Fungal Biology 123 (2019) 151–158

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

Exophiala macquariensis sp. nov., a cold adapted black yeast species recovered from a hydrocarbon contaminated sub-Antarctic soil

Chengdong Zhang [a], Nick Sirijovski [b], Lewis Adler [c], Belinda C. Ferrari [a, *]

[a] School of Biotechnology and Biomolecular Sciences, UNSW Sydney, NSW, 2052, Australia
[b] Division of Pure and Applied Biochemistry, Lund University, Lund, 22100, Sweden
[c] Bioanalytical Mass Spectrometry Facility, UNSW Sydney, NSW, 2052, Australia

ARTICLE INFO

Article history:
Received 15 August 2018
Received in revised form
22 November 2018
Accepted 26 November 2018
Available online 30 November 2018

Corresponding Editor: Nik Money

Keywords:
Bioremediation
Diesel fuel
Macquarie island
Soil fungi
Toluene degradation

ABSTRACT

A new black yeast species, *Exophiala macquariensis* is described that is a member of the ascomycete family *Herpotrichiellaceae*, order *Chaetothyriales*. The genus *Exophiala* is comprised of opportunistic pathogens isolated from clinical specimens as well as species recovered from hydrocarbon contaminated environments. Several species have been reported to be able to degrade benzene, toluene, ethylbenzene and xylenes. Here, a novel species of *Exophiala* (CZ06) previously isolated from a Sub-Antarctic, Macquarie Island soil that was spiked with Special Antarctic Blend diesel fuel (SAB) is described. This isolate has the capacity of toluene biodegradation at cold temperatures. Multilocus sequence typing showed that this fungus was closely related to the pathogenic species *Exophiala salmonis* and *Exophiala equina*. With the capacity to utilise hydrocarbons as a sole carbon source at 10 °C, this fungus has great potential for future bioremediation applications.

1. Introduction

Exophiala is a black yeast genus of the ascomycete family *Herpotrichiellaceae*, order *Chaetothyriales*. Almost all *Exophiala* species form yeast like cells and hypha transitions, and produce budding cells (de Hoog et al., 2011). They have been isolated from clinical specimens (Woo et al., 2013; Yong et al., 2015), animal tissues (de Hoog et al., 2011), environmental samples (de Hoog et al., 2006; Hamada and Abe, 2010; Zhao et al., 2010), plants (Li et al., 2011), arsenic mine soil (Seyedmousavi et al., 2011) and deep sea ocean sediments (Liu et al., 2016).

To date, 51 *Exophiala* species are described and illustrated in MycoBank (www.mycobank.org; Crous et al., 2004). Numerous *Exophiala* species are opportunistic pathogens of immunocompetent humans, causing cutaneous and subcutaneous skin infections (Woo et al., 2013; Yong et al., 2015), and in rare occasions nervous system phaeohyphomycosis (Chang et al., 2000). Furthermore, some *Exophiala* species, such as *Exophilia salmonis*, *Exophiala aquamarina* and *Exophiala equina* may cause cutaneous or disseminated infections of cold-blooded animals (De Hoog et al., 2011). *Exophiala* species have also been frequently isolated from environments rich in hydrocarbons (De Hoog et al., 2006; Isola et al., 2013; Dogen et al., 2013a), with studies showing *Exophiala sp.* can degrade benzene, toluene, ethylbenzene and xylenes (BTEX) (Middelhoven, 1993; Qi et al., 2002; Estevez et al., 2005b). Thus, the genus *Exophiala* has great potential in the bioremediation of hydrocarbon contamination (Cox et al., 1997; Woertz et al., 2001; Estevez et al., 2005a).

Macquarie Island (54°30'S, 159°57'E), is a Sub-Antarctic Island located 1500 km south of Tasmania, Australia (Powell et al., 2010). A permanent Australian Antarctic station operated at the northern isthmus of the island from 1948. The annual temperature range is from 3 °C in June to 7 °C in January, with frequent precipitation. Since the energy supply for the scientific research station is primarily derived from the Special Antarctic Blend diesel fuel (SAB), a large volume of SAB is transported and stored in the Main Power House. Consequently, SAB spills have occurred, leading to several areas of soil pollution comprising petroleum hydrocarbons (Rayner et al., 2007). We recently isolated 91 fungal cultures from Macquarie Island, some of which were capable of growth on media spiked with up to 20,000 mg/kg SAB (Ferrari et al., 2011), including

* Corresponding author. School of Biotechnology and Biomolecular Sciences, UNSW Sydney, NSW 2052 Australia.
E-mail address: b.ferrari@unsw.edu.au (B.C. Ferrari).

https://doi.org/10.1016/j.funbio.2018.11.011
1878-6146/

the isolate designated CZ06, which we present here as a novel species of the genus *Exophiala*.

In this study, we describe CZ06 as a novel species that has the ability to utilise toluene as sole carbon and energy source. The strain was isolated previously from soils spiked with a range of SAB (Ferrari et al., 2011). Phenotypic characteristics indicate that this strain is distinct from other known *Exophiala* species. Phylogenetic analysis based on sequencing of the ITS gene, partial elongation factor 1-α (TEF1) and β-tubulin (TUB2) genes, shows that this *Exophiala* strain (CZ06; CBS 144232) is distinct from all other characterised *Exophiala* species, and is located in a clade composed of *E. salmonis* and *E. equina*. We therefore propose a new species, *Exophiala macquariensis sp. nov.*, to describe this toluene degrading fungus.

2. Materials and methods

2.1. Fungal strain

Exophilia sp. (CZ06) was previously isolated from Macquarie Island soil (Ferrari et al., 2011). Sequencing the internal transcribed space (ITS) region of the isolate revealed a 98 % homology to the corresponding region in *Exophilia salmonis*. This *Exophilia sp*. was recovered from soils spiked with 0–20,000 mg/kg SAB fuel and displayed the potential to grow on toluene as the only carbon source.

