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Review

# *Exophiala spinifera* and its allies: diagnostics from morphology to DNA barcoding

J. S. ZENG*,†,‡ & G .S. DE HOOG*,†

*Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, The Netherlands, †Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands, and ‡Department of Dermatology and Venereology, Union Hospital, Tongji Medical College, Huazhong Science and Technology University, Wuhan, Hubei, P.R. China

Diagnostic features of morphology, physiology, serology and genetics of species belonging to the *Exophiala spinifera* clade (including 11 species: *Exophiala oligosperma, E. spinifera*, *E. xenobiotica*, *E. jeanselmei*, *E. exophialae*, *E. nishimurae*, *E. bergeri*, *E. nigra*, *Rhinocladiella similis*, *Ramichloridium basitonum* and *Phaeoannellomyces elegans*), comprising a large number of human-associated *Exophiala* species, are summarized. Several species have closely similar morphological characters and physiological profiles. Taxonomy is therefore primarily based on sequence diversity of the Internal Transcribed Spacer (ITS) region of ribosomal DNA (rDNA). Multilocus sequencing has shown that ITS is reliable for identification of the species in this clade, and is a therefore a good candidate for barcoding species of *Exophiala*. Species-specific fragments were searched in the ITS region of species in the *Exophiala spinifera* clade and can be used to design probes for diagnosis by hybridization.

**Keywords** black yeasts, *Exophiala*, diagnostics, morphology, physiology, immunology, genetics, barcoding



## Introduction

The black yeast genus *Exophiala* comprises some major agents of human systemic disease. A hallmark of species of the genus is their morphological plasticity, as they tend to pass through complicated life cycles where diagnostic features are variably expressed [1]. Conversely, very similar microscopic structures can be expressed in phylogenetically remote species. Therefore species are notoriously difficult to classify and identify. In the past, diagnostic schemes were morphological, while soon physiological and serological parameters were added [2–6]. In recent years diagnostics has become supplemented by molecular tools, particularly sequence data of the ribosomal DNA (rDNA) Internal Transcribed Spacer (ITS) regions [7–9] and from coding genes such as translation elongation factor 1-α and β-tubulin genes. A large-scale DNA sequencing project known as DNA barcoding has been advocated, which aims to promote rapid and automatic species identification and to provide insight into the evolutionary history of life. It attracted much attention as well as controversy [10–13], but increasingly, techniques are being developed which proved to be powerful in various aspects of molecular identification of unknown samples [14–21].

The '*Exophiala spinifera* clade' is a cluster of taxa that is recognized among members of the Herpotrichiellaceae (black yeasts and relatives) based on SSU rDNA data [22]. At its first recognition it comprised the three previously described species *Exophiala spinifera*, *E. jeanselmei* and *Phaeococcomyces exophialae* (=*Exophiala exophialae*), plus one strain, CBS 725.88 that was later described as *Exophiala oligosperma* and the more distant strain CBS 118157 that was later

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Correspondence: G. S. de Hoog, PO Box 85167, NL-3508 AD, Utrecht, The Netherlands. Tel.: +31 30 2122663. Fax: +31 30 2512097. E-mail: de.hoog@cbs.knaw.nl



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described as the type strain of *Exophiala xenobiotica*. *Exophiala jeanselmei* had long been recognized to be heterogeneous, even on morphological grounds [23] as it showed two dissimilar phenotypes with annellidic *vs.* sympodial conidiogenesis. Since these different types of conidium production may occur in a single species, in a single strain, and even on a single hypha, some preponderantly sympodial taxa such as *Rhinocladiella similis*, *Phaeoannellomyces elegans* and *Ramichloridium basitonum* were included in the study [9,24]. Species of *Exophiala spinifera* clade are defined and identified by phylogeny rather than by morphology. Subsequently some *Exophiala* species (*Exophiala oligosperma*, *E. nishimurae* and *E. xenobiotica*) have been segregated from known species [9,25,26], while *Exophiala nigra* and *E. bergeri* were discovered to be close to *Exophiala jeanselmei* on the basis of their genetic features [9]. So far, a total of eleven of taxa is recognized in the *Exophiala spinifera* clade, viz. *E. oligosperma, E. spinifera*, *E. xenobiotica*, *E. jeanselmei*, *E exophialae*, *E. nishimurae*, *E. bergeri*, *E. nigra*, *Rhinocladiella similis*, *Ramichloridium basitonum* and *Phaeoannellomyces elegans.* A summary of these species in the form of a distance tree is presented in Fig. 1.

In a previous study [27] we found that more than 40% of the *Exophiala* species derived from symptomatic humans in the USA concern species that belong to the *Exophiala spinifera* clade. The predilections of *Exophiala xenobiotica*, *E. oligosperma*, *E. spinifera* and *E. jeanselmei* seem to be slightly different from each other [27]. Despite the paucity of proven case reports, the distinguished entities are likely to be clinically

