Identification and Potential Biotechnological Application of Yeast Isolates in the UNESCO-MIRCEN Biotechnological Yeast Culture Collection of the University of the Free State

By

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Submitted in fulfilment of the requirements for the degree

Magister Scientiae in Biotechnology

In the

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January 2011

Acknowledgements

The structuring and writing of this dissertation was one of the challenging but yet prestigious experiences I have come to face. Without the paramount commitment, endurance, guidance and support of the following people, no doubt that this project would not have reached completion. It is to these people that I pay my deepest gratefulness.

- ♣ **Dr. C. H. Pohl** who undertook the act to be my study leader despite many other academic and professional responsibilities, for her encouragement, empathy and professional commitment.
- ♣ **Prof. J. Albertyn** for recruiting and putting his faith in me and for his guidance, commitment, immense support and on-going patience.
- ♣ **Prof. J. L. F. Kock** who inspired my initial effort by nourishing my background knowledge of some of the concepts applied in this study.
- ♣ **Mrs A. Van Wyk** for providing yeast cultures used in this study and for her sociability and encouragement.
- ♣ **Mr M. F. Maleka** for directing me to this project and believing that I deserve a chance to do a Masters study.
- ♣ My **colleagues** in **Lab 28** and **Lab 49**, for never-ending support and assistance.
- ♣ My father, **Mr M. S. Motaung** for believing that I could go places no matter the situation.
- ♣ The super-women in my life, **Mrs M. Xaba**, **T. S. Motaung**, **T. B. Motaung**, and **T. B. Mbuqa** for unconditional love and support.
- ♣ **National Research Foundation (NRF)** and
- ♣ **TATA Scholarships** for financial support.

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CHAPTER 1

Literature review

1. Motivation

In 1996, the Department of Microbial, Biochemical and Food Biotechnology was awarded the status of MIRCEN (Microbiological Resource Centers) by the United Nations Educational, Scientific and Cultural Organization (UNESCO). This was based on the yeast culture collection housed in the department. The importance of the culture collection is reflected in research performed on cultures including bioprospecting as well as the number of publications from studies performed on yeast cultures.

The culture collection currently accommodates more than 3000 isolates, many of which were isolated from South Africa and other parts of Africa as part of the MIRCEN Fellowship programmes. However, approximately 500 of the 3000 isolates are still unidentified while 2200 have only been conventionally identified using physiological, biochemical or phenotypic methods. This number of unidentified and only conventionally identified isolates is not only considered a challenge by the research team concerned with the collection, but also an opportunity to apply recent molecular advances in yeast systematics. These advances will aid in the exploitation of a wide spectra of biotechnological, clinical, environmental and pharmaceutical importances of isolates held in this culture collection.

Consequently the aims of this study became to identify yeast isolates present in the UNESCO-MIRCEN Biotechnological Yeast Culture Collection, using molecular and conventional identification methods.

2. Introduction

The United Nations (UN) envisioned the idea of a Microbial Resources Center (MIRCEN) to be established in a number of countries around the world (Prior & Kock, 1996). Between 1970 and 1972 the idea was implemented in developed and developing countries in close collaboration with scientists, governmental organizations and academic institutions. Thirty one of these centers were established with each of them housing a collection of important cultures. Microbial Resources Center culture collections were established in part by deposits of cultures from intercontinental culture collections as well as contributing scientists.

The United Nations Educational, Scientific and Cultural Organization (UNESCO) established a MIRCEN at the Department of Microbial, Biochemical and Food Biotechnology in 1996. This formed part of the UNESCO-MIRCEN world-wide network which addresses issues in agriculture, biotechnology and information sciences (Prior & Kock, 1996). In addition, activities undertaken by the UNESCO-MIRCEN Biotechnological Yeast Culture Collection include culture delivery to desired destinations, safe transfer of microorganisms to these destinations and training of young

researchers. These activities are supported by research pertaining to the collection itself, which entails examining microbial strains from different isolation points and assessing their taxonomy and preservation. In southern Africa the collection is not only an operation that concerns microbial and biotechnological resources but also a reservoir for officially preserving the biodiversity of yeasts.

The yeast culture collection of the department had its origin in the early 1980's when a decision was taken by the academic staff that research activities should focus on yeasts. Special attention was given to strains with potential biotechnological applications in the fermentation industry. Consequently, bioprospecting activities from this collection were implemented and initiated between 1982 and 1985 in close collaboration with Professor J. P. van der Walt. At that time a national programme involving the biological utilization of sugarcane bagasse focused on the use of yeasts to ferment D-xylose to ethanol. This attracted most of the departmental research attention. Screening of selected yeasts turned up one strain of *Candida shehatae*, which produced ethanol from D-xylose at a higher rate and yield than reported for *Pachysolen tannophilus* and *Candida tropicalis*, the only other yeasts known at that time to ferment D-xylose (du Preez & van der Walt, 1983).

Subsequent screening identified strains of *Pichia stipitis* (the presumptive teleomorph of *Candida shehatae*) which also showed significant promise in fermenting D-xylose (du Preez & Prior, 1985). The worldwide focus of research aimed at the bioconversion of

lignocellulosic biomass to fuel ethanol rapidly shifted to these latter two species (du Preez *et al*., 1988, 1989).

After this period of research and bioprospecting, the yeast culture collection was expanded when Professor J.P. van der Walt donated yeasts which he collected exclusively from South African sources (e.g. du Preez & Prior, 1985; van der Walt *et al*., 1986; Viljoen *et al*., 1986, 1987). Since 1996 the collection was expanded by isolates obtained from other African countries. This was made possible by UNESCO funding for scientists from this continent to isolate yeasts and deposit them in the UNESCO-MIRCEN Biotechnological Yeast Culture Collection.

Further bioprospecting under the leadership of Professor J.L.F. Kock, director of UNESCO-MIRCEN in South Africa, in partnership with a leading German Medical Research Group resulted in the discovery of a family of oxidized lipids called oxylipins (Sebolai *et al*., 2005). These findings and others reflect the value of this culture collection in bioprospecting research (du Preez & van der Walt, 1983; du Preez *et al*., 1984; du Preez & Prior, 1985; Kock *et al*., 2003; Bareetseng *et al*., 2004; Sebolai *et al*., 2001, 2004).

At present the collection consists of more than 3 000 yeast cultures representing more than 90 genera and 420 species. Yeast cultures in the collection are cryopreserved in liquid nitrogen (-196 ^{0}C) as well as on agar slopes and frozen at -70 ^{0}C.

3. Defining the yeasts

Yeasts are defined as microscopic unicellular fungi, which reproduce asexually by budding or fission (Kreger-van Rij, 1984; Kurtzman & Fell, 1998; Barnett *et al*., 2000; Suh *et al*., 2006). A certain number of these species can be multicellular, developing strands of elongated cells known as pseudohyphae (e.g. *Candida albicans*) or true hyphae (e.g. *Eremothecium ashbyii*). They are classified into two groups, i.e. ascomycetous and basidiomycetous yeasts, each comprising anamorphic and teleomorphic states. Asexual reproduction in the ascomycetous and basidiomycetous yeasts is clearly dissimilar. Anamorphic ascomycetous yeasts stretch all wall layers during asexual reproduction, a process called holoblastic budding (Fig. 1a). Anamorphic basidiomycetous yeasts, on the other hand, grow out the inner wall layer to produce an enteroblastic bud with a neckline or collarette (Fig. 1b) (Kreger-van Rij & Veenhuis, 1971, 1973; von Arx *et al*., 1982; Kreger-van Rij, 1984). Ascospores in teleomorphic ascomycetous yeasts do not develop upon or within fruiting structures as is common in ascomycetous filamentous fungi. Nevertheless, the ascomycetous yeast-like genera (e.g. *Ascoidea, Dipodascus* and *Trichomonascus)* form naked asci carried on hyphae with no discernable yeast phase and are still considered to be yeasts since the asci are naked (Batra & Francke-Grosmann, 1961, 1964; von Arx, 1977; Kurtzman & Fell, 1998;

Kurtzman & Robnett, 2007). On the other hand, teleomorphic basidiomycetous yeasts form basidiospores on the basidium and anamorphic yeast-like taxa such as *Meira argovae* undergo sympodial and acropetal budding with colonies been characterized by fusiform cells (von Arx *et al*., 1982; Tanaka *et al*., 2008). Further dissimilarities observed in these two taxa include the type of cell wall polysaccharide. The predominant polysaccharide in ascomycetous yeasts is the β-glucans and chitin in basidiomycetous yeasts (Suh *et al*., 2006).