2.2. Morphological and physiological characterization

Initial identification of *Exophilia sp*. (CZ06) was based on macroscopic and microscopic features. For microscopic observations, fungi were sub-cultured on PDA and Malt extract agar (MEA) at 25 °C for two weeks. Morphological and microscopic observations were carried out on slide cultures. To do this, one cm^3 agar plugs were cut from the edge of fungal colonies present on PDA plates. The agar plugs were placed upside down onto a sterile glass slide and covered with a cover slip. Then, the microscope slide was placed into a petri dish and incubated at 25 °C for seven days. Before the examination, the agar plugs were removed. Both hyphae and spores were mounted onto a microscope slide in 100 μl lactophenol cotton blue solution (Sigma–Aldrich) and covered with a coverslip. The microscope slides were observed using a BX61 Epi-Fluorescence Microscope equipped with a DP71 digital camera (Olympus, Mt Waverley, Australia) for imaging using the differential interference contrast (DIC) filter. Cardinal growth temperatures were determined by inoculating fungi on MEA culture plates in the dark for 14 d, at temperatures ranging from 4, 10, 20, 25, 30 and 37 °C, all in three simultaneous replicates.

2.3. DNA extraction

Exophilia sp. sub-cultures were maintained on PDA and incubations at 25 °C every two weeks. For DNA extraction, a portion (100 mg) of mycelium was scraped from the surface of agar plates cultures using a sterile blade, for extraction using the DNAsecure Plant Kit (TIANGEN Biotech) according to manufacturer's instructions. A 50 μl eluate was obtained and used for subsequent experiments. The NanoDrop ND-2000 Spectrophotometer (Thermo Scientific) was used for measuring the quality and concentration of DNA. The resulting gDNA extracts was stored in clean 1.5 ml microcentrifuge tubes at −20 °C until required.

2.4. PCR amplification and Sanger sequencing

Three genes were targeted for sequencing: the internal transcribed spacer region (ITS) White et al. (1990), the partial translation elongation factor 1-α gene (TEF1) and the partial β-tubulin gene (TUB2) (De Hoog et al., 2011). PCR was performed in 50 μl reaction volumes comprised of 2× Green Taq Master Mix (Biorise Life Sciences) containing 25 pmol of each primer and 100 ng gDNA template using the JoyTouch Thermocycler (Biorise Life Sciences). Three primer sets ITS1/ITS4, EF1-728F/EF1-986R and Bt-2a/Bt-2b (Table 1) were obtained from Integrated DNA Technologies, which were used to amplify the fungal ITS rDNA region, partial TEF1 and partial TUB2 genes, respectively.

The PCR program for amplification of the fungal ITS and partial TEF1 consisted of an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. Amplification of partial TUB2 was performed by an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min denaturation, 58 °C for 30 s annealing, 72 °C for 1 min extension followed by a final extension step at 72 °C for 5 min. PCR amplicons were run on a 2 % (w/v) agarose gel with the addition of SYBR Safe DNA gel stain (Invitrogen). The gel was visualised using the Safe Imager 2.0 Blue-Light Transilluminator (Invitrogen).

PCR amplicons were purified with the iMag PCR product purification Kit (Biorise Life Sciences) and were cloned into the pGH-TA vector (Biorise Life Sciences), according to the manufacturer's instructions. The DNA sequencing reaction was carried out using the M13 universal sequencing primers (Table 1) and the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Sanger sequencing was performed using the ABI 3730 sequence scanner (Applied Biosystems) at the Ramaciotti Centre for Gene Function Analysis (UNSW, Sydney).

2.5. Phylogenetic analysis

E. macquariensis and fifteen *Exophiala sp*. CBS type strains were used for phylogenetic analysis, with *Cladophialophora mycetomatis* (CBS 454.82) used as an outgroup. The CBS type strains ITS, TEF1 and TUB2 reference sequences were obtained from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). All three loci were combined to produce a concatenated sequence, aligned using ClustalW (MEGA5.05) and a multi locus phylogenetic tree was reconstructed using the Maximum Likelihood statistical method. For this analysis, the nearest neighbour interchange was used as the heuristic method, and gaps were treated as partial deletions with a

Table 1
Primer sets used for amplifying fungal ITS rDNA, TEF1 and TUB2 genes.

Primer	Sequence	Target gene	Reference
ITS1	TCCGTAGGTGAACCTGCGG	ITS	White et al. (1990)
ITS4	TCCTCCGCTTATTGATATGC	ITS	White et al. (1990)
EF1-728F	CATCGAGAAGTTCGAGAAGG	TEF1	De Hoog et al. (2011)
EF1-986R	TACTTGAAGGAACCCTTACC	TEF1	De Hoog et al. (2011)
Bt-2a	GGTAACCAAATCGGTGCTGCTTTC	TUB2	De Hoog et al. (2011)
Bt-2b	ACCCTCAGTGTAGTGACCCTTGGC	TUB2	De Hoog et al. (2011)

95 % site coverage cutoff. The branch robustness was estimated using 1000 Bootstrap replications and the General Time Reversible model was the nucleotide substitutions type (Yong et al., 2015).

2.6. Indole-indigo assay

The indole-indigo assay is based on the principle that during microbial oxidisation of aromatic hydrocarbons, colourless indole is oxidised to indoxyl thus it is converted to red colour, which can be visualised by the naked eye (Hamzah et al., 2010). Fungal mycelia were cultured in 200 ml of 3 % (w/v) Malt Extract (Bacto Laboratories) broth in a 1 L flask and were incubated for 14 d at 25 °C, with shaking at 80 rpm to enrich mycelia. The mycelia were then harvested by centrifugation at 4000 rpm for 10 min, and the mycelia pellet was washed with sterile saline solution (0.9 %) three times by centrifugation (5 min, 12000 rpm). Mycelia pellets were then resuspended with 50 ml Czapek Dox broth without a carbon source (negative control), 3 % glucose (positive control) or 1 % toluene (Sigma–Aldrich) as sole carbon and energy source, in triplicate. The Czapek Dox broth without carbon source was prepared with 2 g $NaNO_3$ (Ajax Finechem), 0.5 g KCl (Ajax Finechem), 1 g K_2HPO_4 (Ajax Finechem), 0.5 g $MgSO_4•7H_2O$ (Ajax Finechem), 0.01 g $FeSO_4•7H_2O$ (Ajax Finechem) and sterile distilled water to a final volume of one litre. Furthermore, the addition of 30 g glucose (Ajax Finechem) to prepare a 3 % glucose containing Czapek Dox broth or 10 ml toluene (Sigma–Aldrich) to prepare a 1 % toluene containing Czapek Dox broth.