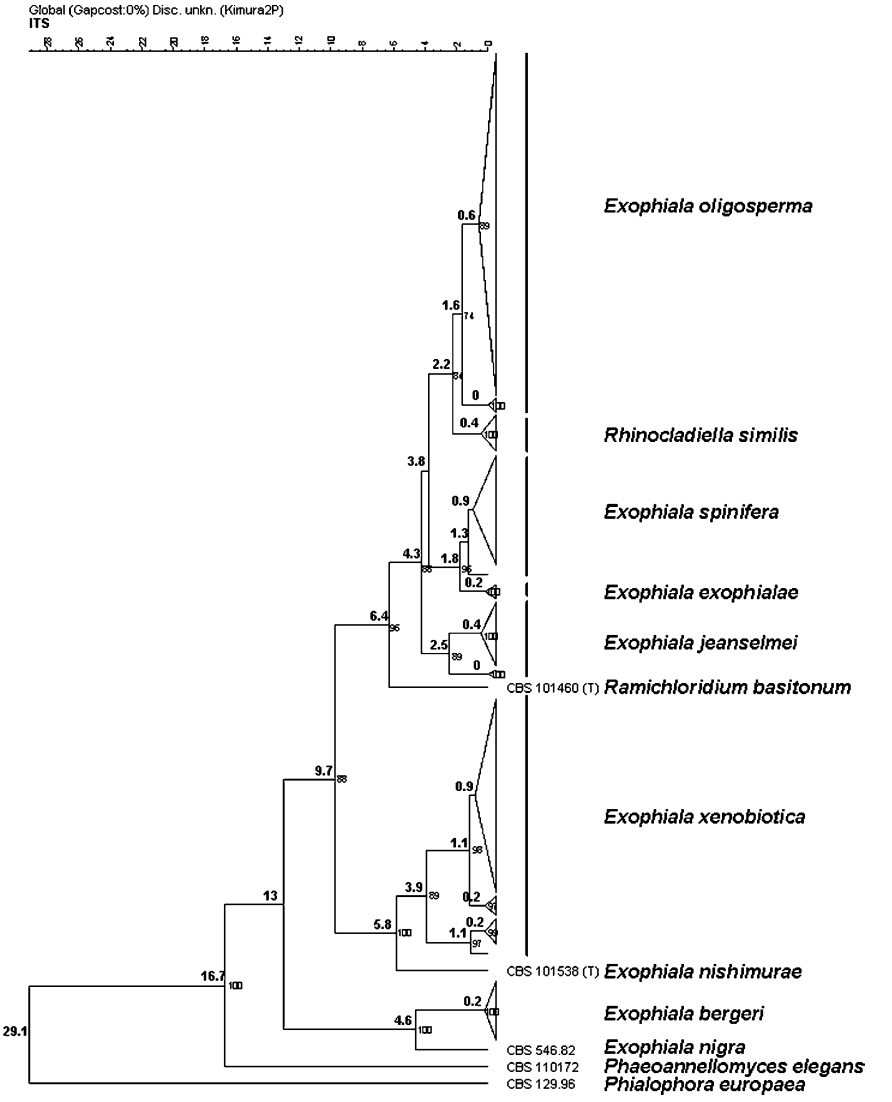


**Fig. 1** The dendrogram based on sequences of ITS region of rDNA gene of species in *Exophiala spinifera* clade was constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method with correction of Kimura 2 parameter (K2P) in the Bionumerics package. The clades comprising taxa with minimum sequence divergence of $<1.0\%$ are collapsed. The number of simulations was 100. The percentages of sequence divergence are indicated over branches in bold, and the bootstrap values of $>70\%$ are located on the right side of nodes. The scale shows percentage of global alignment divergence. *Phialophora europaea* CBS 129.96 was used for comparison. Abbreviations used in the tree, CBS: CBS Fungal Biodiversity Centre, Utrecht, the Netherlands.

significant. Routine characters for identification are mostly inadequate, hampering the development of insight into species-specific pathologies. In this paper we will review the optimal method for identification of these species.

## Morphological identification

On potato dextrose agar (PDA) or Sabouraud's glucose agar (SGA), colonies generally grow restrictedly, are slimy or at least mucous at the centre, olivaceous grey to brownish black, with olivaceous black reverse. Initial propagation is usually with yeast-like cells, which soon transform into germinating cells and to torulose hyphae, prior to forming evenly wide filaments. The presence of torulose mycelium is characteristic for *Exophiala*, however this feature may be insignificant in primarily sympodial species of the clade. *Exophiala* has annellidic conidiogenous cells. Coexisting with annellides may be sympodial and/or phialidic conidiogenous cells as in *Exophiala jeanselmei* (annellidic/sympodial) and *Exophiala spinifera* (annellidic/phialidic). The observation of yeast cells with capsules helps distinguish *Exophiala spinifera* from other species; *Exophiala dermatitidis*, outside the *Exophiala spinifera* clade, is the only other capsule-forming black yeast [28]. Mackinnon *et al.* [29] reported the production of capsules by yeast-like cells of *Exophiala jeanselmei*, but the identities of the strains were not confirmed. *Exophiala spinifera* is also characteristic in its conidiophores and annellated zones, the latter being long-cylindrical with numerous annellations as is clearly observed with scanning electron microscopy [30]. *Exophiala attenuata* has similar conidiophores, but reduced annellated zones [25].

Only a limited number of taxa can be recognized by phenetic features. Diagrams for the identification of human-associated *Exophiala* species and related black yeast-like fungi are given in Figs 2 and 3. *Exophiala nigra* is not included, as this species has never been observed from a human or an animal source. When species-specific microscopic structures are absent or there are no distinguishable differences in morphology, physiology or serology, discriminative sequence data facilitate accurate identification.

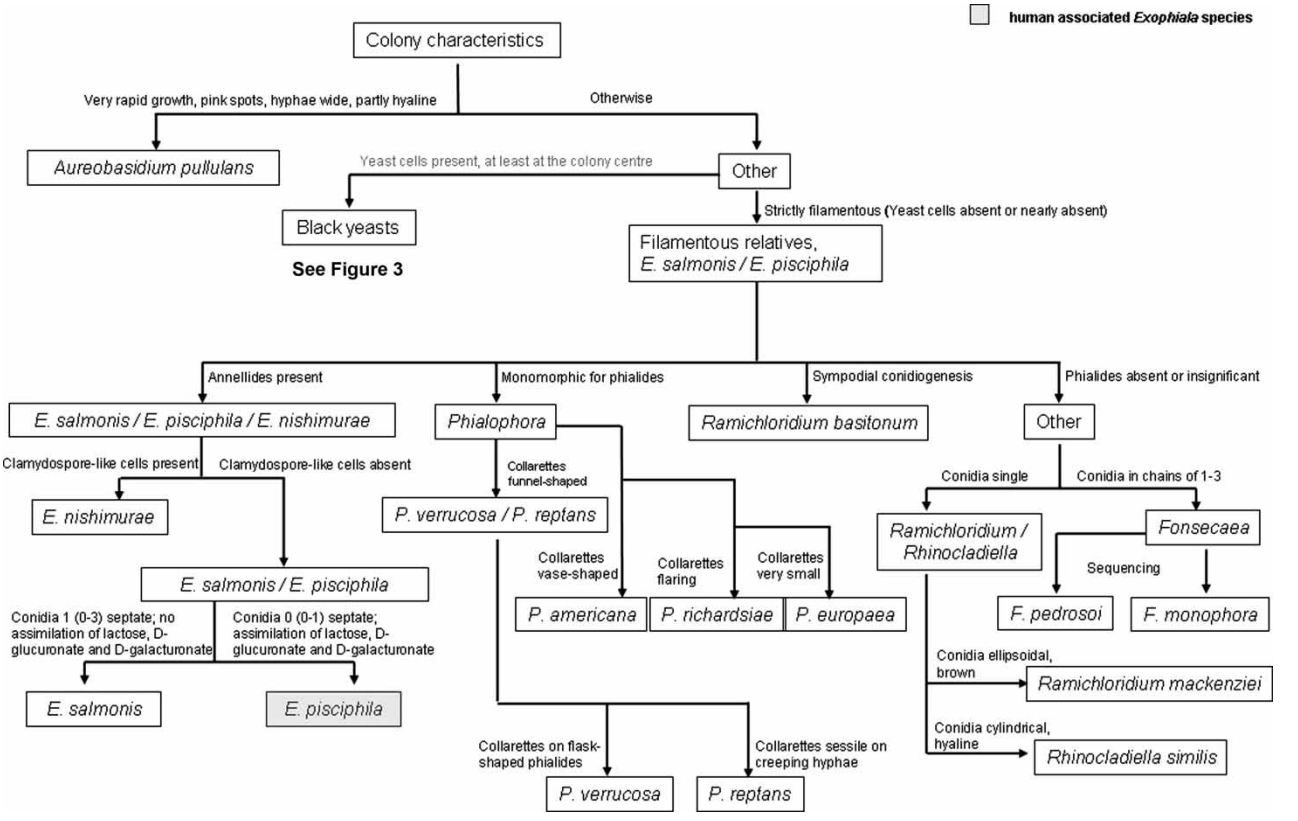


**Fig. 2** Diagram for identification of human-associated *Exophiala* species and related black yeast-like fungi. *Exophiala nigra* is not included due to missing data.

