Exophiala crusticola anam. nov. (affinity Herpotrichiellaceae), a novel black yeast from biological soil crusts in the Western United States

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A novel black yeast-like fungus, Exophiala crusticola, is described based on two closely related isolates from biological soil crust (BSC) samples collected on the Colorado Plateau (Utah) and in the Great Basin desert (Oregon), USA. Their morphology places them in the anamorphic genus Exophiala, having affinities to the family Herpotrichiellaceae (Ascomycota). Phylogenetic analysis of their D1/D2 large subunit nuclear ribosomal RNA (LSU nrRNA) gene sequences suggests that they represent a distinct species. The closest known putative relative to Exophiala crusticola is Capronia coronata Samuel, isolated from decorticated wood in Westland County, New Zealand. The holotype for Exophiala crusticola anam. nov. is UAMH 10686 and the type strain is CP141bT (≡ ATCC MYA-3639T = CBS 119970T = DSM 16793T). Dark-pigmented fungi appear to constitute an important heterotrophic component of soil crusts and Exophiala crusticola represents the first description of a dematiaceous fungus isolated from BSCs.

Members of the family Herpotrichiellaceae exhibit a diverse polymorphic life cycle and, therefore, can be difficult to identify by morphology alone. However, the use of nuclear ribosomal RNA (nrRNA) gene sequence data has facilitated identification and classification of these organisms (see Sapatnora et al., 1995; Untereiner & Naveau, 1999; de Hoog et al., 2003). The family is represented by the teleomorph genus Capronia and the anamorphic genera Cladophialophora, Exophiala, Fonsecaea, Phaeococcomycetes, Phialophora, Ramichloridium and Rhinocladiella (Untereiner & Naveau, 1999). These fungi, commonly called black yeasts, are characterized by the presence of melanin or melanin-like pigments in the cell walls and by a yeast-like growth during part of their life cycles (Haase et al., 1999). Exophiala is also characterized by an annelidic form of blastic conidiogenesis and by conidigenous cells that are often inconspicuously annellate (de Hoog & Hermanides-Nijhof, 1977). The Dictionary of Fungi (Kirk et al., 2001) cites twelve species in the genus Exophiala; however, additional species have been described more recently (e.g. de Hoog et al., 2003). Some species of the genus Exophiala are known as occasional opportunistic human pathogens (de Hoog et al., 2003; Haase et al., 1999).

Capronia coronata Samuel, having an Exophiala anamorph and isolated from decorticated wood in the Westland
County of New Zealand (Müller et al., 1987), was the closest known relative of the BSC isolates CP141bT and OR302-3. However, the sequence divergence value of 3-3% in the divergent D1/D2 domain of the large subunit (LSU) nrRNA gene indicates that our isolates belong to a distinct species and refute an anamorph–teleomorph connection (Kurtzman & Robnett, 1998; Peterson & Kurtzman, 1991). The International Code of Botanical Nomenclature currently allows the use of a separate binary name for the fungal anamorph if the teleomorphic relationship is not firmly established (Greuter et al., 2000); therefore, we propose the name Exophila crusticola for this novel anamorphic species.

**Fungus isolation and characterization**

Strains CP141bT and OR302-3 were isolated from BSC samples collected from the Colorado Plateau (38° 34’ 09-84” N 109° 31’ 0451” W) and the Great Basin desert (44° 29’ 06-03” N 121° 04’ 07-30” W), USA. Initially, 0-5 g crust samples were suspended in Ringer’s solution (Lorch et al., 1995), shaken for 30 min and the suspension was allowed to settle. Subsequently, 100 μl supernatant was placed on plates of BG11 minimal medium (Rippka et al., 1979). The plates were incubated under light at room temperature. Fungal colonies were observed on plates where cyanobacteria were also present. Strains CP141bT and OR302-3 were selected because of their dark pigmentation and replated on a nutrient-rich 10 × PGY-BG11 medium (Lorch et al., 1995). This medium was also used to obtain inoculum for all biochemical tests.