Fungi were incubated at 10 °C with shaking at 80 rpm for 14 d. Sub-samples (1 ml) were taken at days 0, 1, 2, 4, 6, 8, 10, 12 and 14, filtered through a 0.45 μm syringe filter (Millipore) and 200 μl of the filtrate containing secreted enzymes was placed into a well of a 96 well plate. The enzyme reaction was carried out by the addition of 1 μl of 100 mM indole (Sigma–Aldrich) in N,N′-dimethylformamide (Sigma–Aldrich) into the well. Plates were incubated at 10 °C in the dark, and the formation of indigo was observed by naked eye after 12 h.

2.7. Gas chromatography analysis

Toluene standards of 10 mg (0.2 mg/ml), 20 mg (0.4 mg/ml), 30 mg (0.6 mg/ml), 40 mg (0.8 mg/ml) and 50 mg (1 mg/ml) in 50 ml Czapek Dox broth were prepared according to the solubility (0.52 g/L at 20 °C) and density (0.87 g/ml) of toluene. The sterile saline solution (0.9 %) washed fungal mycelia were resuspended in 50 ml Czapek Dox broth without the carbon source (Live mycelia Negative Control), with 3 % glucose + 0.8 mg/ml toluene (Glucose + Toluene) and with 0.8 mg/ml toluene (Toluene only). In addition, a sample of the mycelia was autoclaved and resuspended in 50 ml Czapel Dox broth which contained 0.8 mg/ml toluene (dead mycelia negative control). Six replicates were prepared for each set of samples, three for pre-culture biomass dry weight determination, and three for total biomass after 12 d of incubation. All standards and samples were sealed into 100 ml headspace vials (Thermo Scientific) and stationary incubated at 10 °C.

The toluene measurement was performed using an Agilent 7890 GC instrument installed with a GS-Q column (J&W, 113–3432, 30 m, 0.320 mm). The oven was programmed with the following settings: an initial temperature at 200 °C held for 1 min, followed by a ramp to 245 °C at 25 °C/min rate, held for 1 min at 245 °C, and post-run 1 min at 200 °C. The flame ionisation detector temperature was 250 °C. Helium was used as the carrier gas with a split ratio of 10:1, both column and septum flow was fixed at 3 ml/min. Samples were injected manually using a full 100 μL Gas Tight Syringe (SGE, Australia) each time and the injection temperature set to 250 °C. The toluene amount that remained in each sealed headspace bottle was measured on day 4, 8 and 12 of incubation. The measured peak area was converted into concentration units according to the standard curve of toluene standard. Data analysis was done using Chem station software (Agilent Technologies, USA).

3. Results

The gene sequences obtained for *Exophiala sp*. (CZ06) strain CBS 144232 were deposited in GenBank under the accession numbers: internal transcribed space ITS region (MF619956), partial elongation factor 1-α (MH297439) and partial β-tubulin gene (MH297438). The scientific name and morphological description of the novel species were deposited in MycoBank under accession number CBS 144232.

Three loci of all 17 fungal strains (ITS: 546 characters, TEF1: 204 characters, TUB2: 337 characters) were combined to produce a concatenated sequence, and the multi locus analysis demonstrated that *Exophiala sp*. (CZ06) strain CBS 144232 was nested within the core of the ascomycete order *Chaetothyriales* (Fig. 2). *Exophiala sp*. (CZ06) was located in a clade composed of *Exophiala salmonis* (CBS 157.67) and *E. equina* (CBS 119.23), a sister group of this clade contained *E. aquamarina* (CBS 119918) and *Exophiala pisciphila* (CBS 537.73). The analysis showed that *E. macquariensis* (CBS 144232) was phylogenetically distinct from the other 15 CBS *Exophiala sp*. type strains anlaysed.

3.1. E. macquariensis *Zhang & Ferrari, sp. nov.*

MycoBank No.:CBS 144232 (Fig. 2)

De incubatione CBS 144232 in MEA post duo septimanas in 25 °C, in tenebris.

Coloniae restringitur, rotundum nigrum velutinae olivaceis, margine plana atque cano aerial mycelium ad centrum. Post quindecim coloniarum factus felty griseo substrato et certe in dies 1 mm. Reverse olivaceae nigrum. Diffusible pigmento non producitur. Fermentum cellulis abundant. Cellulae conidiogenae informibus utrem, terminales vel intercalares. Conidia ellipsoidea, 0-1 septatis, 1.5–2.0 × 3.0–5.0 μm, cum vix cernitur. Torsivi compositum est.

Cardinalis temperaturis: Minimum≤4 °C, optimum 20–25 °C, maximum 30 °C. Nec ad incrementum 37 °C.

Description of CBS 144232 on MEA after two weeks incubation at 25 °C in the dark.

Colonies were restricted, circular, olivaceous black with velvety, grey aerial mycelium at the centre and a flat margin (Fig. 1 A&B); After 14 d incubation colonies became felty, containing grey aerial mycelium, with a daily growth rate of approximately 1 mm; Marginal zone with yeast like characteristics; Reverse olivaceous black (Fig. 1C); No diffusible pigments were produced. Yeast cells abundant (Fig. 1H); Conidiogenous cells flask-shaped, intercalary or terminal (Fig. 1D–G). Conidia ellipsoidal, 0-1 septate, 1.5–2.0 × 3.0–5.0 μm, with discernible scars (Fig. 1D–F). Spirally twisted hyphae present (Fig. 1C).

Cardinal temperatures: Minimum ≤4 °C, optimum 20–25 °C, maximum 30 °C. No growth at 37 °C (Fig. 3).

3.2. Toluene utilisation

The indole-indigo assay gave a positive result at 10 °C, with light red colour observed in the toluene samples from days 2–14 (Table 1). In comparison, a dark red colour was observed in the toluene plus glucose samples from day 6–14. No colour change was observed the samples lacking a carbon source. Table 2.