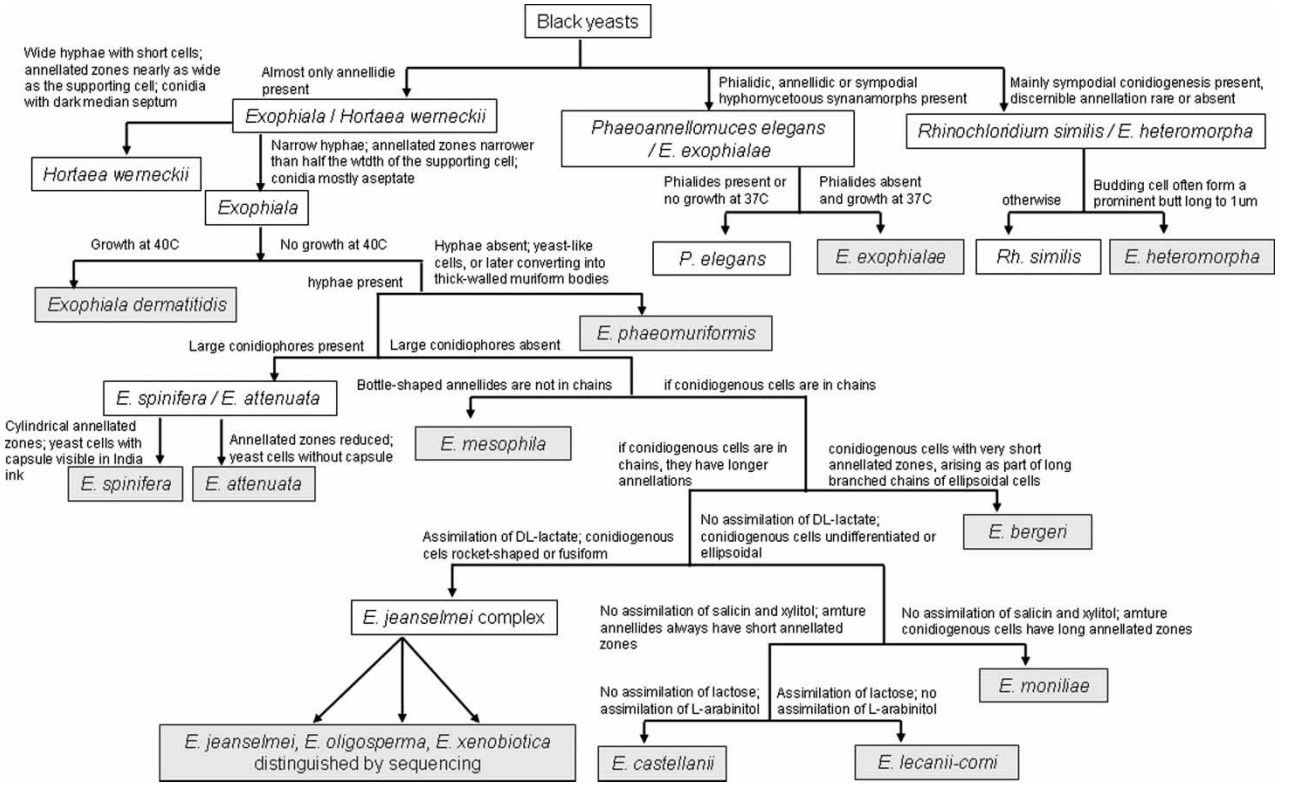


**Fig. 3** Diagram for identification of human-associated *Exophiala* species and related black yeast-like fungi. *Exophiala nigra* is not included due to missing data.

## Physiological identification

Physiological characteristics frequently applied in diagnostics are summarized in Table 1; comparable data are unavailable for *Exophiala xenobiotica*, *Rhinocladiella similis* and *Ramichloridium basitonum*. A major problem with physiological profiles of black yeasts is that as yet reproducibility is low, data sometimes being unclear or even contradictory within species. For many species, profiles have to be re-established on the basis of strains verified by molecular biology. Salient differences are mainly found in assimilation of the carbon compounds D-ribose, methyl-α-D-glucoside, salicin, melibiose, raffinose, soluble starch, meso-erythritol, xylitol, D-glucuronate, DL-lactate, succinate and ethanol. Tolerance of $MgCl_2$, NaCl and cycloheximide at different concentrations, and thermotolerance also showed different profiles between species. Unambiguous differences are lacking to distinguish *Exophiala jeanselmei, E. oligosperma* and *E. spinifera,* as well as *Exophiala spinifera* and *E. exophialae*. Absence of proteolytic activity in *Exophiala jeanselmei* and *E. spinifera* was uncovered by Espinel-Ingroff *et al.* using 26 different formulations of gelatin, milk, casein, and Loeffler media [31]. Other physiological properties including hydrolysis of tyrosine and xanthine were examined in the same study. Kane *et al.* found a consistent difference in sodium chloride tolerance, viz. 7% for *Exophiala spinifera* and 9% for *E. jeanselmei* [32], but this was not reproduced in subsequent studies [6,8]. The ability of species in the *Exophiala spinifera* clade to assimilate melezitose was valuable in differentiating them from *Exophiala dermatitidis* (Table 1) [6,8,25,33].

Biodegradation of low-molecular-weight urethane compounds and cyclohexanone by *Exophiala jeanselmei* CBS 528.76 has been reported [34,35]. *Exophiala oligosperma* strains were repeatedly isolated from air biofilters fed with toluene [36] or styrene [37,38], and raw sewage with high concentrations of ethane, propane and butane [39]. In a survey of fungal assimilation of a wide variety of oxidized aromatic compounds, *Exophiala oligosperma* CBS 658.73 was shown to exhibit a comparatively broad substrate specificity [40]. Environmental strains of *Exophiala xenobiotica* are mostly associated with habitats rich in monoaromatic hydrocarbons, e.g., soil polluted by gasoline, wood treated with phenolic preservatives, creosote-treated railway ties or browncoal rich in phenolic compounds [26]. The remarkable ability to grow at the expense of alkylbenzene hydrocarbons seems to be

**Table 1** Physiological comparison of the main species of the *E. spinifera* clade.

| species name* | | *E. jeanselmei*[a–d] | *E. oligosperma* | *E. spinifera*[a–d, f] | *E. nishimurae*[e] | *E. bergeri*[b, d] | *E. exophialae*[d] | *E. nigra*[d] | *P. elegans* |
|---|---|---|---|---|---|---|---|---|---|
| assimilation | D-glucose | + | + | + | + | + | + | + | + |
| | D-galactose | +,v | + | +,v | + | + | + | + | + |
| | L-sorbose | + | + | + | + | + | + | + | + |
| | D-glucosamine | + | + | +, w | + | + | +, w | w | + |
| | **D-ribose** | +, w | + | + | + | +, w | + | w | − |
| | D-xylose | +, − | + | + | + | + | + | + | + |
| | L-arabinose | + | + | + | + | + | + | + | + |
| | D-arabinose | +, w | + | + | + | +, w | + | + | + |
| | L-rhamnose | + | + | + | + | + | + | + | + |
| | sucrose | + | + | + | + | + | + | w | + |
| | maltose | + | + | + | + | + | + | + | + |
| | α, α-trehalose | + | + | + | + | + | + | + | + |
| | **methyl-α-D-glucoside** | +, − | w | +, w | + | −, w | + | − | w |
| | cellobiose | + | + | + | + | + | + | w | + |
| | **salicin** | +, w | + | + | + | −, w | + | − | + |
| | arbutin | ? | +, − | −, w | + | ? | −, w | ? | ? |
| | **melibiose** | − | − | +, − | w | + | + | − | w |
| | lactose | +, − | − | +, −, w | w | − | −, w | − | w |
| | **raffinose** | −, v | +, − | **+, −, v** | w | + | + | − | w |
| | melezitose | + | + | + | + | + | + | + | + |
| | inulin | − | − | −, w | w | − | −, w | − | w |
| | **soluble starch** | − | − | −, w | w | − | −, w | − | + |
| | glycerol | + | + | + | + | + | + | + | + |
| | **meso-erythritol** | + | + | + | − | − | + | − | + |
| | ribitol | +, w | + | + | w | + | + | w | w |
| | **xylitol** | + , W, V | + | + | − | + | + | + | + |
| | L-arabinitol | + | + | + | + | + | + | w | + |
| | D-glucitol | + | + | + | + | + | + | + | + |
| | D-mannitol | + | + | + | + | + | + | + | + |
| | **galactitol** | w | + | W, V | + | − | +, −, w | − | w |
| | myo-inositol | +, w | +, w | +, w | w | + | +, w | + | + |
| | inositol | +, − | − | − | ? | ? | ? | ? | ? |
| | sorbitol | V | ? | − | ? | ? | ? | ? | ? |
| | glucono-δ-lactone | w | +, w | + | ? | + | + | + | w |
| | D-gluconate | + | + | + | + | +, w | + | w | w |
| | 2-keto-D-gluconate | V | ? | + | ? | ? | ? | ? | ? |
| | **D-glucuronate** | + | + | + | − | + | + | + | + |
| | D-galacturonate | + | + | + | + | +, w | + | w | + |
| | **DL-lactate** | + | + | + | + | −, v | + | − | + |
| | **succinate** | + | +, w | +, w | + | − | +, w | − | w |
| | citrate | **+, −, v** | +, −, w | +,v | − | −, w | +, −, w | − | − |
| | methanol | | − | −, w | − | − | − | − | − |
| | **ethanol** | + | + | + | + | − | +, w | w | − |
| | nitrate | + | + | + | + | w | + | + | + |
| | nitrite | + | + | + | + | + | + | + | + |