After the initial isolation, strains CP141bT and OR302-3 were cultured on standard PDA (Yarrow, 1998) and PGY (Lorch et al., 1995) plates, in addition to 10 × PGY-BG11 plates, in order to observe growth and the formation of hyphal elements. The temperature range for growth was determined on 10 × PGY-BG11 between 10–50°C (in increments of 5°C). The pH range for growth was determined on 10 × PGY-BG11 buffered in 0.1 M phosphate with the pH ranging from 5 to 12 at intervals of 1 pH unit. Catalase activity was determined by bubble production in 30% (v/v) aqueous hydrogen peroxide solution. Oxidase activity was tested by the oxidation of 1% (w/v) tetramethyl-p-phenylenediamine (Merck). Carbon assimilation tests were performed by adding each carbon compound at a final concentration of 0.5% to a base stock of BG11 medium without citric acid (Reddy & Garcia-Pichel, 2005).

Standard methodologies were followed for the maintenance of cultures and the description of yeast-like morphology (Yarrow, 1998). Micromorphological data were gathered using a conventional bright-field microscope (BH2; Olympus) or a microscope in differential interference contrast or bright-field mode (Axio Imager. A1; Zeiss). Microscopic examinations were made from fresh cells in water mounts. Measurements were made at a magnification of ×1000 using a calibrated ocular. Cell and conidia dimension statistics include: x, the arithmetic mean of the cell/conidia length by cell/conidia width (± SD) for n cells/conidia measured; Q, the quotient of cell/conidia length and cell/conidia width in any one cell/conidia, indicated as the range of variation in n cells/conidia measured; Qm the mean of Q values. Colour descriptions adhere to the nomenclature and codification system outlined in the Methuen Handbook of Colour (Kornerup & Wanscher, 1967).

**DNA isolation, PCR and sequence analysis**

A rapid mini-DNA isolation method from yeast-like cells was standardized as follows: a 1 ml suspension of CP141bT culture was centrifuged in an Eppendorf tube at 10,000 r.p.m. for 5 min at room temperature. The cell pellet was suspended in 200 μl lysis buffer (10 mM Tris, 1 mM EDTA pH 8, 1% SDS), 200 μl Tris-saturated phenol was added and the mixture was incubated at 65°C for 60 min. The suspension was then centrifuged at 10,000 r.p.m. for 5 min at room temperature and the supernatant collected. The supernatant was treated with 200 μl chloroform/iso-amyl alcohol mixture (24:1), vortexed and centrifuged at 10,000 r.p.m. for 5 min at room temperature. The supernatant was collected and added to 20 μl 3 M sodium acetate and 600 μl ice-cold alcohol. This mixture was vortexed and incubated at −80°C for 30 min. The suspension was centrifuged at 10,000 r.p.m. for 20 min at 4°C. The resultant DNA pellet was washed with 70% alcohol, vacuum-dried and dissolved in 20 μl TE buffer.

The divergent D1/D2 domain was amplified with the combination of the NL-1 (5’-GATATCAATAGCG-GAGGAAAAG-3’) and NL-4 (5’-GTCGGTTGCTCAG-CAGG-3’) primers of O'Donnell (1993). The ITS4 (5’-TCCTGCTTATTGATATGC-3’) and ITS5 (5’-GGA-AGTTAAAAAGCTGTAACAGG-3’) primers of White et al. (1990) were used to amplify the internal transcribed spacer (ITS1) of the nrRNA gene. PCR was performed with a final reaction mixture (50 μl) containing ~50 ng genomic DNA, 25 pmol each primer, 200 mM dNTPs, 2.5 mM MgCl2, 2.0 U Taq polymerase and 5 μl 10× reaction buffer (Takara). The amplification reactions were performed in a PTC 150 Mini Cycler (MJ Research) with the following cycling parameters; 5 min at 94°C, followed by 30 cycles at 94°C, 30 s at 56°C, and 60 s at 72°C, with a final extension for 5 min at 72°C. The amplified products were separated on a 1% 0.5% agarose gel and visualized by staining with ethidium bromide (0.5 μg ml⁻¹). Amplicons were removed from the gel and purified using a gel extraction kit (Qiagen). Direct sequencing of purified PCR products was performed with the ABI Big Dye Terminator kit (Perkin Elmer Applied Biosystems). Both strands of the PCR product were sequenced for the D1/D2 domain and ITS1 using the NL1 and NL4 and ITS4 and ITS5 primers, respectively.