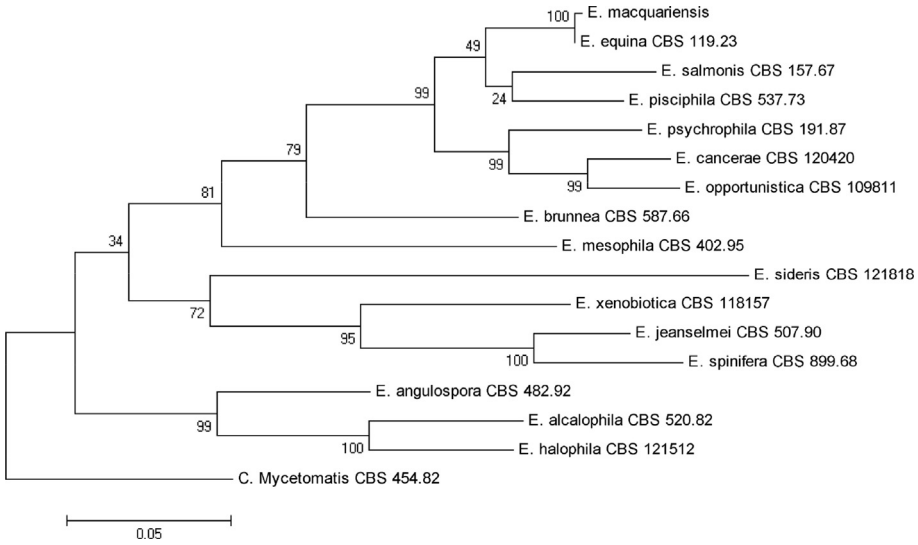

Fig. 1. Phylogenetic reconstruction of *Exophiala macquariensis* (CBS 144232) based on a concatenated sequence (ITS, TEF1 and TUB2) comparison to 15 *Exophiala sp.* CBS type strains and an outgroup (*Cladophialophora mycetomatis*). The Maximum Likelihood tree was constructed using MEGA 5.05 with General Time Reversible model. Bootstrap support values were estimated based on 1000 replicates.

3.3. Gas chromatography analysis and toluene consumption

During 12 d of incubation, the concentration of toluene in the dead mycelia negative control samples were maintained at approximately 0.8 mg/ml throughout the entire incubation period (Fig. 4). The concentration of toluene present in the Toluene only samples gradually decreased to 0.4 mg/ml, with a similar shift observed for the Glucose + Toluene samples.

The dry weight of the live mycelia negative control and dead mycelia negative control samples was similar over the 12 d of incubation (Fig. 5). The average dry weight of the Toluene only samples was 121.9 mg ± 11.4 mg, which was an increase on average of 19.3 mg compared to day 0. In the Glucose + Toluene samples, the average dry weight was 131.4 mg ± 6.7 mg, which was an increase on average of 26.9 mg.

4. Discussion

We propose that the *Exophiala* strain CZ06 isolated from Sub-Antarctic Macquarie Island soil is a novel *Exophiala* species based on the phylogenetic and phenotypic analysis. For phylogenetic analysis, three independent DNA regions widely used for fungal species molecular identification, including ITS rDNA, TEF1 and TUB2 were analysed (Fig. 2). The high bootstrap support of phylogenetic tree showed that *E. macquariensis* is located in the *salmonis*-clade, most closely related to *E. equina* but distinct from all other *Exophiala* species. Both *E. salmonis* and *E. equina* are described as opportunistic pathogens of cold-blood animals (de Hoog et al., 2011). Comparison of phenotypic characteristics of *E. macquariensis* with other closely related *Exophiala* species revealed unique phenotypic characteristics. The optimum growth temperature of *E. macquariensis* was 20–25 °C, and maximum temperature was 30 °C, which was lower than the optimum 24–30 °C and maximum 33–36 °C of *E. equina* (De Hoog et al., 2011). Compared with other waterborne *Exophiala* species, *E. macquariensis* exhibited similar minimum growth temperatures of <4 °C and no growth at 37 °C. Morphological comparisons to *E. equina* and other *salmonis*-clade species showed that the *E. macquariensis* lacks a slimy state observed for the other species at the beginning of growth, with highly abundant yeast type cells which rarely or nearly absent in *E. salmonis* and *E. equina*. Conidia were smaller than other *salmonis*-clade species (De Hoog et al., 2011). Hence, the black yeast selectively isolated from Sub-Antarctic Macquarie Island soil represents a distinct species within *Exophiala*.

Members of the black yeast genus *Exophiala* exhibit a complex ecological behaviour. Strains are frequently isolated from environments rich in hydrocarbons, such as gasoline car tanks (Isola et al., 2013) and creosote treated railway ties (Dogen et al., 2013a). In addition, they are also found in indoor environments where detergents are used, such as inside dishwashers (Zalar et al., 2011; Dogen et al., 2013b) and bathrooms (Hamada and Abe, 2010; Lian and de Hoog, 2010). *Exophiala* species have been previously shown to be enriched in creosote treated railway ties, compared to low levels in untreated, indicating aromatic compounds can promote the growth of these specialised fungi (Zhao et al., 2010; Dogen et al., 2013a). Therefore, selective cultivation with aromatic hydrocarbons can be applied to improve the *Exophiala* isolation from environmental samples (Zhao et al., 2010), and several novel *Exophiala* species have already been charactersied. For example, the isolation and identification of *Exophiala oligosperma* (Estevez et al., 2005b), *Exophiala xenobiotica* (De Hoog et al., 2006) and *Exophiala sideris* (Seyedmousavi et al., 2011).

Many studies have shown that *Exophiala sp.* can readily degrade BTEX. For example, Qi et al. (2002) reported *E. Lecanii-corni* could degrade aromatic hydrocarbons including benzene, ethylbenzene, toluene, and styrene as well as ketones and organic acids. Blasi et al. (2016) screened 163 black yeast like fungi, including *Exophiala* and found they can utilise toluene as the sole carbon and energy source. Exophiala are of interesting to the field of biotechnology, for example in the application of these fungi as biofilters for the removal of volatile aromatic hydrocarbons from polluted air (Woertz et al., 2001; Estevez et al., 2005a,bPrenafeta-Boldu et al., 2012; Rene et al., 2012a,b). Here, the indole-indigo assay showed that *E. macquariensis* was not only tolerant of but capable of utilising toluene as the sole carbon source.

While it is well established that *Exophiala* species, such as *E. oligosperma*, *Exophiala lecanii-corni*, *E. xenobiotica* (Woertz et al., 2001; Estevez et al., 2005a,b; Isola et al., 2013) can assimilate toluene, the toluene biodegradation pathway used is not known.