From this point on, ascomycetous and basidiomycetous yeasts will be referred to as ascomycetes and basidiomycetes respectively. Ascomycetes are mainly classified into the Saccharomycetes and Schizosaccharomycetes, while basidiomycetes are distributed into the following classeses: Hymenomycetes, Urediniomycetes and Ustilaginomycetes (Table 1) (Scorzetti *et al*., 2002; Kurtzman & Fell, 2006). The Schizosaccharomycetes include *Schizosaccharomyces pombe* which is phylogenetically related to members of the family Protomycetaceae (e.g. *Protomyces lactucae-debifis* and *Protomyces inouyei*) and Taphrinaceae (e.g. *Taphrina deformans*, *Taphrina populina* and *Taphrina wiesneri*) in the Archiascomycetes clade (Nishida & Sugiyama, 1994). Recently proposed genera of ascomycetes are *Babjeviella*, *Barnettozyma*, *Lindnera*, *Meyerozyma*, *Millerozyma*, *Peterozyma*, *Priceomyces*, *Scheffersomyces*, *Schwanniomyces*, *Spencermartinsiella*, *Sugiyamaella*, *Trichomonascus* and *Wickerhamomyces* (Table 1) (Kurtzman & Robnett, 2007; Kurtzman *et al*., 2008; Kurtzman & Robnett, 2010; Kurtzman & Suzuki, 2010; Péter *et al*., 2010). Basidiomycetes such as *Cryptococcus curvatus*, *Cryptococcus fragicola*,

Sporobolomyces magnisporus and members of *Rhodotorula* and *Rhodosporidium* are widely distributed in different groups (polyphyly) in their major lineages. As a result, most phylogenetic studies are currently focusing on defining proper phylogenetic positions of polyphyletic taxa in lineages such as the order, Trichosporonales. One of the recently proposed basidiomycete genus is *Cryptotrichosporon* (Table 1) (Type species *C. anacardii* CBS 9551^{T}), which comprises encapsulated anamorphic species (Okoli *et al*., 2007). This genus is phylogenetically assigned to the order Trichosporonales. Current classification of anamorphic and teleomorphic genera of ascomycetes and basidiomycetes (including yeast-like taxa) is provided in Table 1.

Yeasts occupy habitats of aquatic (Chang *et al*., 2008), atmospheric (Pohl *et al*., 2006), plant (Golubev *et al*., 2008) and terrestrial nature (Lee *et al*., 2009). As a result, they play ecological and biotechnological roles worldwide, such as bioremediation of toxic compounds (Kwon *et al*., 2002), fermentation of beverages (Lodolo *et al*., 2008) and food processing (Viljoen *et al*., 2003; Romano *et al*., 2006).

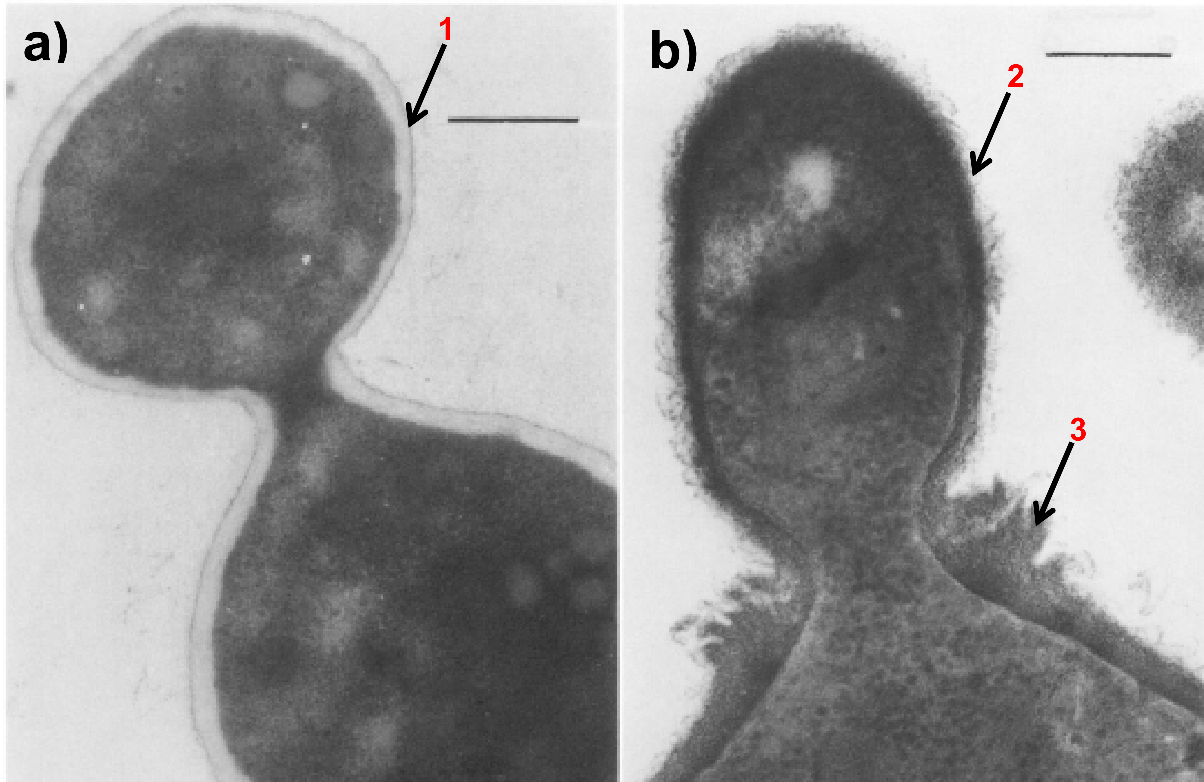

Figure 1: Transmission electron micrograph showing formation of asexual buds during a) holoblastic and b) enteroblastic budding. 1- Holoblastic bud, 2- Enteroblastic bud and 3- Collarette. Bar = 0.5 µm and 0.25 µm respectively. (Taken from von Arx *et al*., 1982).

Table 1: Current classification of ascomycetes and basidiomycetes and yeast-like taxa according to Kurtzman & Fell (2006) and Suh and co-workers (2006).

Ascomycetes
Schizosaccharomycetes
Schizosaccharomycetales Prillinger, Dörfler, Laaser, Eckerlein & Lehle ex Kurtzman
Schizosaccharomycetaceae Beijerinck ex Klöcker
Schizosaccharomyces Lindner (T)
Saccharomycetes
Saccharomycetales Kudryavtsev
Ascoideaceae J. Schröter
Ascoidea Brefeld & Lindau (T)
Cephaloascaceae L.R. Batra
Cephaloascus Hanawa (T)
Dipodascaceae Engler & E. Gilg
Dipodascus Lagerheim (T)
Galactomyces Redhead & Malloch (T)

Geotrichum Link:Fries (A)

Endomycetaceae J. Schröter

Endomyces Reess (T)

Helicogonium W.L. White (T)

Myriogonium Cain (T)

Phialoascus Redhead & Malloch (T)

Eremotheciaceae Kurtzman

Coccidiascus Chatton emend. Lushbaugh, Rowton & McGhee (T)

Eremothecium Borzi emend. Kurtzman (T)

Lipomycetaceae E.K. Novak & Zsolt

Babjevia van der Walt & M.Th. Smith (T)

Dipodascopsis Batra & Millner (T)

Lipomyces Lodder & Kreger van Rij (T)

Myxozyma van der Walt, Weijman & von Arx (A)

Zygozyma van der Walt & von Arx (T)

Metschnikowiaceae T. Kamienski

Clavispora Rodrigues de Miranda (T)

Metschnikowia T. Kamienski (T)

Pichiaceae Zender

Brettanomyces Kufferath & van Laer (A)

Dekkera van der Walt (T)

Peterozyma Kurtzman & Robnett

Pichia Hansen (pro pate) (T)

Saturnispora Liu & Kurtzman (T)

Saccharomycetaceae G. Winter

Kazachstania Zubkova (T)

Kluyveromyces Kurtzman, Lachance, Nguyen & Prillinger (T)

Lachancea Kurtzman (T)

Nakaseomyces Kurtzman (T)

Naumovia Kurtzman (T)

Saccharomyces Meyen ex Reess (T)

Tetrapisispora Ueda-Nishimura & Mikata (T)

Torulaspora Lindner (T)

Vanderwaltozyma Kurtzman (T)

Zygosaccharomyces Barker (T)

Zygotorulaspora Kurtzman (T)

Saccharomycodaceae Kudryavtsev

Hanseniaspora Zikes (T)

Kloeckera Janke (A)

Saccharomycodes Hansen (T)

Saccharomycopsidaceae von Arx & van der Walt

Saccharomycopsis Schiönning (T)

Trichomonascaceae Kurtzman & Robnett

Spencermartinsiella Péter, Dlauchy, Tornai-Lehoczki, M. Suzuki & Kurtzman (T)

Trichomonascus Jackson emend. Kurtzman & Robnett (T)

Wickerhamiella Kurtzman & Robnett

Saccharomycetales *incertae sedis*

Aciculoconidium King & Jong (A)

Ambrosiozyma van der Walt (T)

Arxula van der Walt, M.Th. Smith & Y. Yamada (A)

Ascobotryozyma J. Kerrigan, M.Th. Smith & J.D. Rogers (T)

Babjeviella Kurtzman et M. Suzuki

Barnettozyma Kurtzman, Robnett et Basehoar-Powers (T)

Blastobotrys von Klopotek (A)

Botryozyma Shann & M.Th. Smith (A)

Candida Berkhout (A)

Citeromyces Santa María (T)

Cyniclomyces van der Walt & Scott (T)

Debaryomyces Lodder & Kreger-van Rij (T)

Hyphopichia von Arx & van der Walt (T)

Kodamaea Y. Yamada, T. Suzuki, Matsuda & Mikata emend. Rosa, Lachance, Starmer, Barker, Bowles & Schlag-Edler (T)

Komagataella Y. Yamada, Matsuda, Maeda & Mikata (T)

Kuraishia Y. Yamada, Maeda & Mikata (T)

Lodderomyces van der Walt (T)

Lindnera Kurtzman, Robnett et Basehoar-Powers

Macrorhabdus Tomaszewski, Logan, Snowden, Kurtzman & Phalen (A)

Meyerozyma Kurtzman et M. Suzuki (T)