**Table 1** (*Continued*)

| species name* | | *E. jeanselmei*[a–d] | *E. oligosperma* | *E. spinifera*[a–d, f] | *E. nishimurae*[e] | *E. bergeri*[b, d] | *E. exophialae*[d] | *E. nigra*[d] | *P. elegans* |
|---|---|---|---|---|---|---|---|---|---|
| | ethylamine | + | + | +, w | + | + | + | + | + |
| | L-lysine | + | + | +, w | w | + | + | + | + |
| | cadaverine | + | + | + | + | + | + | + | + |
| | creatine | + | + | + | w | + | + | + | + |
| | creatinine | +, − | +, w | + | w | + | + | + | + |
| tolerance tests | 5% MgC12 | + | + | + | + | ? | + | − | + |
| | 10% MgC12 | w | + | +, w | + | ? | + | − | + |
| | 5% NaCl | w | + | +, w | − | ? | + | − | + |
| | 10% NaCl | − | w | −, w | − | ? | w | − | w |
| | **0.01% cycloheximide** | − | ? | + | ? | − | + | + | − |
| | **0.1 % cycloheximide** | +, − | + | + | + | −, w | + | + | − |
| | 30°C | + | + | + | + | + | + | + | + |
| | 37°C | +, − | + | +, −, w | + | + | + | − | − |
| | 40°C | − | − | −, w | − | − | − | − | − |
| fermentation | | − | − | − | ? | − | − | | − |
| urease acivity | | + | + | + | + | + | + | + | w |
| extracellular DNAse | | − | **−, ?** | − | ? | ? | − | ? | ? |

note: The tests which show different profiles among the species and useful for diagnosis are marked in bold.
*Species names of strains were reassigned on the basis of latest data cited.
+: growth; w: weak growth; −: no growth; v: variable; ?: ambiguous or unknown.
a–f: references from which data were summarized, a: [31], b: [9], c: [33], d: [6], e: [25], f: [8], g: [7].

widespread in species of the *Exophiala spinifera* clade, but thus far this feature has not been used for diagnostic purposes.

Currently available physiological data are insufficient to separate all species of the *Exophiala spinifera* clade, particularly *E. jeanselmei, E. oligosperma* and *E. xenobiotica* which share similar morphologies.

## Serological identification

In animal experiments, antigenic preparations were made from culture filtrates of *Exophiala jeanselmei* by Iwatsu *et al*. [3], and positive delayed-skin reactions were elicited in all tested rats. However, antigens displayed cross-reactivity in 2 of 3 rats tested. These results suggested that a delayed-type skin type using these antigens may be useful not only for the diagnosis of chromoblastomycosis but also for the identification of species of the causative agents [3]. An exoantigen test performed by Espinel-Ingroff *et al*. permitted the differentiation of *Exophiala jeanselmei* and *E. dermatitidis* from one another as well as from *Hortaea werneckii* and other dematiaceous fungi (*Fonsecaea*, *Phialophora*, *Cladosporium* and *Rhinocladiella* species) and *Sporothrix schenckii*, but it failed to distinguish *Exophiala jeanselmei* from *E. spinifera* [4]. This problem was solved by another exoantigen test developed by Standard *et al.* [2]. It was able to differentiate *Exophiala spinifera* not only from *E. jeanselmei*, but also from *Exophiala alcalophila*, *E. moniliae*, *E. pisciphila*, *E. salmonis*, *E. dermatitidis* and *Hortaea werneckii.* While exoantigen tests are promising diagnostic tools, the described sera have not been made publicly available and have not been standardized, thereby excluding use of this method for identification purposes in the clinical laboratory.

## Histopathological identification

Mycoses caused by dematiaceous fungi include chromoblastomycosis, mycetoma and different types of phaeohyphomycosis. Infections may be superficial, cutaneous, subcutaneous, systemic or disseminated. Depending on published case reports, the dominant infections due to species in the *Exophiala spinifera* clade are phaeohyphomycosis [7,41–55], a few of the infections were reported to be chromoblastomycosis [56–61], while mycetoma was uncommon [62–64]. The most frequently reported phaeohyphomycoses are subcutaneous. The main histopathological characters of subcutaneous phaeohyphomycosis with hematoxylin-eosin (HE) or PAS staining are granulomatous inflammation (or accumulation of macrophages, neutrophils, histocytes etc. without granuloma formation) with or without abscess. Melanized fungal elements are found inside or outside granulomata in the dermis and subcutaneous layers [42–44,47,50,51,53–55]. The fungal elements include brownish, yeast-like cells, pseudohyphae and septate, branched or unbranched hyphae. Elements are seen in tissue either in one form or in a combination of these forms. In systemic infections, lymph node biopsy specimens showed giant cells with brown bodies [48,51]. Fungal vasculitis with hyphal elements and fungal nodes with radial orientation of hyphae were found on the sections of brain autopsy from a case infected by *Exophiala oligosperma* [7]. Some fungal cells appeared lightly pigmented or hyaline, lacking melanization. In such cases the application of Fontana-Masson staining is useful to distinguish the infection from those by, e.g., *Aspergillus* [43]. Cells are easily observed with Gomori methenamine silver (GMS) or periodic acid-Schiff stain [50,51]. The manifestation of fungal elements in lesions of skin, lymph nodes or brain is similar, all being filamentous or exhibiting a mixture of hyphal elements and yeast-like cells, occasionally intermingled with torulose hyphae. The histological pictures of subcutaneous phaeohyphomycosis caused by *Exophiala jeanselmei* [43,45,47,53–55], *E. spinifera* [42,49–51] or *Ph. elegans* [44] resemble each other closely. Similarly in chromoblastomycosis, sclerotic bodies produced by a variety of etiologic agents cannot be used for species identification. [56,58,65].

No specific histopathological features are available to differentiate species of the *Exophiala spinifera* clade from other etiologic black yeasts and relatives in infected tissues.