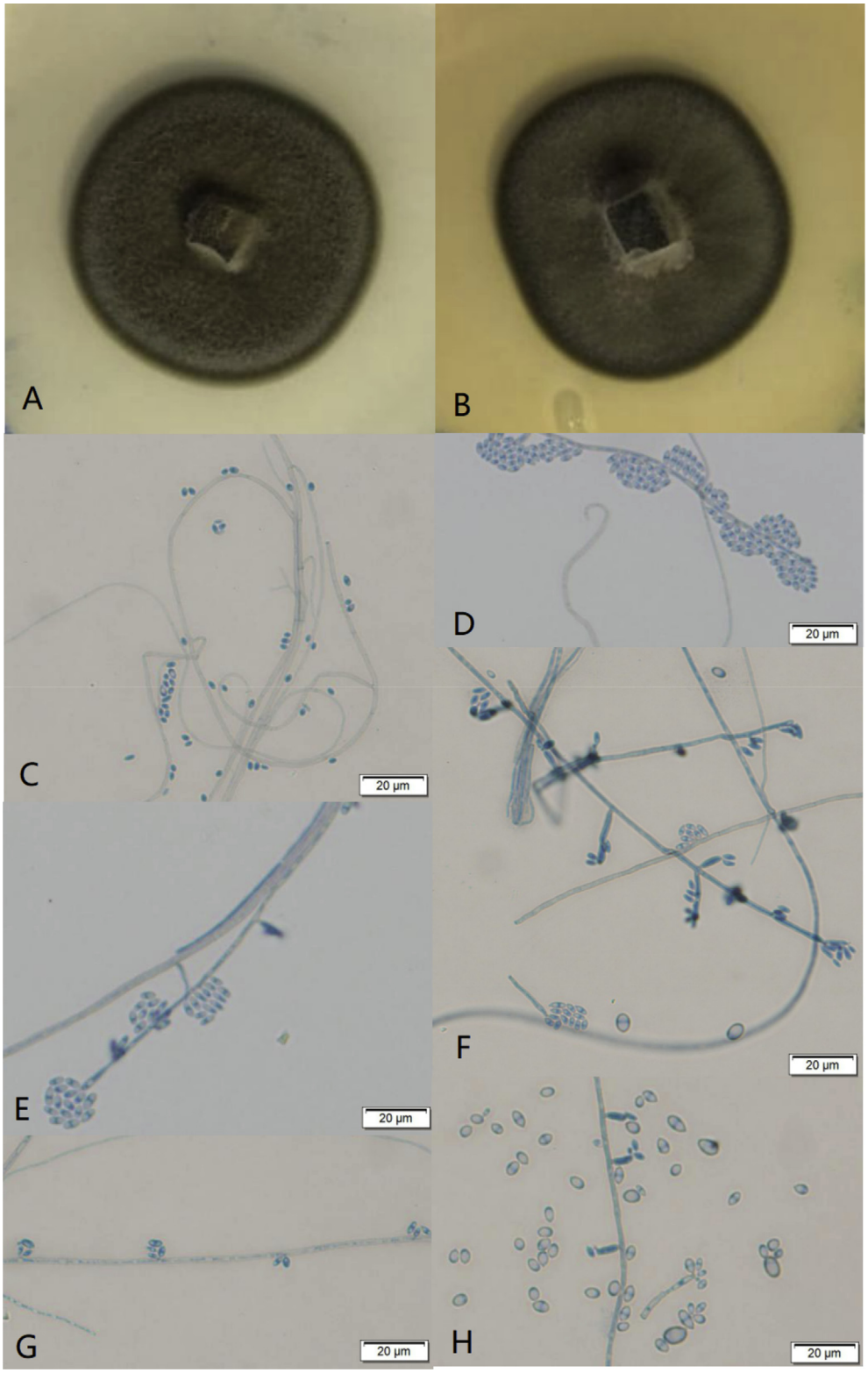

Fig. 2. *Exophiala macquariensis* sp. nov (CBS 144232). A. Cultured on PDA; B. Cultured on MEA; C. Spirally twisted hyphae; D. Multi-celled conidiophores; E. Erect cylindrical multi-celled conidiophores; F, G. Conidiophore with single conidiogenous cell; H. Yeast like cell.

Compared with toluene, more research on the styrene metabolic pathway of *Exophiala jeanselmei*, *E. oligosperma and E. lecanii-corni* is known (Cox et al., 1996; Gunsch et al., 2005; Rene et al., 2012a,b). Based on the analytical results of the HPLC, Cox et al. (1996) deduced the styrene biodegradation pathway of *E. jeanselmei* via styrene oxide, phenylacetaldehyde, phenylacetic acid, 2-hydroxyphenylacetic acid to homogentisic acid. Among them, cytochrome P-450-dependent monooxygenase was involved in the

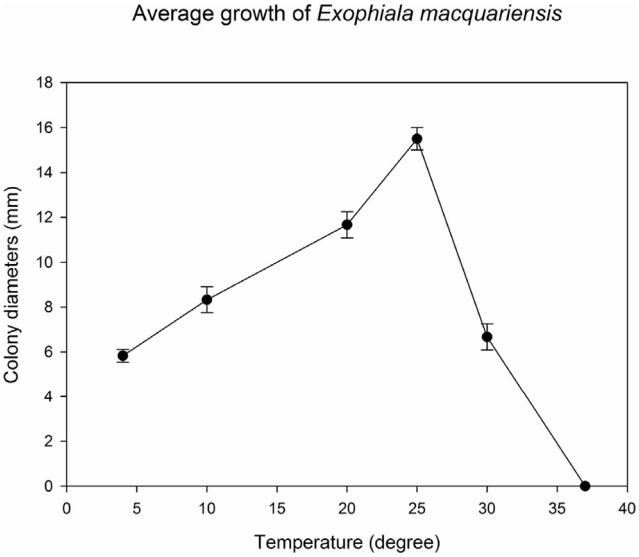

Fig. 3. Optimal growth temperature for *E. macquariensis*. Colony diameters of *E. macquariensis* (CBS 144232) at different growth temperatures ranging from 4 °C to 37 °C, were measured after 14 d incubation on MEA culture plates. Analysis included six independent samples.

oxidation of styrene. In 2012, Rene et al. carried out GC/MS and LC/MS to analyse the intermediate metabolites of styrene and confirmed that *E. oligosperma* uses the same metabolic route of *E. jeanselmei*. According to Gunsch et al. (2005), a homogentisate-1,2-dioxygenase gene was identified in *E. lecanii-corni*, which is involved in ethylbenzene degradation and may be responsible for the aromatic ring cleavage reaction, and the gene expression level induced to ethylbenzene degradation was measured by qRT-PCR assay later (Gunsch et al., 2006). Rustler et al. (2008) confirmed that homogentisate-1,2-dioxygenase catalysed the opening of the homogentisic acid aromatic ring to produce maleylacetoacetic acid. After that, maleylacetoacetic acid is catalysed by glutathione-dependent maleylacetoacetate isomerases into fumarylacetoacetate to acetoacetate and fumarate.