A sequence similarity search was performed using GenBank BLASTN (Altschul et al., 1997) and sequences of closely related taxa were retrieved and aligned using the CLUSTAL W module in the MEGA 3 software package (Kumar et al., 2004).
Pair-wise evolutionary distances between the sequences were calculated using Kimura’s two-parameter model (Kimura, 1980). Phylogenetic trees were constructed using three tree-making algorithms: neighbour-joining (NJ), maximum-parsimony (MP) and the unweighted pair group method with arithmetic mean (UPGMA) in the MEGA 3 software package (Kumar et al., 2004). Clade stability in the phylogenetic trees was assessed using 1000 replicate datasets in a bootstrap analysis.

**Classification, phylogenetic analyses and ecology**

Strains CP141bT and OR302-3 exhibited similar growth and morphological characteristics. Nearly all physiological characteristics exhibited by these isolates were identical; however, a few minor differences were observed (see Supplementary Table S1 in IJSEM online). Sequence data for the D1/D2 domain suggests that the strains are conspecific as the domain contained only one nucleotide substitution (see Supplementary Table S2 in IJSEM online), thus having less than 1% sequence divergence (Peterson & Kurtzman, 1991). Strain CP141bT was chosen as the representative strain for further characterization and as the basis for the species description as it was the first to be isolated.

Specific characteristics (morphological, growth-related and biochemical) of *Exophiala crusticola* anam. nov. are given in the species description. In general, the isolates were metabolically versatile in their use of sugars and small-molecular mass organics, with the exception of amino acids, and were not very proficient at metabolizing complex organics. This may indicate that their ecological role may be as osmotrophs specialized on the photosynthetic exudates from cyanobacteria. Recurrent wetting and drying cycles in soil crusts promote a chronic leaching of organics from these primary producers, creating a significant concentration of dissolved organics in the soil solution, which are then available to consumers such as *E. crusticola*. Both isolates were recovered from minimal media on which cyanobacteria were growing and they appeared to be thriving on cyanobacterial exudates. This type of trophic role was alluded to by de Hoog (1993) who described black yeasts of the family *Herpotrichiellaceae* as secondary saprophytes that are known to inhabit ‘bacterial mats’.

Strains CP141bT and OR302-3 exhibit morphological characters, including a dimorphic habit and inconspicuously annellate conidiogenous cells, which support their placement within the genus *Exophiala*. Morphologically, *E. crusticola* is characterized by mucoid colonies that exhibit very slow growth. Conidiogenous cells are produced on torulose hyphae, normally terminal, and on unswollen hyphae, normally as singular, intercalary, fertile loci. Conidia are globose, subglobose to ovoid (Fig. 1). The ability of *E. crusticola* to produce submerged hyphae that, in turn, produce conidia (Fig. 1) is not common within the genus *Exophiala* and may reflect a growth habit that is compatible with the BSC habitat. The manner in which *E. crusticola* produces conidia is similar to that found in *Exophiala lecanii-corni* (Benedek & G. Specht) Haase & de Hoog. *E. lecanii-corni*, however, tends to be more conspicuously hyphal, whereas *E. crusticola* exhibits a more consistent yeast-like phase. In addition, the former produces ellipsoidal to reniform conidia that are narrower (1·5–2·4 μm in width) and have a broader range of lengths (3·3–5·9 μm) than conidia found in *E. crusticola* (de Hoog & Hermanides-Nijhof, 1977). Other species of the genus *Exophiala* are more readily distinguished from *E. crusticola*. For example, the elongated, lateral conidiogenous cells found in species such as *Exophiala salmonis* and *Exophiala jeanselmei* var. *jeanselmei* are lacking in *E. crusticola*.