Millerozyma Kurtzman et M. Suzuki (T)

Nadsonia Sydow (T)

Nakazawaea Y. Yamada, Maeda & Mikata (T)

Ogataea Y. Yamada, Maeda & Mikata (T)

Pachysolen Boidin & Adzet (T)

Phaffomyces Y. Yamada, Higashi, S. Ando & Mikata (T)

Priceomyces M. Suzuki et Kurtzman (T)

Scheffersomyces Kurtzman et M. Suzuki (T)

Schizoblastosporion Ciferri (A)

Schwanniomyces Klocker emend. M. Suzuki & Kurtzman (T)

Sporopachydermia Rodrigues de Miranda (T)

Starmerella Rosa & Lachance (T)

Starmera Y. Yamada, Higashi, S. Ando & Mikata (T)

Sympodiomyces Fell & Statzell (A)

Trigonopsis Schachner emend. Kurtzman & Robnett (A)

Wickerhamia Soneda (T)

Wickerhamomyces Kurtzman, Robnett et Basehoar-Powers

Yamadazyma Billon-Grand emend. M. Suzuki, Prasad & Kurtzman emend. Kurtzman & Robnett (T)

Yarrowia van der Walt & von Arx (T)

Zygoascus M.Th. Smith emend. Kurtzman & Robnett (T)

Basidiomycetes

Hymenomycetes

Cystofilobasidiales Boekhout & Fell

<u>Cystofilobasidiaceae</u>

Cystofilobasidium Oberwinkler & Bandoni (T)

Cryptococcus Vuillemin (*pro parte*) (A)

Guehomyces Fell & Scorzetti (A)

Itersonilia Derx (A)

Mrakia Y. Yamada & Komagata (T)

Phaffia Miller, Yoneyama & Soneda (A)

Tausonia Bab'eva (A)

Udeniomyces Nakase & Takematsu (A)

Xanthophyllomyces Golubev (T)

Filobasidiales Julich

Filobasidiaceae

Cryptococcus Vuillemin (*pro parte*) (A)

Filobasidium Olive (T)

Trichosporonales Boekhout & Fell

Trichosporonaceae

Cryptococcus Vuillemin (*pro parte*) (A)

Cryptotrichosporon Okoli & Boekhout (A)

Trichosporon Behrend (A)

Tremellales Rea emend. Bandoni

Tremellaceae

Auriculibuller Sampaio (T)

Bullera Derx (A)

Bulleribasidium Sampaio, Weiss & Bauer (T)

Bulleromyces Boekhout & Fonseca (T)

Cryptococcus Vuillemin (*pro pate*) (A)

Cuniculitrema Sampaio & Kirschner (T)

Dioszegia Zsolt emend.Takashima, Deak & Nakase (A)

Fellomyces Y. Yamada & Banno (A)

Filobasidiella Kwon-Chung (T)

Holtermannia Saccardo & Traverso (T)

Kockovaella Nakase, Banno & Y. Yamada (A)

Sirobasidium Lagerheim & Patouillard (T)

Sterigmatosporidium Kraepelin & Schulze (T)

Tremella Persoon (T)

Trimorphomyces Bandoni & Oberwinkler (T)

Tsuchiyaea Y. Yamada, Kawasaki, M. Itoh, Banno & Nakase (A)

Uredinomycetes

Agaricostilbales Oberwinkler & Bauer

Agaricostilbaceae

Agaricostilbum Wright emend. Wright, Bandoni & Oberwinkler (T)

Bensingtonia Ingold emend. Nakase & Boekhout (A)

Chionosphaera Cox (T)

Kondoa Y. Yamada, Nakagawa & Banno emend. Fonseca et al. (T)

Kurtzmanomyces Y. Yamada, M. Itoh, Kawasaki, Banno & Nakase emend. Sampaio (A)

Sporobolomyces Kluyver & van Niel (A)

Sterigmatomyces Fell emend. Y. Yamada &Banno (A)

Microbotryales

Microbotryaceae

Bensingtonia Ingold emend. Nakase & Boekhout (A)

Curvibasidium Sampaio & Golubev (T)

Leucosporidiella Sampaio (A)

Leucosporidium Fell, Statzell, Hunter & Phaff (T)

Mastigobasidium Golubev (T)

Reniforma Pore & Sorenson (A)

Rhodosporidium Banno (*pro parte*) (T)

Rhodotorula Harrison (*pro parte*) (A)

Sporobolomyces Kluyver & van Niel (*pro parte*) (A)

Naohideale

Bannoa Hamamoto (T)

Erythrobasidium Hamamoto, Sugiyama & Komagata (T)

Naohidea Oberwinkler (T)

Rhodotorula Harrison (*pro parte*) (A)

Sakaguchia Y. Yamada, Maeda & Mikata (T)

Sporobolomyces Kluyver & van Niel (*pro parte*) (A)

Sporidiobolales Sampaio,Weiss & Baue

Sporidiobolaceae

Rhodosporidium Banno (*pro parte*) (T)

Rhodotorula Harrison (*pro parte*) (A)

Sporidiobolus Nyland (T)

Ustilaginomycetes

Malassezia Baillon (A)

Pseudozyma Bandoni emend. Boekhout (A)

Rhodotorula Harrison (*pro parte*) (A)

Sympodiomycopsis Sugiyama, Tokuoka & Komagata (A)

Tilletiopsis Derx ex Derx (A)

(A) – Anamorphic genera, (T) – Teleomorphic genera

Some genera appear in more than one order (e.g. *Cryptococcus* and *Rhodotorula*), indicating polyphyly. Ascomycetous yeasts with ambiguous phylogenetic positions are placed in the Saccharomycetales *incertae sedis*.

4. Yeast identification

4.1 Phenotypic identification

The process of identifying yeasts is normally achieved using a battery of phenotypic tests which are standardized, following isolation and purification of cultures (Kurtzman & Fell, 1998). These combined characters or physiological and biochemical profiles are used for the description of new species which is translated into Latin in a publication format following rules outlined by the International Code of Botanical Nomenclature

(Kurtzman & Fell, 1998; Barnett, 2004). This system of identification is sometimes perceived as artificial because a combination of characters is used for demarcation of species.

4.1.1 Growth on malt/yeast extract agar

Growth may be examined on malt or yeast extract prepared as liquid or solid media. Morphology of cells, whether they occur in pairs, clusters or are scattered and the sizes of asexual structures (e.g. hyphae) are examined in detail microscopically. These examinations are performed after three to five days and they may be accompanied by other examinations such as mode of asexual reproduction and morphology of colonies (e.g. surface, texture or colour). Incubation time and temperature of strains differ depending on the species studied. For instance, *Eremothecium* strains may be incubated for ten days at 22 ^{0}C before examination (Kurtzman & Fell, 1998). Some strains of *Cryptococcus* (e.g. *C. podzolicus*) may be incubated for three to seven days at 18-20 ^{0}C before examination (de Garcı´a *et al*., 2010; Russo *et al*., 2010).

4.1.2 Dalmau plate technique

The Dalmau plate technique is performed on a medium which can promote formation of hyphae. Commonly used media for this technique include cornmeal or potato dextrose agar (Glushakova *et al*., 2010; Péter *et al*., 2010). Presence or absence of pseudo or true hyphae is observed after a week or two at 25 ^{0}C (de Garcı´a *et al*., 2010). This

follows inoculation of cultures where they are streaked as dots and lines and covered with a sterile cover slip.

4.1.3 Ascospore induction

Induction of ascospores is performed only if the strain of interest is known or suspected to have a teleomorphic (sexual) state. This is performed by mixing actively growing cultures of different strains, followed by incubation at 15 and 25 ^{0}C (Péter *et al*., 2010). This is followed by microscopic examination that is performed after three weeks to observe any sporulation (Yarrow, 1998). However, ascospores may develop within the first week onwards and examination can also proceed at this point (e.g. Glushakova *et al*., 2010). Morphology of the ascospores, including size and number of ascospores per ascus as well as ascus ornamentation, is then recorded.

4.1.4 Assimilation and fermentation tests

In assimilation tests, a carbon compound is added to a liquid basal medium, such as Yeast Nitrogen Base, where it serves as sole source of energy. Common laboratory carbon compounds include glucose, sucrose, raffinose, starch, and xylose. In addition the use of alcohols (e.g. methanol, adonitol and erythritol), organic acids (e.g. succinic and citric acid) and glycosides (e.g. glucoside, arbutin and salicin) is also common for assimilation properties of strains. Nitrogen compounds also form part of assimilation

tests where common nitrogen compounds assimilated by most yeasts include glucosamine, nitrate and nitrite (van der Walt & Yarrow, 1984).

Fermentation of sugars in yeasts ranges from weak to vigorous (van der Walt & Yarrow, 1984). However, certain ascomycetes and basidiomycetes, such as species of *Lipomyces* and *Cryptococcus* respectively, lack fermentative ability (Fell & Statzell-Tallman, 1998; Thanh, 2006). During fermentation, carbon dioxide is produced from carbon compounds under favourable conditions. Durham tubes are commonly used to observe carbon dioxide liberation (seen as a bubble).

4.1.5 Additional tests

Additionally, yeast strains are examined for formation of starch-like (amyloid) compounds, growth on high sugar and salt concentration as well as in vitamin-free medium (Yarrow, 1998). Starch formation is observed by staining yeast cells with iodine solution. In addition, urease tests are performed in order to determine the presence of enzymes that act upon urea (usually on 20% urea), while gelatine liquefaction test determines presence of enzymes involved in liquefying gelatine. The Diazonium blue B (DBB) test is also conducted to confirm the affinity of a strain, whether it belongs to ascomycetes or basidiomycetes. Ascomycetes normally produce no colour while basidiomycetes produce a dark red or purple colour after staining of cells by drops of DBB solution.

