas, and thus a relatively rapid vector of dispersal has to be hypothesised enabling the fungus to cross climate zones where the saprobic phase is unable to survive. Kawasaki *et al*. (1993) analysed three further loci in mtDNA using RFLP. Only some of their strains were available for sequencing. These had all identical mtDNA profiles, with the exception of IFM 4808 = CBS 160.54, that differed in two markers (Table 1). If we assume that there is no real separation of ITS groups (A) and (C) (see above), the conclusion is warranted that mtDNA allows distinction of polymorphism at the same level of diversity as detected in this study with ITS, EF1 and BT2.

South America harbours group (D) which represents a second species, *C. yegresii*. This species thus far has not been found on humans, and seems to be restricted to living *Stenocereus* cactus plants. Nishimura *et al*. (1989) published a strain from chromoblastomycosis in China which matched the morphology of strains now classified as *C. yegresii*, but as far as we are aware this strain has not been sequenced.

Ecology and virulence of *Cladophialophora carrionii / C. yegresii*

Cladophialophora carrionii was preponderantly found as an agent of human infection and only occasionally on dead plant debris, mainly seceded cactus needles. The only three strains available of *C. yegresii* were isolated from living, asymptomatic *Stenocereus* plants surrounding the cabin of a symptomatic patient from whom *C. carrionii*, CBS 114402 was isolated. Although in some publications convincing evidence was presented that infections originate from puncture by plant material (e.g., Salgado *et al*. 2004), it now becomes clear that the environmental look-alikes of clinical strains do not necessarily belong to the same species (Crous *et al.* 2006, Mostert *et al*. 2006), but may be members of other, related taxa with slightly different ecology; an unambiguous connection between a clinical and an environmental strain still has to be proven.

The endemic area of the two species, *C. carrionii* and *C.*

yegresii, has a semi-arid climate, with average yearly temperatures of 24 ºC, scarce rainfall (up to 600 annual mL) and is located at moderate altitude (up to 500 m) (Borelli 1979, Richard-Yegres & Yegres 1987). The landscape is dominated by large cacti and other xerophytes. *Stenocereus griseus* is a columnar American cactus with a very strong, protective external epidermis that allows the accumulation of water in the shaft and enables tolerance of extreme drought. The species produces ovoidal, thorny fruits of about 5 cm diam, which are commonly eaten by the local population. It has therefore been suggested that patients with chromoblastomycosis acquire their infection by traumatic inoculation with cactus spines, similar to the supposed infection process of *Madurella mycetomatis* (Laveran) Brumpt in the arid climate of Africa (Ahmed *et al*. 2002). The frequent occurrence of 16 / 1 000 for chromoblastomycosis in areas endemic for *Cladophialophora* in Venezuela (Yegres *et al*. 1985; Yegűez-Rodriguez *et al*. 1992) indicates a marked invasive potential for *C. carrionii*. Local goat-keepers are particularly at risk: in 1984, 14 of 18 patients investigated had these occupational characteristics (Yegres *et al*. 1985). Nevertheless, virulence of *C. carrionii* is low when inoculated into the footpads of mice (Yegres *et al*. 1998); also an environmental strain of *C. carrionii* failed to produce lesions in mice and in a volunteer (Richard-Yegres & Yegres 1987).

We performed inoculation experiments with *C. carrionii* and *C. yegresii* using freshly grown, healthy cacti in the greenhouse. The plants were followed over a 1-yr period; during all this time the control plants remained without lesions. Both *Cladophialophora* strains were able to produce infection when syringe-inoculated deep into young cactus tissue. Histopathology showed septate hyphae between host cells, and the shaft was maintained over prolonged periods without causing visible damage. This absence of appreciable destruction would categorise them as endophytes. Cactus tissue is rich in carbohydrates, vitamins and minerals (Vélez & Chávez 1980) which may promote endophyte growth.

In contrast, suspensions applied superficially lead to growth on and in spines only. The absence of infection after superficial application indicates that the fungi are unable to invade healthy plant tissue from the surface and thus they cannot be characterised as obligatory phytopathogens.

The two species differed in the degree of scarring after traumatic inoculation into mature plants: the clinical strain *C. carrionii* was consistently more virulent than *C. yegresii* that originated from the same host plant. Both species showed the same viability in re-isolated cultures. In nature, the fungi are likely to invade only when the integrity of the epidermis is broken, as happens e.g. by goat feeding or transmission by sap-sucking birds or piercing insects. They also show the same transformation to meristematic morphology (González *et al*. 1990) when entering hard spine tissue. A possible trigger for this conversion is the dominance of lignin in the spines. Survival on and in spines is enhanced by their capturing of atmospheric water formed after nightly condensation. The fact that superficial application leads to colonisation around and inside the spines suggests that the spines play a role in mechanic dispersion of the fungi.