In recent years, some preliminary studies have shown that *Exophiala* species has broader industrial applications. Elmi et al. (2015) reported that *Exophiala spinifera* could desulfurise 99 % of 0.3 mM dibenzothiophene within seven days, and therefore can be used as a complementary method of biodesulfurization. In agriculture, *Exophiala* species that are plant endophytes have been isolated and used to improve agricultural productivity. For example, the presence of *E. pisciphila,* an endophyte of maize roots, increases host plant tolerance to heavy metal contaminated soils by limiting the transport of heavy metal ions from roots to shoots (Li et al., 2011). An endophyte *Exophiala* strain isolated from *Cucumis sativus* roots secreted phytohormones capable significant promote rice seedling, and improve cucumber tolerate to salinity and drought stresses (Khan et al., 2011). Additionally, studies have shown that *E. pisciphila* produce useful secondary metabolites, with exophillic acid and derivatives displaying functional inhibition of HIV-1 integrase activity (Ondeyka et al., 2003). While the exophillic acid derivatives isolated from another *Exophiala sp.* strains exhibit antiparasitic activity (Cheikh-Ali et al., 2015).

To date, there are only limited fungal species described from the Antarctic and Sub-Antarctic regions (Kerry, 1990; Ferrari et al., 2011; Godinho et al., 2013; Dreesens et al., 2014; Ji et al., 2016; Pudasaini et al., 2017). To our knowledge, the present work provides the first description of a novel *Exophiala* species isolated from Sub-Antarctic Macquarie Island soils polluted with petroleum hydrocarbons. *E. macquariensis* is a cold-adapted yeast, that is tolerant to petroleum hydrocarbons at growth limiting concentrations (Ferrari et al., 2011). Here we show that this novel species also has the capacity to utilise toluene as sole carbon and energy source at 10 °C (Figs. 4 and 5). Phylogenetic analysis showed that *E. macquariensis* is closely related to the cold-blood animal pathogenic species *E. salmonis* and *E. equina* (Fig. 1). Given the capacity to degrade toluene under cold conditions *E. macquariensis* will be of major interest to future bioremediation efforts.

Table 2
Indole-indigo assay of *E. macquariensis* sp. nov. with toluene as the sole carbon and energy source.

	Day 0	Day 1	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
No carbon source	–	–	–	–	–	–	–	–	–
Glucose samples	–	–	–	–	+	++	++	++	++
Toluene samples	–	–	+	+	+	+	+	+	+

(–: colourless; +: light red; ++: dark red).

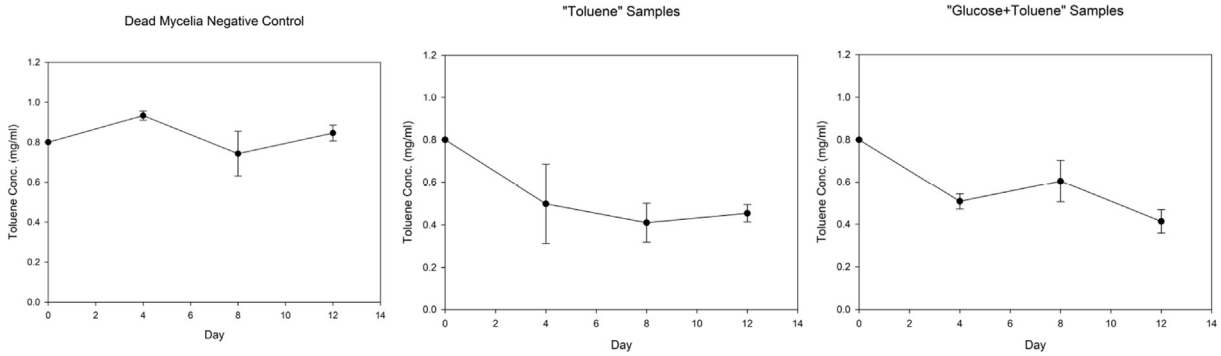

Fig. 4. GC–MS measurements of toluene remaining in *E. macquariensis* cultures monitored over 12 d of incubation at 10°C. Left: dead mycelia negative control, Middle: toluene only, Right; glucose + toluene. Cultures containing toluene as the only carbon source showed significant reductions in total toluene present after 8 d of incubation. Analysis was performed using three replicates.

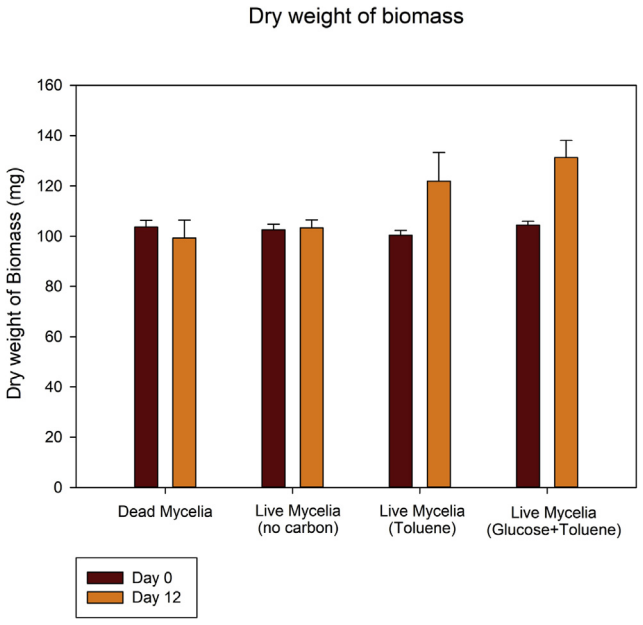

Fig. 5. Measured dry weight of *E. macquariensis* when cultured with/out toluene as the sole carbon source. An increase in biomass was observed between day 0 and 12 for samples containing toluene as an only carbon source, as well as samples containing toluene and glucose. All analysis was performed in triplicate.