4.2 Molecular identification

Molecular characteristics are important to properly assign strains, species and to an extent, genera to their respective phylogenetic groups. The D1/D2 domain, internal transcribed spacers (ITS) and 18S small subunit are some of the important regions of the ribosomal RNA (or rRNA) used for molecular characterization of ascomycete and basidiomycete taxa (Kurtzman, 1992). These rRNA regions have slow and different rates of mutations and are regarded as a collection of evolutionary chronometers. They are also perceived as molecular barcodes. Consequently they contribute largely in the natural identification system or molecular identification of yeasts.

The ribosomal RNA molecule comprises the 18S rRNA (SSU), internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA between these spacers as well as the 26S rRNA (LSU) (Fig. 2). The D1/D2 domain is located on the 5' side of the 26S rRNA (Guadet *et al*., 1989; Sugita & Nishikawa, 2003) and is approximately 600 bases long (Fig. 3). The tandem repeats of the rRNA are separated by the nontranscribed (or intergenic) spacers (NTS or IGS1 and IGS2, separated by the 5S) while the 3′ and 5′ side of the molecule are flanked by the externenal transcribed spacers (ETS) (Richard *et al*., 2008). The D1/D2 domain is amplified using polymerase chain reaction (PCR) and sequenced with universal primers, NL-1 and NL-4 following nucleic acid extraction from yeast strains. Primers, ITS-4 and ITS-5 have been designed to amplify the internal transcribed spacers (approx. 600 base pairs) for most basidiomycetes (Fell *et al*., 2000). After DNA amplification, PCR products are sequenced and analysed using relevant

molecular analysis tools. Sequences are then compared with available yeast sequences on the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). The D1/D2 domain and ITS regions are commonly used in combination with the 18S small subunit rRNA in most recent studies (Glushkova *et al*., 2010; Kurtzman & Robnett, 2010; Péter *et al*., 2010).

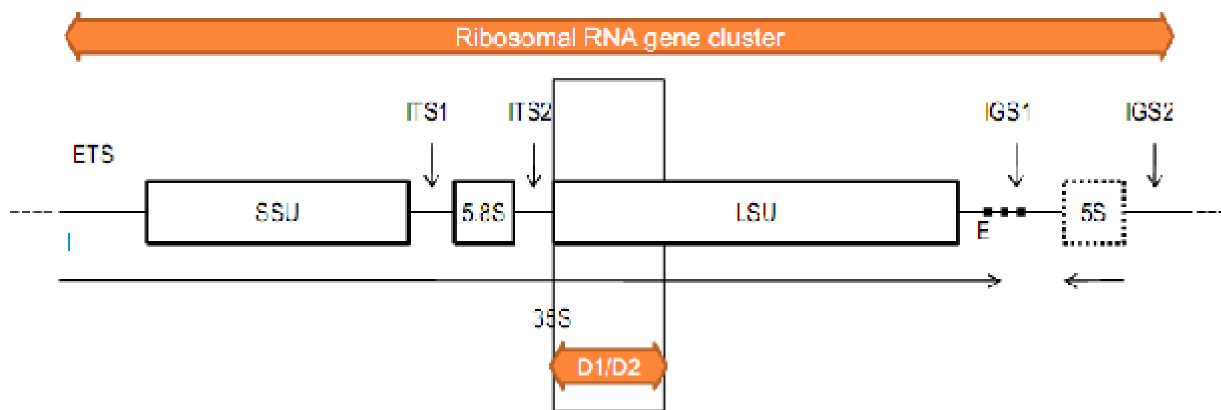

Figure 2: Schematic representation showing a single copy of ribosomal RNA. (Taken from Lachance, 2010).

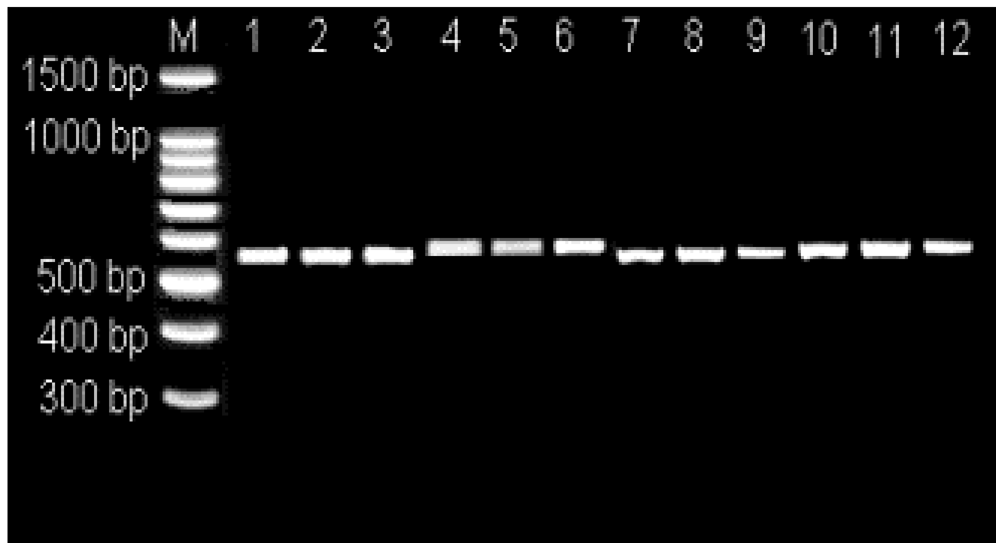

Figure 3: Electrophoregram indicating the approximate size of the D1/D2 region following gel electrophoresis using DNA standard. (Taken from Abd-Elsalam *et al*, 2003).

4.2.1 D1/D2 domain of rRNA

Sequencing of the D1/D2 domain of the rRNA gene has been evaluated for almost three decades. Guadet and co-workers (1989) compared gene sequences from *Saccharomyces cerevisiae*, *Neurospora crassa* and *Fusarium oxysporum* to show bases that were conserved in the D1/D2 domain. These species showed dissimilar nucleotide sequence in this domain which confirmed that they belonged to separate genera. This was one of the first uses of D1/D2 sequences in different fungal groups (Table 2). As a result molecular taxonomists focused more on the use of these sequences for identification of yeasts (Yamada & Kawasaki, 1989).

Further studies by Peterson & Kurtzman (1990) compared partial sequences from the D1/D2 domain using only yeast taxa, *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Schizosaccharomyces pombe* and species of *Issatchenkia* (Table 2). Species in these genera were well resolved from sequence comparison in this region between species. Studies that followed increased the number of ascomycete taxa that can be resolved from rate of mutations in the D1/D2 domain of the rRNA (Gueho *et al*., 1990; Kurtzman & Liu, 1990; Kurtzman & Robnett, 1991; Kurtzman, 1993, 1995). The D1/D2 domain of 26S rRNA is mostly analyzed together with the 18S small subunit (SSU) rRNA to resolve yeast taxa in a number of studies. For instance, nitrate-assimilating *Pichia* species with hat-shaped ascospores were reassigned into the genera *Kuraishia, Nakazawaea* and *Ogataea* using these sequences (Yamada *et al*., 1994). As a result, the genus *Ogataea* was emended to include some members of *Pichia* and neighbouring taxa that were found to be related to the genus *Ogataea* (e.g. methanol-assimilating yeasts) (Nagatsuka *et al*., 2008; Péter *et al*., 2007, 2008; Kurtzman & Robnett, 2010).

Table 2: Some of the published impacts in yeast systematics resulting from sequence analysis of D1/D2 domain rRNA alone and when combined with other regions (18S and/or ITS rRNA) from 1989-2000.

Impact/(s)	**Taxa studied**	**Reference**
One of first publications describing sequencing of D1/D2 domain for demarcation of sibling species in fungi.	*Saccharomyces cerevisiae* and other fungi.	Guadet *et al*., 1989
Close species relatedness determined in *Issatchenkia* species. This genus was found to be the most divergent among other genera of yeasts.	*S. cerevisiae*, *Debaryomyces hansenii*, *Schizosaccharomyces pombe* and *Issatchenkia* spp.	Peterson & Kurtzman, 1990
Re-assignment of some *Pichia* species to *Saturnospora* gen. nov.	*Pichia* and *Williopsis* spp.	Liu & Kurtzman, 1991
Use of rRNA for discrimination of species of yeasts.	Saccharomycetes and yeast-like taxa	Kurtzman, 1992
Evidence that yeast-like genera are not monophyletic to filamentous fungi but to budding yeasts. Introduction of the order	Some yeasts and yeast-like genera	Kurtzman, 1993

Impact/(s)	Taxa studied	Reference
Schizosaccharomycetales.		
Proposal of the family Eremotheciaceae	*Ashbya, Eremothecium, Holleya, Nematospora* and *Pichia* spp.	Kurtzman, 1995
Proposal of the genera *Ogataea*, *Kuraishia* and *Nakazawaea*	*Pichia* and *Hansenula* spp.	Yamada *et al*., 1994
Proposal of new genus *Komagataella*	Methanol-assimilating yeasts	Yamada *et al*., 1995
rDNA/RNA database completed for ascomycetous yeasts identification.	Ascomycetous yeasts	Kurtzman & Robnett, 1998
rDNA/RNA database completed for basidiomycetous yeasts identification.	Basidiomycetous yeasts	Fell *et al*., 2000

Studies of identification are based on guidelines formulated in literature for separating taxa using sequence divergence in the D1/D2 domain of rRNA (Kurtzman & Robnett, 1998; Kurtzman & Fell, 2006). Strains of a species are separated by 0.5% substitutions

(i.e. three nucleotide differences). On the other hand, strains showing 1-2% substitutions (i.e. six nucleotides or more) are likely to be separate species. Péter and co-workers (2010) were able to identify a strain representing a hetrothalic yeast species, which was found to be distant from all members of the Trichomonascaceae clade, using more substitutions (7%) than previously reported. As a result of these substitutions in the D1/D2 domain of this strain, it represented a new genus in the Trichomonascaceae clade (Péter *et al*., 2010). A public database (NCBI, http://www.ncbi.nlm.nih.gov/) containing yeast sequences has been developed and made available for identification means. This database is continuously updated by authors contributing sequences of novel yeasts isolated from different habitats around the globe. However, taxonomic impacts brought by sequence analysis were well recognized even before the development of this database (see Table 2).