Phylogenetic studies based on nrRNA gene sequences suggest that the family *Herpotrichiellaceae* constitutes a monophyletic entity (Untereiner & Naveau, 1999). Our analysis included sequence data from seven taxa representing the teleomorph genus *Capronia* and twelve taxa representing the anamorphic genera *Cladophialophora, Exophiala, Fonsecaea* and *Phialophora*, which were extracted from GenBank. *Phaeococcomyces catenatus* (Chaetothyriales, Ascomycetes, Ascomycota) and *Saccharomycyes cerevisiae* strain S288C (Saccharomycetales, Saccharomycetes, Ascomycota) were selected for the outgroup. Alignment of the D1/D2 LSU nrRNA gene sequences revealed 40 unique nucleotide signatures for *E. crusticola* in comparison with other closely related anamorphic (*E. jeanselmei*, *Exophiala moniliae* and *Exophiala spinifera*) and teleomorphic (*Capronia acutiseta* and *Capronia coronata*) taxa.

![Exophiala crusticola anam. nov. CP141bT on PDA agar at 25 °C. (a) Annellate yeast-like cell; (b) conidiogenous cells producing conidia; (c) torulose hyphae; (d) fascicle of submerged hyphae protruding from colony margin. Bars, 1 μm (a), 2 μm (b–c), 1 mm (d).](http://ijs.sgmjournals.org)
(see Supplementary Table S2 in IJSEM online). Neighbour-joining and maximum-parsimony analyses (data not shown) placed *E. crusticola* deep within the *Herpotrichiellaceae* clade. Both *C. acutiseta* and *C. coronata* showed a 3-3% sequence divergence from *E. crusticola*. Although analysis using UPGMA, maximum-parsimony (data not shown) and neighbour-joining (Fig. 2) criteria revealed the close relationship between *C. coronata* and *E. crusticola*, the nodes were not strongly supported by bootstrap values. However, the analyses overwhelmingly supported this relationship over that of *C. acutiseta* with *E. crusticola*. When compared with *E. crusticola*, the sequence of *E. jeanselmei* showed a 3-8% sequence divergence and those of *E. moniliae* and *E. spinifera* showed a 4-0% sequence divergence. The topology of trees recovered in a phylogenetic analysis using the ITS sequence (see Supplementary Fig. S1 in IJSEM online) also supported our results. Neighbour-joining and maximum-parsimony analyses (data not shown) and neighbour-joining (Fig. 2) criteria revealed the close relationship between *E. crusticola* and *C. acutiseta* (e.g. Spatafora et al., 1995; Untereiner & Naveau, 1999; de Hoog et al., 2003).

Isolation of strains of *E. crusticola* from Colorado Plateau and Great Basin desert BSCs samples suggests that this anamorphic fungus may be common in BSCs and that it plays a role in crust ecology. Cultivation-independent studies of field samples would be necessary to reinforce this conclusion. However, these results certainly support States and Christensen's (2001) conclusion, based on cultivation, that dark-pigmented fungi are prominent components of BSCs. Furthermore, finding *E. crusticola* associated with BSCs in desert localities is not surprising considering that black yeasts have been isolated from extreme environments and they are known to be able to survive severe drought, excessive temperatures and UV irradiation because of their robust, melanized cell walls (de Hoog, 1993). Extracellular polysaccharides (EPS) produced by prokaryotic organisms are known to bind soil particles, thus aiding in the establishment of BSCs and slowing erosion in desert soils (Garcia-Pichel et al., 2001). Black yeasts are also known to produce EPS (de Hoog, 1993) and, in culture when supplied with plenty of organics, isolates of *E. crusticola* produce copious amounts of EPS. This suggests that eukaryotic fungal organisms associated with BSCs also contribute to soil stability in desert systems through EPS production in addition to the aggregation of soil particles by hyphae. The isolation of other EPS-producing yeasts from BSCs, such as *Rhodotorula* (R. Potrafka, personal communication), lends further credence to this hypothesis.