Acknowledgments

The authors would like to thank Dr Josie van Dorst for soil sample collection from Sub-Antarctic Macquarie Island. Funding was obtained from UNSW Sydney for this project.

References

Blasi, B., Poyntner, C., Rudavsky, T., Prenafeta-Boldu, F.X., de Hoog, S., Tafer, H., Sterflinger, K., 2016. Pathogenic yet environmentally friendly? Black fungal candidates for bioremediation of pollutants. Geomicrobiol. J. 33 (3–4), 305–317.

Chang, C.L., Kim, D., Park, D.J., Kim, H.J., Lee, C.H., Shin, J.H., 2000. Acute cerebral phaeohyphomycosis due to *Wangiella dermatitidis* accompanied by cerebrospinal fluid eosinophilia. J. Clin. Microbiol. 38 (5), 1965–1966.

Cheikh-Ali, Z., Glynou, K., Ali, T., Ploch, S., Kaiser, M., Thines, M., Bode, H.B., Macia-Vicente, J.G., 2015. Diversity of exophillic acid derivatives in strains of an endophytic *Exophiala sp.* Phytochemistry 118, 83–93.

Cox, H.H.J., Faber, B.W., van Heiningen, W.N.M., Radhoe, H., Doddema, H.J., Harder, W., 1996. Styrene metabolism in *Exophiala jeanselmei* and involvement of a cytochrome P-450-dependent styrene monooxygenase. Appl. Environ. Microbiol. 62 (4), 1471–1474.

Cox, H.H.J., Moerman, R.E., van Baalen, S., van Heiningen, W.N.M., Doddema, H.J., Harder, W., 1997. Performance of a styrene-degrading biofilter containing the yeast *Exophiala jeanselmei*. Biotechnol. Bioeng. 53 (3), 259–266.

Crous, P.W., Gams, W., Stalpers, J.A., Robert, V., Stegehuis, G., 2004. MycoBank: an online initiative to launch mycology into the 21st century. Stud. Mycol. 50, 19–22.

De Hoog, G.S., Zeng, J.S., Harrak, M.J., Sutton, D.A., 2006. *Exophiala xenobiotica* sp. nov., an opportunistic black yeast inhabiting environments rich in hydrocarbons. Antonie van Leeuwenhoek 90, 257–268.

De Hoog, G.S., Vicente, V.A., Najafzadeh, M.J., Harrak, M.J., Badali, H., Seyedmousavi, S., 2011. Waterborne *Exophiala* species causing disease in cold-blooded animals. Persoonia 27, 46–72.

Dogen, A., Ilkit, M., de Hoog, G.S., 2013a. Black yeast habitat choices and species spectrum on high altitude creosote-treated railway ties. Fungal Biol. 117, 692–696.

Dogen, A., Kaplan, E., Oksuz, Z., Serin, M.S., Ilkit, M., de Hoog, G.S., 2013b. Dishwashers are a major source of human opportunistic yeast-like fungi in indoor environments in Mersin, Turkey. Med. Mycol. 51, 493–498.

Dreesens, L.L., Lee, C.K., Cary, S.C., 2014. The distribution and identify of edaphic fungi in the McMurdo dry valleys. Biology 3, 466–483.

Elmi, F., Etemadifar, Z., Emtiazi, G., 2015. A novel metabolite (1,3-benzenediol, 5-hexyl) production by *Exophiala spinifera* strain FM through dibenzothiophene desulfurization. World J. Microbiol. Biotechnol. 31, 813–821.

Estevez, E., Veigh, M.C., Kennes, C., 2005a. Biofiltration of waste gases with the fungi *Exophiala oligosperma* and *Paecilomyces variotii*. Appl. Microbiol. Biotechnol. 67, 563–568.

Estevez, E., Veigh, M.C., Kennes, C., 2005b. Biodegradation of toluene by the new fungal isolates *Paecilomyces variotii* and *Exophiala oligosperma*. J. Ind. Microbiol. Biotechnol. 32 (1), 33–37.

Ferrari, B.C., Zhang, C., van Dorst Josie, 2011. Recovering greater fungal diversity from pristine and diesel fuel contaminated sub-Antarctic soil through cultivation using both a high and a low nutrient media approach. Front. Microbiol. 2, 217.

Godinho, V.M., Furbino, L.E., Santiago, I.F., Pellizzari, F.M., Yokoya, N.S., Pupo, D., Alves, T.M., Junior, P.A.S., Romanha, A.J., Zani, C.L., Cantrell, C.L., Rosa, C.A., Rosa, L.H., 2013. Diversity and bioprospecting of fungal communities associated with endemic and cold-adapted macroalgae in Antarctica. ISME J. 7, 1434–1451.

Gunsch, C.K., Cheng, Q., Kinney, K.A., Szaniszlo, P.J., Christian, P.W., 2005. Identification of a homogentisate-1,2-dioxygenase gene in the fungus *Exophiala lecaniicorni*: analysis and implications. Appl. Microb. Biotechnol. 68, 405–411.

Gunsch, C.K., Kinney, K.A., Szaniszlo, P.J., Whitman, C.P., 2006. Quantification of homogentisate-1,2-dioxygenase expression in a fungus degrading ethylbenzene. J. Microbiol. Methods 67, 257–265.

Hamada, N., Abe, N., 2010. Growth characteristics of four fungal species in bathrooms. Biocontrol Sci. 15 (3), 111–115.

Hamzah, A., Tavakoli, A., Rabu, A., 2010. Detection of toluene degradation in bacteria isolated from oil contaminated soils. Sains Malays. 40 (11), 1231–1235.

Isola, D., Selbmann, L., de Hoog, G.S., Fenice, M., Onofri, S., Prenafeta-Boldu, F.X., Zucconi, L., 2013. Isolation and screening of black fungi as degraders of volatile aromatic hydrocarbons. Mycopathologia 175 (5–6), 369–379.

Ji, M., van Dorst, J., Bissett, A., Brown, M.V., Palmer, A.S., Snape, I., Siciliano, S.D., Ferrari, B.C., 2016. Microbial diversity at mitchell peninsula, eastern Antarctica: a potential biodiversity "hotspot". Polar Biol. 39, 237–249.