4.2.2 18S SSU rRNA

The 18S small subunit of the ribosomal RNA has been analyzed mostly for basidiomycetes. For instance, Nakase and co-workers (1993) suggested that some phenotypic characters were not informative in assigning phylogenetically related taxa. As means to support this suggestion, it was observed that the presence of a filobasidiaceous basidium and the Q-10 isoprene unit in the type species of *Erythrobasidium* could not validly place this genus in the family Filobasidiaceae (Sugiyama & Suh, 1993). As a result, the 18S SSU phylogenetic data analysis revealed that species of this genus cluster with teliospore-forming yeasts, i.e. *Rhodosporidium*

toruloides and *Leucosporidium scottii*. This phylogenetic study also supported the ultra-structural finding that *E. hasegawianum* and members of *Rhodosporidium* share similar septal pore structures (Suh *et al*., 1993). In addition, 18S SSU has been studied for the assessment of phylogenetic relationships between species of *Saccharomyces*. The inclusion of *S. kunashirensis* and *S. martiniae* into this genus was entirely based on phylogenetic analysis of 18S SSU gene sequences (James *et al*., 1997). Similarly, *Saccharomyces* species were proven to be polyphyletic as they clustered in the *Kluyveromyces*, *Torulaspora*, and *Zygosaccharomyces* clades in the 18S SSU phylogenetic tree.

4.2.3 Internal transcribed spacers (ITS) of rRNA

The internal transcribed spacers also had important impacts on yeast systematics. Fell and co-workers (2000) and Scorzetti and co-workers (2002) proposed that not all basidiomycetes can be resolved solely from sequencing the D1/D2 domain. Consequently, the domain was compared to the ITS region and found to be less variable in species of the genera *Filobasidium*, *Phaffia*, *Rhodotorula*, *Sporobolomyces*, *Mrakia* and *Xanthophyllomyces* (Diaz & Fell, 2000; Fell *et al*., 2000; Scorzetti *et al*., 2002; Fell *et al*., 2007). Gene sequence data from this analysis indicated that more variability was only observed with the ITS and other regions [e.g. intergenic spacers (IGS)]. This prompted several studies to incorporate both the ITS and D1/D2 regions in identification of basidiomycetes since sequence variability might be compromised if sequence and phylogenetic analysis is performed based on only D1/D2 sequences

(Landell *et al*., 2009; de Garcı´a *et al*., 2010). For instance, Fell and co-workers (2000) showed that *Cryptococcus ater, Filobasidium elegans and F. floriforme* possessed similar D1/D2 sequences and can only be separated with ITS sequences.

The use of combined sequence data (e.g. 18S, D1/D2 rDNA and ITS sequences) is common practice in current phylogenetic studies of ascomycetes and basidiomycetes. This multigenic approach increases support of species in respective ascomycete and basidiomycete lineages by strengthening basal branches and also enables the detection of those clades of ambiguity in the phylogenetic trees (Kurtzman & Robnett, 2003; Kurtzman *et al*., 2007; Kurtzman, 2010). In addition, a multigenic approach combined with morphological, physiological and biochemical studies maintain a reliable identification system for both ascomycete and basidiomycete taxa (Suh *et al*., 2006; Pagnocca *et al*., 2010).

5. Purpose of study

With the above as background the purpose of this study became the following:

1. To identify and characterize yeast isolates present in the UNESCO-MIRCEN biotechnological yeast culture collection, using:

   - D1/D2 domain of 26S rDNA (**Chapter 2)**

   - ITS sequencing of rDNA (**Chapter 3, 4**) and

2. To describe new species of yeasts using standardized techniques (**Chapter 3, 4**)

3. To note possible biotechnological applications of species (**Chapter 2**)

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CHAPTER 2

Molecular identification of unidentified yeast isolates

1. Introduction

Ribosomal DNAs such as the 18S small subunit (SSU), the internal transcribed spacers (ITS) and the D1/D2 domain of 26S rDNA have gained recognition in yeast taxonomy as identification tools during the past years (Kurtzman, 1995; Kurtzman, 2003; Kurtzman & Robnett, 2007, 2008). As a result, yeasts of biotechnological, clinical and industrial importance are identified more rapidly than previously. In addition, ribosomal genes contribute largely to studies involving the discovery of previously uncharacetrized species. Combined data analysis from these ribosomal genes (e.g. ITS-D1/D2 or ITS-18S SSU or ITS-D1/D2-18S or combined with other genes excluding rDNA genes) is largely been applied in a number of studies involving delineation and description of new species (Pagnocca *et al*., 2010; Péter *et al*., 2010). Nevertheless, the use of the D1/D2 domain sequences alone has initially paved a way through understanding molecular systematics of yeasts. Therefore is always incorporated with other genes in the analysis and demercatin of separate species and their close relationships.

The D1/D2 domain is located on the 5' side of the rDNA before the external and non-transcribed spacers (Lachance, 2010). Because eukaryotic ribosomal DNA is present as tandem repeats, the D1/D2 DNA sequence is in abundance and thus easily extracted for use in identification. Primers designed for this region are not species specific and can be used, during polymerase chain reaction (PCR), to target the D1/D2 region for almost all species of yeasts. These are some of the primary reasons this region is considered a universal tool for the taxonomy of yeasts (Kurtzman & Fell, 1998).

Since this region has come to be recognized, the discovery of novel ascomycetes and basidiomycetes has been increasing and this is based on comparisons between sequences of type strains of all yeast genera, used as reference sequences for identification, and sequences of strains under study. Type strain sequences have been deposited and made available from the extensive database on the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) or others such as DNA Data Bank of Japan (DBBJ) (http://www.ddbj.nig.ac.jp/). In this way the task of yeast identification is facilitated. The current study reports on the identification of ascomycetes and basidiomycetes present in the UNESCO-MIRCEN biotechnological yeast culture collection, using D1/D2 sequence based analysis in the 26S region of large subunit rDNA.

2. Materials and methods

2.1 Identification of yeast isolates using D1/D2 domain of 26S rDNA

2.1.1 Strains examined

During this study, 197 unidentified strains deposited and maintained in the UNESCO-MIRCEN Biotechnological Yeast Culture Collection of the University of the Free-State were used.

2.1.2 Nucleic acid isolation

Nucleic acid isolation from yeast cells followed growth for approximately 24 h at 30 ^{0}C in 50 ml yeast malt extract (YM) broth (3 g yeast extract, 3 g malt extract, 5 g peptone,

and 10 g glucose per litre of distilled water). Cells were suspended in 20 µl triple distilled water and lysed with a pipette tip followed by boiling for 10 min at 96 ^{0}C. Genomic DNA extraction was performed as described by Labuschagne and Albertyn (2007) with certain modifications. Yeast strains were cultivated on 5 ml yeast extract (10 g l^{-1}), peptone (20g l^{-1}) plus D-glucose (20g l^{-1}) (YPD) media in 16 mm capped test tubes at 30 ^{0}C for 24-48 hours while shaking. Cells were harvested by centrifugation following addition of 500 µl DNA lysis buffer (100 mM Tris–HCl, pH 8.0, 50 mM EDTA, pH 8.0, and 1 % SDS) and glass beads. This solution was vigorously mixed and cooled on ice for 5 min. Ammonium acetate (275 µl, 7 M, pH 7.0) was added, followed by incubation at 65 ^{0}C for 5 min and cooling for 5 min on ice. Chloroform was added and this mixture was centrifuged at 20 000 × *g* for 2 min at 4 ^{0}C. The supernatant was transferred to a new tube and DNA was precipitated with isopropanol and centrifuged at 20 000 × *g* for 5 min at 4 ^{0}C. The pellet was washed with 70% (v/v) ethanol, dried and re-dissolved in 100 µl TE (10 mM Tris–HCl and 1 mM EDTA, pH 8.0).

2.1.3 Amplification and sequencing of D1/D2 domain

Polymerase chain reaction (PCR) was performed for the 26S rDNA D1/D2 domain using fungal primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3' (Kurtzman & Robnett, 1998). Sequence pairs for rDNA were obtained with the ABI BigDye Terminator Cycle sequencing kit (Applied Biosystems®) and resulting sequences were analysed using GenePro 4.8 (Drummond *et al*., 2009) and compared with sequences on GenBank (http://www.ncbi.nlm.nih.gov/).