Although other unidentified 'yeasts' have been isolated from BSCs (States & Christensen, 2001), this publication appears to be the first to clearly identify a BSC-associated black yeast. The fact that our isolates represent a novel anamorphic species underscores the need for further investigation into the diversity and ecology of fungal components of BSCs. Overall, the paucity of literature related to fungi associated with BSCs reflects the fact that these organisms are inadequately known and indicates that the field is ripe for future investigation.

**Latin diagnosis of Exophiala crusticola Bates, Gundlapally & Garcia-Pichel anam. nov.**

*Colonia ex CP141b<sup>†</sup> in PGY ad 25 °C (±2 °C) tardae crescentes, 2-0-3-0 mm diametro ad 14 dies attingentes. In PDA ad 25 °C (±2 °C) tardae crescentes, 0-5-1-0 mm diametro ad 14 dies at 25 °C (±2 °C) attingentes. Videor mucosae quam primum, deinide laeves ut verruculosae, siccae et firmae, elevatae ex agarum usque 1 mm ad limites brunneae (6E4), fusco-brunneae (6F4) ut griseo-brunneae (6F3), partes interiores nigellae. Margo integer et quam primum acutus designo, inde hyphae submersae lineares vel

![Fig. 2. Neighbour-joining tree based on the analysis of the sequence of the D1/D2 domain of the LSU rRNA gene (approximately 540 bases) showing the relationship between *Exophiala crusticola* anam. nov. CP141b<sup>†</sup> and related taxa. GenBank accession numbers are given in parentheses. Bootstrap values are expressed as percentages of 1000 replications and clades supported by bootstrap values over 65% are indicated (branches in bold). The outgroup, *Phaeococcomyces catenatus* strain CBS 650.76 (AF050277) and *Saccharomyces cerevisiae* strain S288C (NC001144), was trimmed from the figure without modifying the tree topology. Bar, 0-01 substitutions per nucleotide position.](image-url)

Cellulae fermentiformis commune, cellulae-singulae, parietes-latae, globosae, subglobosae, ovoidea ut limoniformes, 5–6–9 × 4–8–7–2 μm [x=6.5±0.7×5.9±0.9 μm, Qm=1.1, n=20], subhyaline, olivacea pallidae ut brunnea pallidae, ad apex expediciones conidia secundariae ex singulae, minutae, inconspicuus annelatus clavae conidiiferiae. Hyphae exinflatae vel torulose, parietes-latae, septatae, subglobosae, ovoidea, ellipsoidae ut cylindriceae et elongatae, ramorae interdum vel regularae, 3–0–8 μm diametro, subhyaline, olivacea pallidae ut brunnea pallidae; cellulae conidiogenitae non disimiles, parietes-latae, ellipsoidae ut cylindriceae et elongatae, subhyaline, olivacea pallidae ut brunnea pallidae, ramorae raro vel interdum; conidia gentiae ex minutae, inconspicuus annelatus clavae conidiiferitae, terminales ex hyphae torulose vel laterales ex singulae, intercalares loci ex hyphae exinflatae (raro terminales). Conidia cellulae-singulae, parietes-teniae, laeves, globosae, subglobosae ut ovoidea, 4–0–4–8 × 3–2–4–0 μm [x=4.3±0.4×3.5±0.4 μm, Qm=1.2, n=20], subhyaline, olivacea pallidae ut brunnea pallidae. Teleomorphus incognitus.

CP141bT erat positus pro catalasa, oxidasa, ureasa et inители pro lipasa, phosphatasa, gelatinasa, ADNasa, β-galactosidasa, deaminasa phenylalaninum, dihydrodrolasa argininae, decarboxylasae lysinae et decarboxylasae ornithinae, non poterat uter caseinum vel aesculinum. Fungo poterat uter salis acetii, arabinosum, fructose, galactosum, glucosum, glycerinem, inulimum, acidum lacticum, lactosum, levulosum, maltosum, mannitum, rafinodum, saccharosum, sucrosum et xylosum per origo ex carbonis, autem non poterat uter acidum citricum, dextrinum, ethanolaminum, vel acidum succinicum. Cycloheximidum cellularum ex CP141bT venum est.