## Genetic identification

For classification, phylogeny and identification of the species in the *Exophiala spinifera* clade and epidemiological research of the infections due to these species, a large diversity of molecular biological methods has been applied. Initial methods concerned DNA-DNA hybridization, and banding methods such as restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD). More recently, DNA sequencing was applied to mitochondrial DNA (mtDNA), partial mitochondrial cytochrome b gene, internal transcribed spacer (ITS) region, small subunit (SSU) of ribosomal DNA (rDNA), partial Elongation Factor 1-α (EF 1-α), β-Tubulin (β-TUB), Chitin Synthase (CHS) and Actin (ACT) genes.

The *Exophiala spinifera* clade was first defined by Haase *et al*. [22] based on an analysis of SSU rDNA of the Herpotrichiellaceae. The group was phylogenetically

clearly delimited and distinct from the remaining species of *Exophiala*. The clade comprised the known species *Exophiala spinifera*, *Exophiala jeanselmei*, *Phaeococcomyces exophialae* (=*Exophiala exophialae*), strain CBS 725.88 later described as *Exophiala oligosperma*, and strain CBS 118157 later described as the type strain of *Exophiala xenobiotica*. *Exophiala jeanselmei* had long been recognized to be heterogeneous. On the basis of morphology, three varieties were distinguished [23]. This was confirmed by DNA-DNA hybridization, RFLP analysis of mtDNA, and sequencing of partial mitochondrial cytochrome b gene and ITS rDNA, leading to the distinction of 6–18 genetically different entities [66–68]. The varieties of *Exophiala jeanselmei* were attributed the status of individual species, and the typical variety was found to contain cryptic but distantly related species, defined by sequences of SSU, ITS and partial mitochondrial cytochrome b genes, and RFLP of mtDNA and ITS [22,66,68]. The biodiversity of *Exophiala spinifera* was exhibited in mtDNA using RFLP and sequencing of ITS rDNA [69]. Strains morphologically classified as *Exophiala jeanselmei* proved to be highly diverse upon sequencing ITS rDNA, leading to species being newly introduced or redefined [9,25,26]. Multilocus studies [87] showed that species are preponderantly clonal, and consequently that additional genes do not provide more phylogenetic information than acquired on the basis of separate datasets. For this reason ITS rDNA has remained a very useful parameter of the distinction of entities [70]. In the absence of sexuality, and given slight ecological differences between the entities, we refer to these taxa as species. Modern taxonomy of species in the *Exophiala spinifera* clade is therefore based on sequence diversity of ITS rDNA, after confirmation by additional genes (EF 1-α, β-TUB and ACT) [70].

With the aid of RFLP profiles of mtDNA and SSU and ITS sequencing it is possible to separate species within the *Exophiala spinifera* clade from those outside the clade [69,71–73]. RFLP failed to distinguish some closely interrelated species, e.g., *Exophiala bergeri* and *Phaeoannellomyces elegans* [72]. Sequencing of less variable genes is optimal for recognition of species belonging to main clades in the black yeasts [9,22,24,74]. Among these genes, sequence data of mtDNA and CHS are not available for all species. Partial SSU sequences have been applied for species distinction, but they are insufficiently variable for this purpose. Abliz *et al.* reported that intra-species sequence diversity of the D1/D2 domains of large subunit (26S) rDNA was very small in *Exophiala dermatitidis*, *E. jeanselmei*, *E. spinifera* and *E. moniliae*, while interspecies differences were consistently larger [75]. These data suggested that the D1/D2 domain may prove to be a useful tool for identification of these species, but studied data set outside the *Exophiala spinifera* clade was limited. Therefore, optimal molecular diagnostics of the species in the *E. spinifera* clade through the sequencing of the rDNA ITS regions needs to be undertaken [8,9,25,26]. Fig. 1 shows a dendrogram based on sequences of the ITS region of the rDNA gene of all species in the *E. spinifera* clade, constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method with correction of Kimura 2 parameter (K2P) in the Bionumerics package v. 4.0 (Applied Maths, Kortrijk, Belgium). The tree generated on the basis of the sequence data obtained with the method described in [27] in our previous [26,27,70] and present studies, and the sequences downloaded from GenBank. The matrix associated with the dendrogram is available in TreeBASE (P.I.N. 14616). The sources of strains in the tree are shown in Table 2. Strains of each species formed a reciprocal monophyletic clade with a high bootstrap value. The sequence divergence within a species varied from 0.2% (*Exophiala bergeri* and *E. exophialae*) to 3.9% (*E. xenobiotica*), and distances between the two closest species in the tree varied from 1.8% (*E. spinifera* and *E. exophialae*) to 5.8% (*E. xenobiotica* and *E. nishimurae*) (Fig. 1). Though there is overlap between intra- and interspecific divergences, most of the intraspecies diversities are lower than 2% except those of *Exophiala xenobiotica* (3.9%) and *E. jeanselmei* (2.5%), while most of the interspecies diversities are larger than 2% except those between *E. spinifera* and *E. exophialae* (1.8%). Sequence alignments of partial ITS 1 and 2 regions of rDNA of representative strains of the *E. spinifera* clade is exhibited in Fig. 4. Variabilty is obvious through the whole ITS1 region and in 3 locations in ITS2 region.

DNA barcoding is a standardized approach to identify species by a short gene sequence from a uniform region in the genome [http://www.barcoding.si.edu]. The technique allows rapid and unambiguous definition and recognition, and has phylogenetical implications as it is directly based upon the evolutionary history of life. Barcoding has been applied to resolve species boundaries in populations of apparently similar organisms and discover possible new species [17,76–79]. There is no universal barcoding gene, as no single gene that is conserved in all domains of life and exhibits enough sequence divergence for species discrimination. Cytochrome c oxidase subunit I (COI) region of mitochondrial DNA was proposed as the barcode gene in the animal kingdom [80]. A chloroplast gene such as maturase K, or a nuclear gene such as ITS, may be an effective target for barcoding in plants [13].