3. Results and discussion

3.1 Strain identification based on D1/D2 rDNA sequences

Hundred and ninety seven strains (138 ascomycetes and 59 basidiomycetes) were identified in the current study using D1/D2 rDNA sequencing. After performing PCR on D1/D2 domain rDNA, extracted from yeast strains, fragment sizes ranged from 500-612 base pairs for this region (Fig. 1) as expected (Kurtzman & Robnett, 1998). All strains were identified by sequencing this domain and doing a BLAST search on NCBI. The species names are listed in Table 1, with their representative strain numbers, sequence *E*-values, and percentage identity (Pi) (percentage nucleotide difference is indicated in brackets) as well as abriviated isolation locations.

Most of the sequences exhibited sequence identities of 98-100% (i.e. 0-1% substitutions or one to six nucleotide differences) during comparison with sequences available on the NCBI yeast sequence database. Only four strains showed significantly low identities. Three of these strains (UOFS Y-2225, UOFS Y-2262 and UOFS Y-2244) were found to be novel, belonging to *Cryptococcus*, and represented a news species closely related to *Cryptococcus curvatus* CBS 570^T (96-97% sequence identity). These strains came from a group of isolates obtained from cyanide contaminated soils (Table 1, isolation location abbreviated with CCSS). The fourth strain (UOFS Y-1920) came from a group of isolates obtained from soil, in South Africa. This strain showed 99-100% D1/D2 rDNA sequence identity with five undescribed basidiomycete species on GenBank.

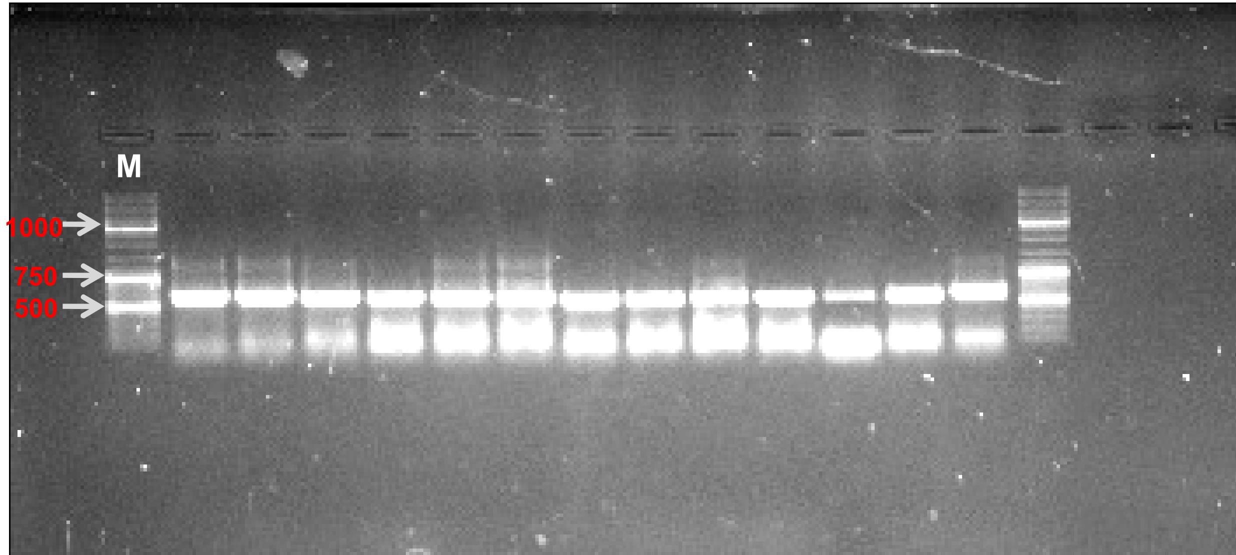

Figure 1: Gel photo (1% agarose) showing the D1/D2 26S rDNA domain located between the 750 and 500 base pair bands of the marker (indicated by M, DNA ladder, O'GeneRuler™ DNA Ladder = 1kb).

Table 1: Yeast isolates as predicted from D1/D2 domain sequence divergence.

Species name	Strain	*E*-values	Pi (% nt)	Location
Ascomycetes				
Candida aaseri	UOFS Y-2121	0.0	100% (0)	SSA
Candida aaseri	UOFS Y-1949	0.0	100% (0)	WDSA
Candida aaseri	UOFS Y-1969	0.0	98% (1)	SAM
Candida blankii	UOFS Y-1771	0.0	100% (0)	BTSA
Candida blankii	UOFS Y-1772	0.0	100% (0)	BTSA
Candida blankii	UOFS Y-1770	0.0	100% (0)	BTSA
Candida blankii	UOFS Y-1929	0.0	99% (1)	GPSA
Candida boidinii	UOFS Y-0719	0.0	99% (1)	SPSA
Candida boidinii	UOFS Y-0654	0.0	99% (1)	STMK
Candida catenulata	UOFS Y-2442	0.0	99% (1)	DBAE
Candida catenulata	UOFS Y-2445	0.0	98% (1)	DBAE
Candida conglobata	UOFS Y-0656	0.0	99% (1)	ESNK
Candida intermedia	UOFS Y-0649	0.0	99% (1)	MCK
Candida michaelii	UOFS Y-0568	0.0	99% (1)	SLK
Candida parapsilosis	UOFS Y-0572	0.0	99% (1)	GIMK
Candida parapsilosis	UOFS Y-0569	0.0	99% (1)	PBNK
Candida parapsilosis	UOFS Y-0645	0.0	100% (0)	SSA

Table 1: Yeast isolates as predicted from D1/D2 domain sequence divergence.

Species name	Strain	*E*-values	Pi (% nt)	Location
Candida pseudolambica	UOFS Y-2827	0.0	100% (0)	UMSA
Candida rugosa	UOFS Y-2435	0.0	100% (0)	HRAE
Candida rugosa	UOFS Y-2229	0.0	99% (1)	CCSS
Candida shehatae var. lignosa	UOFS Y-0264	0.0	99% (1)	DUSA
Candida silvae	UOFS Y-0659	0.0	99% (1)	FHIK
Candida silvae	UOFS Y-0549	0.0	98% (1)	SSA
Candida silvae	UOFS Y-0660	0.0	99% (1)	FHLK
Candida silvae	UOFS Y-1855	0.0	99% (1)	SSA
Candida silvae	UOFS Y-1854	0.0	99% (1)	SSA
Candida silvae	UOFS Y-0648	0.0	98% (1)	SCK
Candida sorbophila	UOFS Y-2372	0.0	98% (1)	CCSS
Candida sorboxylosa	UOFS Y-0634	0.0	98% (1)	LCEK
Candida sorboxylosa	UOFS Y-0636	0.0	99% (1)	CPMK
Candida sorboxylosa	UOFS Y-0630	0.0	99% (1)	FPCK
Candida tropicalis	UOFS Y-2509	0.0	100% (0)	O
Candida tropicalis	UOFSY-2380	0.0	98% (1)	SABG
Candida tropicalis	UOFS Y-2826	0.0	98% (1)	UMSA
Candida viswanathii	UOFS Y-1917	0.0	98% (1)	OSSA

Table 1: Yeast isolates as predicted from D1/D2 domain sequence divergence.

Species name	Strain	*E*-values	Pi (% nt)	Location
Clavispora lusitaniae	UOFS Y-2629	0.0	99% (1)	JFA
Clavispora lusitaniae	UOFS Y-0655	0.0	100% (0)	EAMK
Clavispora lusitaniae	UOFS Y-2625	0.0	99% (1)	PA
Clavispora lusitaniae	UOFSY-2634	0.0	99% (1)	JSA
Clavispora lusitaniae	UOFS Y-2634	0.0	98% (1)	JFA
Clavispora lusitaniae	UOFS Y-2628	0.0	99% (1)	SGSA
Clavispora lusitaniae	UOFS Y-2625	0.0	100% (0)	PASA
Clavispora lusitaniae	UOFS Y-2629	0.0	100% (0)	JSA
Clavispora lusitaniae	UOFS Y-2631	0.0	99% (1)	JSA
Clavispora lusitaniae	UOFS Y-2632	0.0	99% (1)	JSA
Clavispora lusitaniae	UOFS Y-2628	0.0	98% (1)	SGSA
Debaryomyces hansenii	UOFS Y-2438	0.0	100% (0)	HRAE
Debaryomyces hansenii	UOFS Y-2428	0.0	99% (1)	LVAE
Debaryomyces hansenii	UOFS Y-2427	0.0	100% (0)	HCAE
Debaryomyces hansenii	UOFS Y-2452	0.0	100% (0)	HAAE “a.a”
Debaryomyces nepalensis	UOFS Y-2508	0.0	99% (1)	O
Debaryomyces pseudopolymorphus	UOFS Y-0720	0.0	99% (1)	SPSA

Table 1: Yeast isolates as predicted from D1/D2 domain sequence divergence.