Holotypus: Colorado Plateau (38° 34' 09"-84° N 109° 31' 04'-51" W), USA – segregatus ex collecto crustae biologicae terrarum. Cultura sica ex Exophiala crusticola (isolatus typus CP141bT) ad UAMH cum numerus accessionem 10686 deposita est. Isolatus typus CP141bT (= ATCC MYA-3639 = CBS 119970 = DSM 16793) modus lyophilizatus conservatus est.

**Description of Exophiala crusticola Bates, Gundlapally & Garcia-Pichel anam. nov.**

*Exophiala crusticola* (crus.ti' cola. L. n. crusta crust; L. suff. -cola from L. n. incola an inhabitant; N.L. n. crusticola a crust inhabitant).

Colonies of CP141bT on PGY at 25 °C (± 2 °C) are slow-growing, attaining 2–0–3–0 μm diameter at 14 d. On PDA at 25 °C (± 2 °C), colonies are slow-growing, attaining 0.5–1.0 mm diameter at 14 d. Appear mucoid at first, soon becoming smooth to verruculose, dry and firm, elevated from media up to 1 mm, outer part brown (6E4), dark brown (6F4) to greyish brown (6F3) and inner parts blackish. Margin entire and, at first, sharply defined, then submerged linear or branching hyphae gradually develop that emerge randomly around the margin and form abundant fascicles after 14 d on PDA. Aerial hyphae eventually develop over the surface of the colony. Hyphae lacking after 14 d on PGY. Lacking exudates and odour. Growth observed at temperatures ranging from 5–30 °C, growth not observed at or above 37 °C.

Yeast-like cells are abundant, single-celled, thick-walled, globose, subglobose, ovoid to lemon-shaped, 5–6–8–0 × 4–8–7–2 μm [x=6.5±0.7×5.9±0.9 μm, Qm=1.1, n=20], subhyaline, pale olivaceous to pale brown, developing secondary conidia at the apex from a single, minute, inconspicuously annelate conidiferous peg. Hyphae unswollen or torulose, thick-walled, septate, subglobose, ovoid, elliptical to cylindrical and elongate, occasionally or regularly branched, 3–0–8 μm in diameter, subhyaline, pale olivaceous to pale brown; conidiogenous cells undifferentiated, thin-walled, elliptical to cylindrical and elongate, subhyaline, pale olivaceous to pale brown, rarely to occasionally branched; conidia produced from a minute, inconspicuously annelate conidiferous peg, terminal on torulose hyphae or laterally as single, intercalary loci on unswollen hyphae (rarely terminally). Conidia single-celled, thin-walled, smooth, globose, subglobose to ovoid, 4–0–4–8 × 3–2–4–0 μm [x=4.3±0.4×3.5±0.4 μm, Qm=1.2, n=20], subhyaline, pale olivaceous to pale brown. The teleomorph is unknown.

CP141bT is positive for catalase, oxidase and urease activities and negative for lipase, phosphatase, gelatinase, DNNase, β-galactosidase, phenylalanine deaminase and arginine dihydrodrolase activities, as well as lysine and ornithine decarboxylases. Is not able to hydrolyse casein or aesculin. The fungus is able to utilize acetate, arabinose, fructose, galactose, glucose, glyceral, inulin, lactic acid, lactose, levalose, maltose, mannitol, raffinose, saccharose, sucrose, et xyllosum per origo ex carbonis, autem non poterat uter acidum citricum, dextrinum, ethanolaminum, vel acidum succinicum. Cycloheximidum cellularum ex CP141bT venum est.

Holotype: Colorado Plateau (38° 34' 09"-84° N 109° 31' 04'-51" W), USA, isolated from a biological soil crust sample. A dried culture of *Exophiala crusticola* (type strain CP141bT) is deposited at UAMH (accession no. 10686). The type strain CP141bT is also maintained freeze-dried at the ATCC (ATCC MYA-3639), CBS (CBS 119970) and DSMZ (DSM 16793) culture collections.

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References