A possibly coincidental mechanism of dispersal might be traumatic inoculation into living tissue of humans or animals, where the same muriform cells are formed, defining the skin disease chromoblastomycosis. It may be questioned whether animal/human inoculation plays a role in the evolution of the fungus. ITS differences between the two species are observed in 23 positions, with a ratio of transitions : transversions of 2 : 1 (Table 3). Thus no saturation of mutations has taken place and the diversification can be regarded as an example of recent sympatric speciation. *Cladophialophora carrionii* is widely distributed, and shows a higher degree of diversity than *C. yegresii*. This would be suggestive for a longer evolutionary time span of existence and *C. carrionii* then should be regarded as ancestral to *C. yegresii*, with the latter showing a founder effect due to the absence of polymorphisms. However, such an order of event (a host jump from humans to cactus) is difficult to imagine. It is more likely that *C. yegresii* is the original cactus endophyte exhibiting extremotolerance via its muriform cells. T-rex data suggested a more direct connection of *C. yegresii* with African and Australian rather than Venezuelean strains of *C. carrionii*. We suppose that the low degree of observed variation in *C. yegresii* is not a founder effect, but rather a sampling effect, as living cacti have thus far not been studied outside the framework of our study on the patient with *Cladophialophora* chromoblastomycosis. The difference in virulence may be simply explained by *C. carrionii,* which lives as a saprobe on dead cactus debris for part of its life cycle, and is less adapted to an endophytic life style.

Cladophialophora cf. carrionii is known to occur on lignified materials, such as wood chips of *Eucalyptus crebra* and wooden remains of *Prosopis juliflora* and *Stenocereus griseus* (Riddley 1957, Yegres *et al*. 1985, Fernández-Zeppenfeldt *et al*. 1994). This does not exclude a certain degree of pathogenicity to humans, as also pathogens like *Cryptococcus neoformans* (Sanfelice) Vuill. are known to have an essential part of their life cycle in hollows of *Eucalyptus* trees. *Cryptococcus neoformans* produces diphenol oxidase to degrade lignin, an aromatic polymer in the cell wall of plants and a component of wood (Cabral 1999). Similar degradation pathways are present in *Cladophialophora carrionii* (Prenafeta-Boldú *et al.* 2006).

The natural occurrence of *C. carrionii* and *C. yegresii* in association with xerophytes has been proven, but their environmental route of dispersal is still unknown. As transformation to meristematic cells takes place when the hyphae reach the spines and on dead spines, the muriform cell apparently is the extremotolerant phase of the species. The conidial anamorph can be found sporulating on rotten spines directly after rainfall (Richard-Yegres & Yegres 1987), but as the fungus has thus far never been isolated from outside air, it is still unclear how a new host plant is reached.

The behaviour of *C. carrionii* on humans, provoking the very characteristic disease, chromoblastomycosis, of which the agents are limited to the ascomycete family *Herpotrichiellaceae* (de Hoog *et al*. 2000) is puzzling. In humans, the extremophilic muriform anamorph is expressed rather than hyphae, and thus humans do not seem a natural reservoir of the fungus. Nevertheless some acquired cellular immunity seems to be involved. Albornoz *et al*. (1982) demonstrated that a significant share of the local population of goat keepers (Yegres *et al*. 1985) is asymptomatically infected with *C. carrionii*; Iwatsu *et al.* (1982) detected cutaneous delayed hypersensitivity in rats experimentally-infected with agents of chromoblastomycosis. With murine experimental infection of the related fungus *Fonsecaea pedrosoi*, Ahrens *et al*. (1989) found enlargement and metastasis of lesions in athymic but not in normal mice, or in mice with defective macrophage function. Several authors (Kurita 1979, Nishimura & Miyaji 1981, Polak 1984) observed a significant role of acquired cellular immunity in *F. pedrosoi*, while Cardona-Castro & Agudelo-Flórez (1999) obtained chronic infection in immunocompetent mice when inoculated intraperitoneally. Garcia Pires *et al*. (2002) noted an unbalance between protective Th1 and less efficient Th2 responses. The possible host response leads to different clinical types, referred to as tuberculoid and suppurative granuloma, respectively. The existence of genetic constitutional factors in susceptibility is underlined by a marked frequency of family relationships among symptomatic individuals (Yegűez-Rodriguez *et al*. 1992). The disease is not observed in local animals such as goats, possibly due to their high body temperature (≈ 39 °C). Nevertheless, hyphal fragments artificially inoculated into goats led to transformation into muriform cells, but the lesions disappeared within 60 d (Martínez *et al*. 2005). Further animal experiments using strains identified according to new taxonomy will be necessary to answer questions on the role of the fungus on warm-blooded animals.

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