Species name	Strain	*E*-values	Pi (% nt)	Location
Exophiala dermatitidis	UOFS Y-2113	0.0	100% (0)	S
Exophiala dermatitidis	UOFS Y-2115	0.0	98% (1)	S
Exophiala dermatitidis	UOFS Y-2116	0.0	100% (0)	SSA
Galactomyces geotrichum	UOFS Y-2462	0.0	99% (1)	HSAE
Hanseniaspora guilliermondii	UOFS Y-2447	0.0	99% (1)	HPAE
Hanseniaspora uvarum	UOFSY-2468	0.0	99% (1)	FMBE
Hanseniaspora uvarum	UOFS Y-658	0.0	98% (1)	RCNK
Hanseniaspora uvarum	UOFS Y-2465	0.0	99% (1)	RSAE
Hanseniaspora uvarum	UOFSY-2522	0.0	99% (1)	PBSA
Hanseniaspora uvarum	UOFS Y-2461	0.0	99% (1)	HSAE
Hanseniaspora uvarum	UOFS Y-2521	0.0	100% (0)	SMSA
Issatchenkia orientalis	UOFSY-2470	0.0	99% (1)	FMBE
Issatchenkia orientalis	UOFS Y-2471	0.0	98% (1)	FMBE
Issatchenkia orientalis	UOFS Y-2408	0.0	99% (1)	JSAE
Issatchenkia orientalis	UOFS Y-2381	0.0	99% (1)	SABG
Issatchenkia orientalis	UOFS Y-2389	0.0	99% (1)	SBAE
Issatchenkia orientalis	UOFS Y-2540	0.0	99% (1)	SBAE
Issatchenkia orientalis	UOFS Y-2541	0.0	98% (1)	SBAE

Table 1: Yeast isolates as predicted from D1/D2 domain sequence divergence.

Species name	Strain	*E*-values	Pi (% nt)	Location
Issatchenkia orientalis	UOFSY-2469	0.0	100% (0)	FMBE
Issatchenkia orientalis	UOFS Y-2492	0.0	100% (0)	HAAE “p.p”
Issatchenkia orientalis	UOFSY-2383	0.0	100% (0)	SBAE
Issatchenkia terricola	UOFS Y-2537	0.0	99% (1)	SSLT
Lipomyces mesembrius	UOFS Y-1779	0.0	100% (0)	SKSA
Lipomyces starkeyi	UOFS Y-2823	0.0	98% (1)	BNP
Lipomyces tetrasporus	UOFS Y-2822	0.0	100% (0)	BNP
Lipomyces tetrasporus	UOFS Y-2813	0.0	100% (0)	BNP
Lipomyces yamadae	UOFS Y-2824	0.0	100% (0)	BNP
Lodderomyces elongisporus	UOFS Y-2398	0.0	100% (0)	SBAE
Lodderomyces elongisporus	UOFS Y-2394	0.0	100% (0)	PWAE
Lodderomyces elongisporus	UOFS Y-2395	0.0	99% (1)	PWAE
Lodderomyces elongisporus	UOFS Y-2394	0.0	99% (1)	PWAE
Lodderomyces elongisporus	UOFS Y-2396	0.0	99% (1)	SABG
Lodderomyces elongisporus	UOFS Y-2401	0.0	98% (1)	SBAE
Lodderomyces elongisporus	UOFS Y-1842	0.0	98% (1)	SPOB
Lodderomyces elongisporus	UOFS Y-1841	0.0	99% (1)	SPOB
Lodderomyces elongisporus	UOFS Y-2402	0.0	99% (1)	HGAE

Table 1: Yeast isolates as predicted from D1/D2 domain sequence divergence.

Species name	**Strain**	***E*-values**	**Pi (% nt)**	**Location**
Myxozyma udenii	UOFS Y-0500	0.0	99% (1)	CBFN
Myxozyma udenii	UOFS Y-0499	0.0	99% (1)	CBFN
Myxozyma vanderwaltii	UOFS Y-2814	0.0	100% (0)	BNP
Myxozyma vanderwaltii	UOFS Y-2815	0.0	100% (0)	BNP
Meyerozyma guilliermondii	UOFS Y-1930	0.0	99% (1)	SSA
Pichia anomala	UOFSY-2344	0.0	99% (1)	BSA
Pichia anomala	UOFS Y-2479	0.0	99% (1)	NABE “b”
Pichia anomala	UOFS Y-2464	0.0	100% (0)	RSAE
Pichia anomala	UOFS Y-2504	0.0	99% (1)	ECGE
Pichia anomala	UOFS Y-1960	0.0	99% (1)	BSA
Pichia anomala	UOFS Y-0657	0.0	99% (1)	MCK
Pichia anomala	UOFS Y-2497	0.0	100% (0)	BZAE
Pichia capsulata	UOFS Y-2512	0.0	100% (0)	PT
Pichia ciferrii	UOFS Y-0687	0.0	100% (0)	FMK
Pichia deserticola	UOFS Y-2504	0.0	99% (1)	ECGE
Pichia fermentans	UOFS Y-2419	0.0	99% (1)	HAAE “p.a”
Pichia galeiformis	UOFS Y-0633	0.0	99% (1)	MKAK
Pichia guilliermondii	UOFS Y-1934	0.0	100% (0)	SSA

Table 1: Yeast isolates as predicted from D1/D2 domain sequence divergence.

Species name	Strain	*E*-values	Pi (% nt)	Location
Pichia guilliermondii	UOFS Y-0638	0.0	99% (1)	PJNK
Pichia guilliermondii	UOFS Y-0623	0.0	99% (1)	FWKK
Pichia guilliermondii	UOFS Y-1933	0.0	100% (0)	S
Pichia guilliermondii	UOFS Y-2399	0.0	99% (1)	SBAE
Pichia guilliermondii	UOFS Y-0640	0.0	99% (1)	FK
Pichia guilliermondii	UOFS Y-2385	0.0	100% (0)	PWAE
Pichia guilliermondii	UOFS Y-2472	0.0	98% (1)	NABE “f”
Pichia guilliermondii	UOFS Y-1936	0.0	99% (1)	SSA
Pichia kluyveri	UOFS Y-0625	0.0	99% (1)	LCEK
Pichia kluyveri	UOFS Y-0577	0.0	100% (0)	KPK
Pichia kluyveri	UOFS Y-0663	0.0	99% (1)	RCNK
Pichia kluyveri	UOFS Y-0625	0.0	99% (1)	LCEK
Pichia kluyveri	UOFS Y-0625	0.0	99% (1)	LCEK
Pichia kudriavzevii	UOFS Y-2457	0.0	98% (1)	BRKE
Pichia kudriavzevii	UOFS Y-0637	0.0	98% (1)	GIMK
Pichia membranifaciens	UOFS Y-0269	0.0	98% (1)	SI
Pichia mexicana	UOFS Y-1916	0.0	100% (0)	PC
Saccharomyces cerevisiae	UOFS Y-2517	0.0	100% (0)	CBAB

Table 1: Yeast isolates as predicted from D1/D2 domain sequence divergence.

Species name	Strain	*E*-values	Pi (% nt)	Location
Saccharomyces cerevisiae	UOFS Y-2484	0.0	100% (0)	PFTE
Saccharomyces cerevisiae	UOFS Y-2488	0.0	100% (0)	HFGE
Saccharomyces uvarum (bayanus)	UOFS Y-0912	0.0	100% (0)	UFCC
Saccharomycopsis vini	UOFS Y-0893	0.0	99% (1)	UFCC
Schizosaccharomyces pombe	UOFS Y-1740	0.0	98% (1)	Dr. O. Augustyn
Schwanniomyces polymorphus	UOFS Y-1966	0.0	99% (1)	SESA
Torulaspora delbrueckii	UOFS Y-2414	0.0	99% (1)	RMAE
Torulaspora delbrueckii	UOFS Y-2412	0.0	99% (1)	ROAE
Yarrowia lipolytica	UOFS Y-2828	0.0	99% (1)	UMSA
Yarrowia lipolytica	UOFS Y-2829	0.0	98% (1)	UMSA
Yarrowia lipolytica	UOFS Y-2830	0.0	100% (0)	UMSA
Zygosaccharomyces bailii	UOFSY-2327	0.0	99% (1)	EWSA
Zygosaccharomyces rouxii	UOFS Y-0763	0.0	98% (1)	UFCC
Zygosaccharomyces rouxii	UOFS Y-0688	0.0	99% (1)	HJB
Basidiomycetes				
Cryptococcus albidosimilis	UOFS Y-2604	0.0	98% (1)	D
Cryptococcus albidosimilis	UOFS Y-2605	0.0	99% (1)	D

Table 1: Yeast isolates as predicted from D1/D2 domain sequence divergence.

Species name	Strain	*E*-values	Pi (% nt)	Location
Cryptococcus albidosimilis	UOFS Y-2616	0.0	100% (0)	TGDF
Cryptococcus albidus	UOFS Y-1843	0.0	99% (1)	SPOB
Cryptococcus curvatus	UOFS Y-2254	0.0	99% (1)	CCSS
Cryptococcus curvatus	UOFS Y-2263	0.0	99% (1)	CCSS
Cryptococcus flavescens	UOFS Y-2606	0.0	99% (1)	DH
Cryptococcus flavescens	UOFS Y-2665	0.0	99% (1)	CT
Cryptococcus flavescens	UOFS Y-2663	0.0	99% (1)	CNB
Cryptococcus flavescens	UOFS Y-2421	0.0	99% (1)	RMAE
Cryptococcus flavescens	UOFS Y-2655	0.0	99% (1)	CTB
Cryptococcus macerans	UOFS Y-2543	0.0	99% (1)	Prof. B. Viljoen
Cryptococcus saitoi	UOFS Y-2627	0.0	99% (1)	SGSA
***Cryptococcus* sp. nov.**	UOFS Y-2262	0.0	97% (3)	CCSS
***Cryptococcus* sp. nov.**	UOFS Y-2225	0.0	97% (3)	CCSS
***Cryptococcus* sp. nov.**	UOFS Y-2244	0.0	97% (3)	CCSS
Cryptococcus terreus	UOFS Y-0516	0.0	99% (1)	SVSA
Cryptococcus wieringae	UOFS Y-2633	0.0	99% (1)	JSA
Cryptococcus wieringae	UOFS Y-2636	0.0	98% (1)	JSA
Cryptococcus wieringae	UOFS Y-2633	0.0	99% (1)	JFA

Table 1: Yeast isolates as predicted from D1/D2 domain sequence divergence.