Kerry, E., 1990. Effects of temperature on growth rates of fungi from subantarctic maquarie island and casey, antarctics. Polar Biol. 10, 293–299.

Khan, A.L., Hamayun, M., Ahmad, N., Waqas, M., Kang, S., Kim, Y., Lee, I., 2011. *Exophiala sp.* LHL08 reprograms *Cucumis sativus* to higher growth under abiotic stresses. Physiol. Plantarum 143, 329–343.

Li, T., Liu, M.J., Zhang, X.T., Zhang, H.B., Sha, T., Zhao, Z.W., 2011. Improved tolerance of maize (Zea mays L.) to heavy metals by colonization of a dark septate endophyte (DSE) *Exophiala pisciphila*. Sci. Total Environ.Environ. 409, 1069–1074.

Lian, X., de Hoog, G.S., 2010. Indoor wet cells harbour melanized agents of cutaneous infection. Med. Mycol. 48, 622–628.

Liu, C., Huang, X., Xie, T., Duan, N., Xue, Y., Zhao, T., Lever, M.A., Hinrichs, K., Inagaki, F., 2016. Exploration of cultivable fungal communities in deep coal-bearing sediments from ~1.3 to 2.5 km below the ocean floor. Environ. Microbiol. 19 (2), 803–818.

Middelhoven, W.J., 1993. Catabolism of benzene compounds by ascomycetous and basidiomycetous yeasts and yeastlike fungi. Antonie van Leeuwenhoek 63, 125–144.

Ondeyka, J.G., Zink, D.L., Dombrowski, A.W., Polishook, J.D., Felock, P.J., Hazuda, D.J., Singh, S.B., 2003. Isolation, structure and HIV-1 integrase inhibitory activity of exophillic acid, a novel fungal metabolite from *Exophiala pisciphila*. J. Antibiot. 56 (12), 1018–1023.

Powell, S.M., Bowman, J.P., Ferguson, S.H., Snape, I., 2010. The importance of soil characteristics to the structure of alkane-degrading bacterial communities on sub-Antarctic Macquarie Island. Soil Biol. Biochem. 42, 2012–2021.

Prenafeta-Boldu, F.X., Guivernau, M., Gallastegui, G., Vinas, M., de Hoog, G.S., Elias, A., 2012. Fungal/bacterial interactions during the biodegradation of TEX hydrocarbons (toluene, ethylbenzene and *p*-xylene) in gas biofilters operated under xerophilic conditions. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 80 (3), 722–734.

Pudasaini, S., Wilson, J., Ji, M., van Dorst, J., Snape, I., Palmer, A.S., Burns, B.P., Ferrari, B.C., 2017. Microbial diversity of Browning Peninsula, Eastern Antarctica revealed using molecular and cultivation methods. Front. Microbiol. 8, 591.

Qi, B., Moe, W.M., Kinney, K.A., 2002. Biodegradation of volatile organic compounds by five fungal species. Appl. Microb. Biotechnol. 58, 684–689.

Rayner, J.L., Snap, I., Walworth, J.L., Harvey, P.M., Ferguson, S.H., 2007. Petroleum-hydrocarbon contamination and remediation by microbioventing at sub-Antarctic Macquarie Island. Cold Reg. Sci. Technol. 48, 139–153.

Rene, E.R., Bernat, P., Dlugonski, J., Veiga, M.C., Kennes, C., 2012a. Use of styrene as sole carbon source by the fungus *Exophiala oligosperma*: optimization and modeling of biodegradation, pathway elucidation, and cell membrane composition. Appl. Biochem. Biotechnol. 168 (6), 1351–1371.

Rene, E.R., Mohammad, B.T., Veiga, M.C., Kennes, C., 2012b. Biodegradation of BTEX in a fungal biofilter: influence of operational parameters, effect of shock-loads and substrate stratification. Bioresour. Technol. 116, 204–213.

Rustler, S., Chmura, A., Sheldon, R.A., Stolz, A., 2008. Characterisation of the substrate specificity of the nitrile hydrolyzing system of the acidotolerant black yeast Exophiala oligosperma R1. Stud. Mycol. 61, 165–174.

Seyedmousavi, S., Badali, H., Badali, H., Chlebicki, A., Zhao, J., Prenafeta-Boldu, F.X., de Hoog, G.S., 2011. *Exophiala sideris*, a novel black yeast isolated from environments polluted with toxic alkyl benzenes and arsenic. Fungal Biol. 115, 1030–1037.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H.,

Sninsky, J.J., White, T.J. (Eds.), PCR Protocols. A Guide to Methods and Applications. Academic Press, San Diego, pp. 315–322.
Woertz, J.R., Kinney, K.A., McIntosh, N.D.P., Szaniszlo, P.J., 2001. Removal of toluene in a vapor-phase bioreactor containing a strain of the dimorphic black yeast *Exophiala lecanii-corni*. Biotechnol. Bioeng. 75 (5), 550–558.
Woo, P.C.Y., Ngan, A.H.Y., Tsang, C.C.C., Ling, I.W.H., Chan, J.F.W., Leung, S.Y., Yuen, K.Y., Lau, S.K.P., 2013. Clinical spectrum of *Exophiala* infections and a novel *Exophiala* species, *Exophiala hongkongensis*. J. Clin. Microbiol. 51 (1), 260–267.
Yong, L.K., Wiederhold, N.P., Sutton, D.A., Sandoval-Denis, M., Lindner, J.R., Fan, H., Sanders, C., Guarro, J., 2015. Morphological and molecular characterization of *Exophiala polymorpha* sp. nov. isolated from sporotrichoid lymphocutaneous lesions in a patient with myasthenia gravis. J. Clin. Microbiol. 53 (9), 2816–2822.
Zalar, P., Novak, M., de Hoog, G.S., Gunde-Cinerman, N., 2011. Dishwashers-A man-made ecological niche accommodating human opportunistic fungal pathogens. Fungal Biol. 115, 997–1007.
Zhao, J., Zeng, J., de Hoog, G.S., Attili-Angelis, D., Prenafeta-Boldu, F.X., 2010. Isolation and identification of black yeasts by enrichment on atmospheres of monoaromatic hydrocarbons. Microb. Ecol. 60, 149–156.