**Table 2** Source of strains tested.

| species name | strain number | region | source | GenBank accession nr. |
|---|---|---|---|---|
| *E. jeanselmei* | CBS 677.76 | UK | mycetoma | AY163553 |
| | CBS 119095 | USA | foot skin | |
| | UTHSC R-2968 | USA | skin scraping | |
| | UTHSC 88-402 | USA | skin | |
| | UTHSC 94-28 | USA | knee | EF025410 |
| | UTHSC 01-2688 | USA | finger | |
| | UTHSC R-3338 | USA | foot | EF025411 |
| | CBS 117.86 | Japan | mycetoma (isolated from CBS 116.86) | |
| | CBS 116.86 | Japan | mycetoma | |
| | CBS 507.90 (T) | Uruguay | mycetoma | AY156963 |
| | CBS 528.76 | USA | skin | AY857530 |
| | UTHSC R-2666 | Australia | ankle | |
| | UTHSC R-1922 | USA | foot lesion biopsy | |
| | CBS 109635 | USA | arm lesion | |
| *E. nishimurae* | CBS 101538 (T) | Venezuela | bark (isolated from IFM 41855) | AY163560 |
| *E. oligosperma* | CBS 725.88 (T) | Germany/Philippines | sphenoid | AY163551 |
| | dH 12971 | Finland | insulation material | |
| | dH 13019 | Spain | toluene | |
| | dH 13308 | Austria | steambath | |
| | dH 12236 | Ukraine | forest litter | |
| | UTHSC 97-2226 | Brazil | human | |
| | UTHSC 96-2015 | USA | duodenal aspirate | |
| | UTHSC 93-271 | USA | maxillary sinus | |
| | UTHSC 93-2599 | USA | spleen | |
| | UTHSC 92-2007 | USA | lung autopsy | EF025414 |
| | UTHSC R-768 | USA | skin | |
| | UTHSC 94-1531 | USA | knee tissue | |
| | UTHSC 97-474 | USA | throat | |
| | UTHSC 98-697 | USA | valve (heart?) | EF025413 |
| | UTHSC 92-85 | USA | maxillary sinus | |
| | UTHSC 02-2072 | USA | lung | |
| | UTHSC R-680 | USA | subcutaneous lesion | |
| | UTHSC 01-593 | USA | lung | |
| | UTHSC 04-46 | USA | sputum | |
| | UTHSC 01-2053 | USA | tissue | |
| | UTHSC 93-2598 | USA | lung tissue | |
| | UTHSC 93-2310 | USA | lymph node | |
| | UTHSC 94-1756 | USA | pleural fluid | |
| | UTHSC 94-2548 | USA | lung | |
| | UTHSC 89-254 | USA | human | |
| | UTHSC 01-1205 | USA | pleural fluid | |
| | UTHSC 00-1921 | USA | foot | |
| | dH 13321 | Austria | Sauna floor | |
| | CBS 115966 | Netherlands | process water | |
| | dH 13304 | Austria | Sauna | |
| | dH 13314 | Austria | steambath | |
| | dH 13320 | Austria | steambath | |
| | UTHSC 91-870 | USA | hand | |
| | UTHSC 02-45 | USA | animal | |
| | UTHSC 01-597 | USA | nail | |
| | dH 13579 | Austria | steambath floor | |
| | UTHSC 95-2350 | USA | middle finger | EF025386 |
| | CBS 109807 | Brazil | fungemia | |
| | UTHSC R-2997 | Brazil | human | |
| | UTHSC R-3000 | Brazil | human | |
| | UTHSC R-2999 | Brazil | human | |
| | UTHSC R-2998 | Brazil | human | |
| | UTHSC R-2977 | Brazil | human | |
| | UTHSC R-2976 | Brazil | human | |
| | UTHSC R-2993 | Brazil | human | |

**Table 2** (*Continued*)

| species name | strain number | region | source | GenBank accession nr. |
|---|---|---|---|---|
| | UTHSC R-2989 | Brazil | human | |
| | UTHSC R-2991 | Brazil | human | |
| | UTHSC R-2987 | Brazil | human | |
| | UTHSC R-2979 | Brazil | human | |
| | UTHSC R-2981 | Brazil | human | |
| | UTHSC R-2984 | Brazil | human | |
| | UTHSC R-2980 | Brazil | human | |
| | UTHSC 88-209 | USA | lymph node | |
| | UTHSC R-2975 | Brazil | human | |
| | UTHSC R-2988 | Brazil | human | |
| | UTHSC R-2995 | Brazil | human | |
| | CBS 265.49 | France | honey | AY163555 |
| | UTHSC R-2996 | Brazil | human | |
| | UTHSC 95-416 | USA | foot lesion | |
| | UTHSC 95-2041 | USA | foot lesion | EF025415 |
| | UTHSC 96-968 | USA | leg | |
| | CBS 537.76 | Italy | human | |
| | CBS 538.76 | unknown | branchus | |
| | CBS 634.69 | Baltic Sea | wood, ship resting at sea bottom | |
| *E. spinifera* | CDCB-5383 | USA | elbow lesion | |
| | UTHSC R-1443 | UK | phaeohyphomycotic cyst | |
| | CBS 194.61 | India | systemic mycosis | |
| | CBS 101537 | Venezuela | cactus | |
| | CBS 236.93 | Germany | apple juice | |
| | CBS 269.28 | Germany | skin | |
| *E. spinifera* | CBS 356.83 | Egypt | skin | AJ244246 |
| | CBS 425.92 | Germany | apple juice | |
| | CBS 101644 | USA | maize kernel | |
| | CBS 899.68 (T) | USA | nasal granuloma | AY156976 |
| | CBS 110628 | Venezuela | bark | |
| | UTHSC R-2959 | China | human | |
| | UTHSCR-773 | USA | human | |
| | UTHSC R-2955 | USA | human | |
| | UTHSC 88-15 | USA | human | EF025419 |
| | UTHSC R-2870 | USA | subcutaneous cyst | EF025418 |
| | UTHSC 91-188 | USA | upper thigh | EF025417 |
| | UTHSC 97-2073 | USA | skin | EF025416 |
| | CBS 667.76 | Uruguay | fallen palm | |
| | CBS 670.76 | Uruguay | nest of Anumbis anumbi | |
| | CBS 102179 | Senegal | skin | |
| *E. xenobiotica* | CBS 117650 | USA | arm abscess | |
| | CBS 117641 | USA | knee cyst | DQ182591 |
| | CBS 117655 | USA | buttock | |
| | CBS 117676 | USA | finger | DQ182592 |
| | CBS 117649 | USA | wound | |
| | CBS 117654 | USA | total knee | |
| | CBS 204.50 | Switzerland | apple juice | |
| | CBS 117671 | USA | eye vitreous tab | |
| | CBS 117662 | USA | leg tissue | |
| | CBS 117648 | USA | sclera | EF025407 |
| | CBS 117646 | USA | finger | |
| | CBS 118157 (T) | Venezuela | oil-spilled soil | DQ 182587 |
| | CBS 117669 | USA | cyst in elbow | |
| | CBS 117667 | USA | arm biopsy | |
| | CBS 642.82 | Australia | treated Eucalyptus pole | |
| | CBS 102455 | Brazil | eye | |
| | CBS 119306 | USA | animal | |
| | CBS 117674 | USA | blood | DQ 182589 |
| | CBS 117647 | USA | wrist wound | |
| | CBS 101271 | Netherlands | skin | |

**Table 2** (*Continued*)

| species name | strain number | region | source | GenBank accession nr. |
|---|---|---|---|---|
| | CBS 522.76 | UK | timber | |
| | CBS 117754 | Germany | benzene-contaminated ground water | |
| | CBS 117672 | USA | scalp | |
| | CBS 117673 | USA | scalp | DQ 182590 |
| | CBS 117753 | USA | leg wound | |
| | CBS 648.76A | Canada | sputum | |
| | CBS 718.76 | Canada | foot | |
| | CBS 117661 | USA | eye vitreous fluid | |
| | CBS 117652 | USA | human | |
| | CBS 117657 | USA | knee tissue | |
| | CBS 117665 | USA | tissue | DQ 182588 |
| | CBS 117645 | USA | human | |
| | CBS 117651 | USA | forearm | |
| | CBS 117659 | USA | human | |
| | CBS 117644 | USA | foot abscess | |
| | CBS 117658 | USA | dialysis fluid | |
| | CBS 117663 | USA | forearm | |
| | CBS 102606 | USA | bathroom | |
| | CBS 527.76 | Sweden | culture contaminant of *Hyphodontia breviseta*, on *Picea abies* | |
| | CBS 117235 | USA | sputum | |
| | CBS 117656 | USA | foot sinus | |
| | CBS 117642 | USA | foot wound | DQ182593 |
| | CBS 117675 | USA | great toe | |
| | CBS 117643 | USA | hand | |
| | CBS 117653 | USA | peritoneal dialysis fluid | DQ182594 |
| *E. exophialae* | CBS 101542 | Colombia | soil | AY156967 |
| | CBS 671.76 | Uruguay | nest | AY156975 |
| | CBS 668.76 (T) | Uruguay | *Dasypus septemcinctus*, straw in burrow, armadillo | AY156973 |
| *Ramichloridium basitonum* | CBS 101460 (T) | Japan | skin | AY163561 |
| *Rhinocladiella similis* | dH 14724 | Austria | industrial indoor air | |
| | dH 13054 | Slovenia | CSF | AY857529 |
| | UTHSC R-2978 | USA | human | |
| | UTHSC R-3002 | USA | human | |
| | UTHSC R-3003 | USA | human | |
| | CBS 116299 | France | aspirate of bronchus | |
| | CBS 111763 (T) | Brazil | foot lesion | |
| *Phaeoannellomyces elegans* | CBS 110172 | Netherlands | railway tie | EF551549 |
| *Exophiala nigra* | CBS 546.82 | USSR | unknown | EF551550 |
| *Exophiala bergeri* | CBS 353.52 (T) | Canada | skin | |
| | UTHSC R-2664 | USA | leg wound | |
| | UTHSC 93-1707 | USA | hand | |
| | UTHSC 00-1119 | USA | lesion in forearm | EF025403 |
| | UTHSC 99-211 | USA | knee | EF025406 |
| | UTHSC 94-540 | USA | index finger | EF025404 |
| | UTHSC 99-1723 | USA | penis | EF025405 |
| *Phialophora europaea* | CBS 129.96 | Germany | chromoblastomycosis of toe | EF551553 |

CBS: CBS Fungal Biodiversity Centre, Utrecht, the Netherlands;
UTHSC: University of Texas Health Science Center, San Antonio, TX, USA;
CDC: Centers for Disease Control and Prevention, Atlanta, U.S.A;
dH: working number of strains in Department of Ecology of Clinical Fungi in CBS Fungal Biodiversity Centre, Utrecht, the Netherlands.

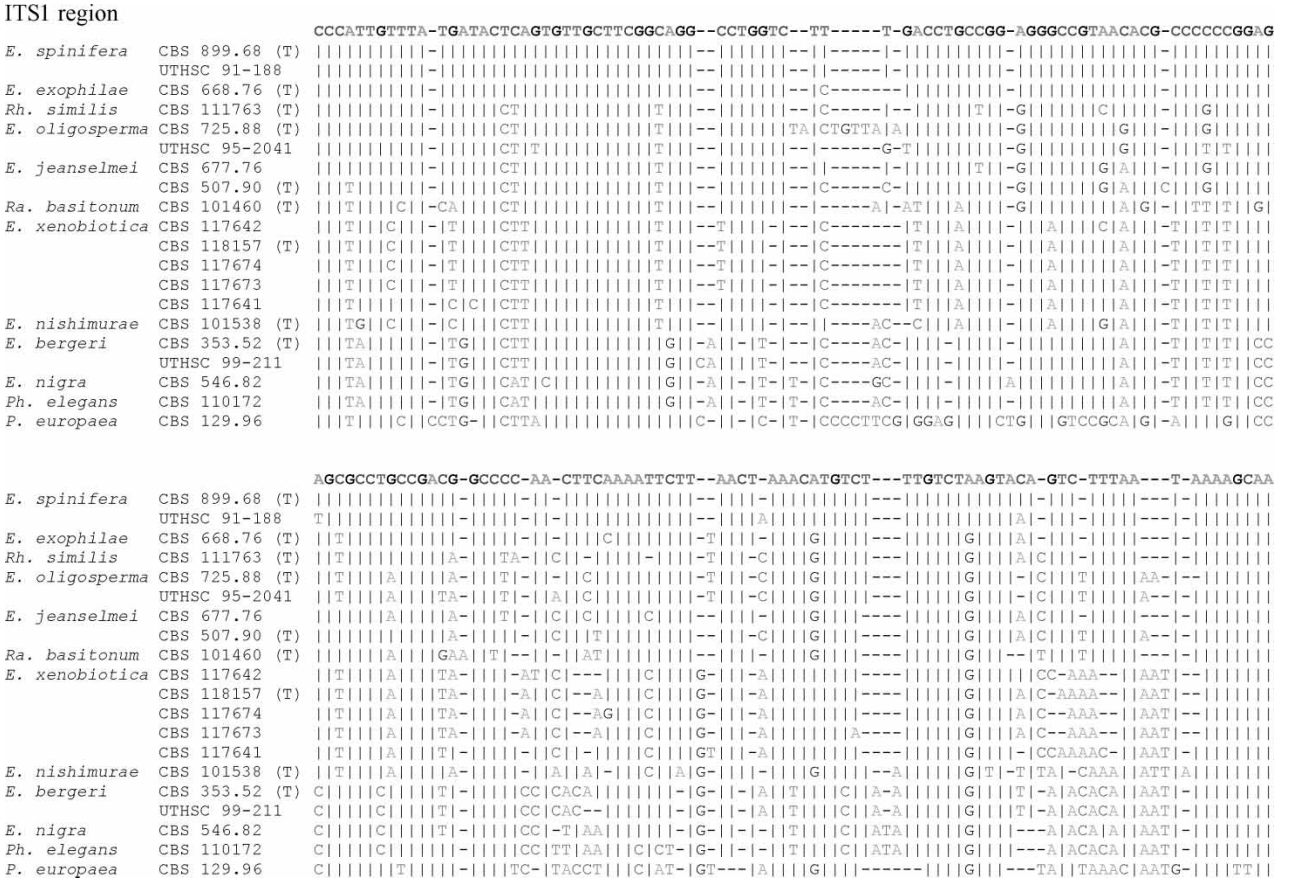


**Fig. 4** Additive variation pattern of ITS regions of rDNA of representative strains of the *Exophiala spinifera* clade. *Exophiala spinifera*, CBS 899.68 is used as leading strand. T, ex-type strain; I, invariant; -, absent.

The utility of COI was tested in fungi with strains from 58 species of *Penicillium* subgenus *Penicillium* and 12 allied species [19]. The COI sequence divergences within species were less than those of ITS and beta-tubulin sequences, while the divergences between species were comparable to ITS. This is a good prospect for fungus identification using COI DNA barcodes. The development of a barcoding system for fungi that shares a common gene target with other kingdoms would be a significant advantage. As we know, the mitochondrial genes of fungi contain many introns. Though this limitation can be circumvented by employing reverse transcription in conjunction with PCR [13], reference sequences from taxonomically confirmed specimens of fungi are limited.

Sequencing ITS rDNA has been widely used for studies of fungal diversity and phylogeny. In many groups traditional taxonomy is basically congruent with the main traits of phylogeny reconstructed with ITS sequences. In black yeasts, however, molecular diversity appears to be very much larger than was observed on the basis of phenetics; novel species are continually being described. The ITS region is a tandem repeat with a length about 500–600 bp in most fungi. It can easily be amplified from many taxa using a limited set of primers designed from adjacent exons. Based on the fact that differences among species are consistently larger than those within species, ITS sequences have been frequently utilized for fungal identification [25,81–88]. ITS currently is a logical candidate for DNA barcoding of fungi, and certainly for black yeasts, as long as no other gene has been sequenced at a comparatively wide scale.

In conclusion, 'accuracy of barcoding for identification depends on the ratio between intraspecific variation and interspecific divergence. The more overlap there is between these categories, the less effective barcoding becomes' [11]. We observed distinct overlap between intraspecific and interspecific divergence values of ITS sequences of species in the *Exophiala spinifera* clade, which seems to imply difficulties in the definition of threshold values to identify candidate

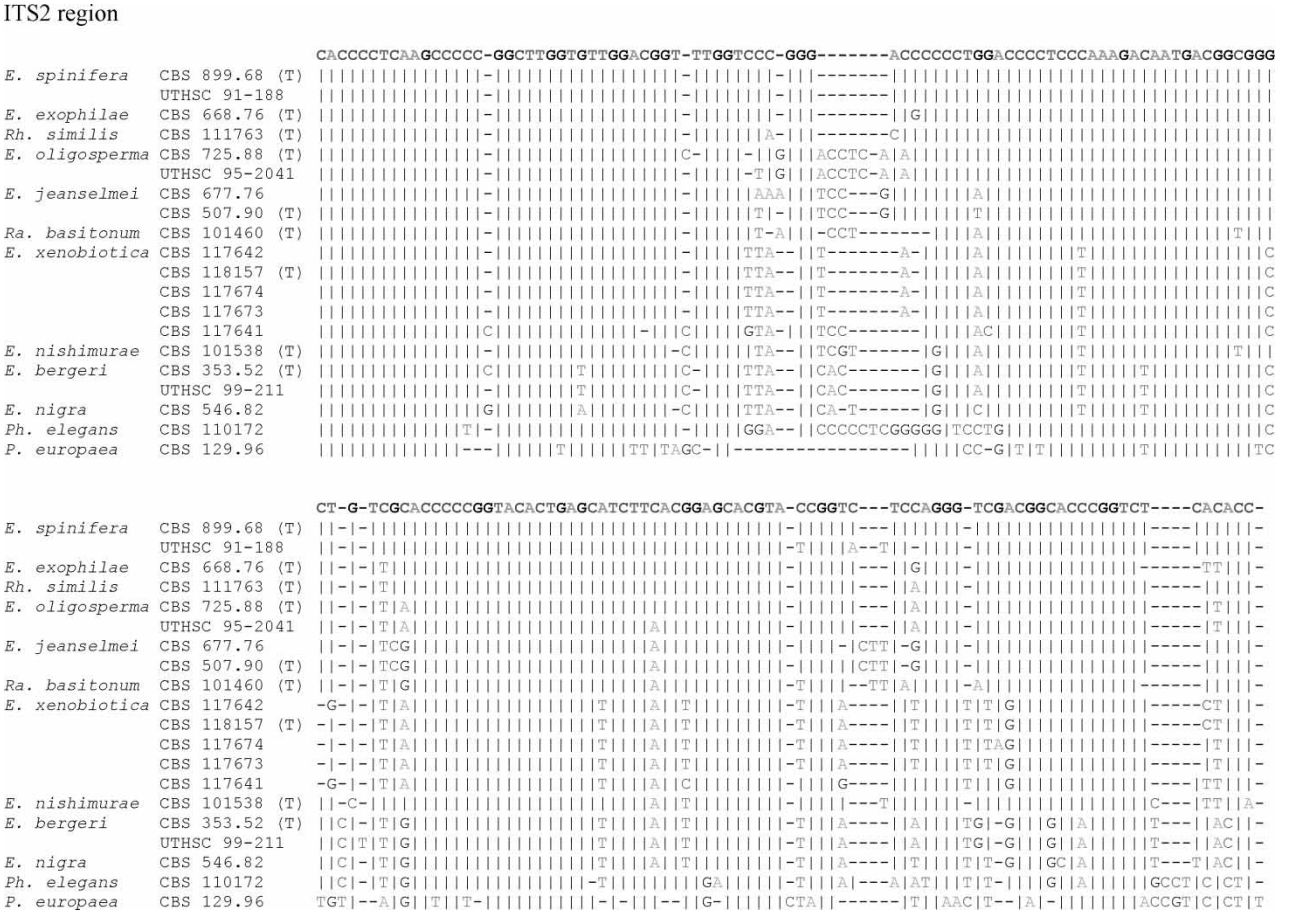


**Fig. 4** Additive variation pattern of ITS regions of rDNA of representative strains of the *Exophiala spinifera* clade (*Continued*).

species. Using a 2% threshold for species diagnosis in our data set, 7 out of 11 described species would be accurately identified. Fortunately, the overlap occurred in the tree where each species was reciprocally monophyletic to all others. Intraspecific variation locally exceeded interspecific divergence in other parts

**Table 3** Sequences of species-specific fragments for species in *E. spinifera* clade.

| Species name | Strain number | GenBank accession nr. | Sequence of species-specific fragment | Position of fragment in ITS region[d] |
|---|---|---|---|---|
| *Exophiala bergeri* | CBS 353.52 (T) | EF551462 | ACAAAATTCTGAATAAATCATGCCT | 140-164 in ITS 1 |
| *Exophiala exophialae* | CBS 668.76 (T) | AY 156973[c] | CAGGCCTGGTCTCGACCTGCCGGAG | 69-93 in ITS 1 |
| *Exophiala jeanselmei* | CBS[a] 507.90 (T) | AY 156963 | AAGACAATGACGGCGGGCTGTTCGA | 71-95 in ITS2 |
| *Exophiala nigra* | CBS 546.82 | EF551550 | TTAAAAAATTCTGAATAATCATGCC | 139-163 in ITS 1 |
| *Exophiala nishimurae* | CBS 101538 (T) | [b] | AAACTACAAACTCATGAACTAAACG | 137-161 in ITS1 |
| *Exophiala oligosperma* | CBS 725.88 (T) | AY163551 | CTCCAAAATTCTTTAACCAAACGTG | 147-171 in ITS1 |
| *Exophiala spinifera* | CBS 899.68 (T) | AY 156976 | GGCCCCAACTTCAAAATTCTTAACT | 129-153 in ITS1 |
| *Exophiala xenobiotica* | CBS 118157 (T) | DQ182587 | CTGGCTCGTCCTACCGGAGGACCGT | 74-98 in ITS 1 |
| *Phaeoannellomyces elegans* | CBS 110172 | EF551549 | TTTAAAAACTCTTGAATAATCATGC | 143-167 in ITS 1 |
| *Ramichloridium basitonum* | CBS 101460 (T) | AY163561 | ATAAAATTCTTAACTAAACGTGTCT | 140-164 in ITS 1 |
| *Rhinocladiella similis* | CBS 111763 (T) | EF551461 | ACCTCAAAATCTTTAACCAAACGTG | 136-160 in ITS 1 |

[a]CBS: Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands.
[b]The sequenc deposited as AY163560 in GenBank is incorrect.
[c]The species name was *E. spinifera* when the sequence was deposited in GenBank.
[d]Based on sequences of the strains listed in the Table.

of the tree. Such overlap will not affect identification of unknown specimens in a thoroughly sampled tree [11]. To test accuracy of ITS sequences for identification, the sequences used in this study were blasted against our local database which contains almost 6000 entities of human associated black yeasts, and against GenBank. If taxonomic names would have been updated in GenBank, the tested samples almost always showed highest sequence similarities to the entities which were same species or nearest neighbours in the *Exophiala spinifera* clade in both databases. Therefore, ITS rDNA works well as a barcode for species of the *E. spinifera* clade.

Detecting species-specific oligo-nucleotide fragments is also useful for diagnosis by hybridization. The species-specific fragments were searched in the ITS region of species in *Exophiala spinifera* clade and listed in Table 3. Sequences of the fragments are identical within a species, and divergent between species (at least 8% except for that between *E. spinifera* and *E. exophialae*). Specificity of the fragments was also proved by blasting the sequences in GenBank and our local database. In conclusion, the specific sequences in the ITS region can be used for designing probes for identifying species in *Exophiala spinifera* clade.



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