Species name	Strain	*E*-values	Pi (% nt)	Location
Rhodosporidium kratochvilovae	UOFS Y-2529	0.0	99% (1)	WAGP
Rhodosporidium toruloides	UOFS Y-2253	0.0	99% (1)	CCSS
Rhodosporidium toruloides	UOFS Y-2245	0.0	100% (0)	CCSS
Rhodosporidium toruloides	UOFS Y-2233	0.0	99% (1)	CCSS
Rhodosporidium toruloides	UOFS Y-2257	0.0	99% (1)	CCSS
Rhodosporidium toruloides	UOFS Y-2265	0.0	99% (1)	CCSS
Rhodosporidium toruloides	UOFS Y-2234	0.0	100% (0)	CCSS
Rhodosporidium toruloides	UOFS Y-2373	0.0	99% (1)	CCSS
Rhodosporidium toruloides	UOFS Y-2261	0.0	99% (1)	CCSS
Rhodosporidium toruloides	UOFS Y-2247	0.0	98% (1)	CCSS
Rhodosporidium toruloides	UOFS Y-2255	0.0	99% (1)	CCSS
Rhodosporidium toruloides	UOFS Y-2231	0.0	99% (1)	CCSS
Rhodosporidium toruloides	UOFS Y-2370	0.0	99% (1)	CCSS
Rhodosporidium toruloides	UOFS Y-2371	0.0	98% (1)	CCSS
Rhodosporidium toruloides	UOFS Y-2237	0.0	99% (1)	CCSS
Rhodotorula glutinis	UOFS Y-0629	0.0	99% (1)	FWKK
Rhodotorula mucilaginosa	UOFS Y-2603	0.0	100% (0)	D
Rhodotorula mucilaginosa	UOFSY-2550	0.0	99% (1)	BMG

Table 1: Yeast isolates as predicted from D1/D2 domain sequence divergence.

Species name	Strain	*E*-values	Pi (% nt)	Location
Rhodotorula mucilaginosa	UOFS Y-2219	0.0	98% (1)	DASA
Rhodotorula mucilaginosa	UOFS Y-2603	0.0	99% (1)	D
Rhodotorula mucilaginosa	UOFS Y-0634	0.0	100% (0)	FNK
Rhodotorula mucilaginosa	UOFS Y-0662	0.0	98% (1)	KPK
Rhodotorula mucilaginosa	UOFS Y-0657	0.0	99% (1)	MCK
Rhodotorula mucilaginosa	UOFS Y-0565	0.0	98% (1)	SIMK
Rhodotorula mucilaginosa	UOFS Y-0664	0.0	98% (1)	SCK
Rhodotorula mucilaginosa	UOFS Y-2466	0.0	98% (1)	TBAE
Trichosporon asahii	UOFS Y-2518	0.0	99% (1)	Prof Smit
Trichosporon asahii	UOFS Y-0571	0.0	99% (1)	CIOM
Trichosporon asahii	UOFS Y-2664	0.0	100% (0)	CBB
Trichosporon asahii	UOFS Y-0647	0.0	99% (1)	FMK
Trichosporon asahii	UOFS Y-1202	0.0	99% (1)	SE
Trichosporon asahii	UOFS Y-1947	0.0	98% (1)	SE
Trichosporon asahii	UOFS Y-2273	0.0	99% (1)	SE
Trichosporon coremiiforme	UOFS Y-0639	0.0	98% (1)	SANK
Trichosporon coremiiforme	UOFS Y-0644	0.0	99% (1)	UK
Trichosporon montevideense	UOFS Y-2266	0.0	99% (1)	CCSS

Table 1: Yeast isolates as predicted from D1/D2 domain sequence divergence.

Species name	**Strain**	***E*-values**	**Pi (% nt)**	**Location**
Trichosporon mycotoxinivorans	UOFS Y-0689	0.0	99% (1)	SD
***Trichosporon* sp. nov.**	UOFS Y-1920	0.0	100% (0)	SSA
Ustilago triodiae	UOFSY-0117	0.0	98% (1)	SSA

Legend:

***E*-value** – reliability measure of similarity of sequences with significant aligment (query and subject)

Pi – Percentage identity, **nt** – percentage nucleotide difference

Key to isolation locations

BMG - Biofilm from Merriespruit goldmine, South Africa, site no MS149160704; **BNP**- Banks of the Napo River, Ecuador; **BRKE** - *Beta vulgaris* (suge beet) rhizo plane, Kafr-Elshekh, Egypt; **BSA** - Banana, South Africa; **BTSA** - Bread tree, South Africa; **BZAE** - Baker's yeast from domestic bread, El- Zawia Village, Assuit, Egypt; **CBAB** - Coopers Brewery, Australia. Brewer's yeast for bitter draught; **CBB** - Conifer bark, Bloemfontein, South Africa; **CBFN** - Compost soil, Bloemfontein, South Africa; **CCSS** - Cyanide contaminated soil, Sasolburg, South Africa; **CIOM** - Clean sea sand, shores of Indian Ocean, Mombasa, Kenya; **CNB** - Conifer needles, Bloemfontein, South Africa; **CPMK** - Crocodile pond, Mombasa, Kenya; **CT** - Conifer tree, South Africa; **CTB** - Conifer tree, Bloemfontein, South Africa; **DASA** - Dung of aardvark, South Africa; **DBAE** - Healthy *Musa nana* (dwarf banana) fruit, Assiut, Egypt; **DF** - Drakensberg, flower, South Africa; **D** – Drakensberg, South Africa; **Dr. O. Augustyn** - Received from Dr. O. Augustyn; **DUSA** - *Drosophila pseudobscura* (fruit fly), USA; **EAMK** - Exudates from *Acacia* sp., Mombosa, Kenya; **ECGE** - El-Hwamdia sugar company, Giza, Egypt; **ESNK** - Exudate, *Schinus* sp, Nairobi, Kenya; **EWSA** - "Esprit, kiwi" wine, SA; **FHIK** - Flower of *Hymenocallis litovalis*, Kenya; **FHLK** - Flower, *Hymenocallis litovalsi*, Kenya; **FK** - Flower, Kenya; **FMBE** - "Fayroz" non-alcoholic malt beverage, Egypt; **FMK** - Finger millet, Kenya; **FNK** - Fruit, Nairobi, Kenya; **FPCK** - Fish pond, Chiromo, Kenya; **FWKK** - Fresh river water, Kirinyaga, Kenya; **GIMK** - Grass inflorescence, Diani Beach, Mombasa, Kenya; **GPSA** - Garden soil, Pretoria, South Africa; **HAAE "p.m"** - Healthy *Pyrus malus* (apple) Fruit, Assiut, Egypt; **HAAE"p.p"**- Healthy *Prunus persica* (peach) Fruit, Assiut, Egypt; **HAAE "a.a"** - Healthy *Ananas amosus* (pine apple), Assuit, Egypt;

HCAE - Healthy *Citrus limon* (lemon), Assuit Egypt; **HFGE** - Sugar cane molasses, El - Hwamdia sugar factory, Giza, Egypt; **HGAE** - Healthy *Vitus vinifera* (grape) berry, Assiut, Egypt; **HJB** - Home made fig jam, Bloemfontein, South Africa; **HPAE** - Healty *Psidium guajava* (guava) fruit, Assiut, Egypt; **HRAE** - Healthy and rotten *Vitus vinifera* (grape) leaves, Assiut, Egypt; **HSAE** - Healthy *Fragaria vesca* (strawberry) fruit, Assiut, Egypt; **JFA**- Juice from *Agave* sp., South Africa; **JSA** - Juice pressed from *Agave* sp. leaf, South Africa; **JSAE** - Juice from *Saccharum officinarium* (Sugar cane), public drink Assuit, Egypt; **KPK** - Kitchen table, primary school, Kenya; **LCEK** - Leaves of *Catha edulis* (Miraa), Kenya; **LVAE** - Leaves of *Vitus vinifera* (grape), Assiut, Egypt; **MCK** - Maize cernels, Kenya; **MKAK** - *Kigelia africana (*Muratina fruit), Kenya; **NABE "b"** - Non-alcoholic beverage "Birell", Egypt; **NABE "f"** - Non-alcoholoc beverage "Fayroz", Egypt; **O** - Orange; **OSSA** - Oil sample, South Africa; **PA** - Pulp from *Agave* sp., South Africa; **PASA** - Pulp from *Agave* sp. leaf, South Africa; **PBNK** - Peanut butter, Tru factory, Nairobi, Kenya; **PBSA** - Podzolic soil, Bettiesbaai, South Africa; **PC** – Prof. Prior collection; **PFTE** - Public fermented drink "Boza" made from barley bread, Tanta, Egypt; **PJNK** - Passion fruit juice, Tru-Fruit, factory, Nairobi, Kenya; **Prof. Smit** - Received from Prof. M. Smith; **Prof. B. Viljoen** - Received from Prof. B. Viljoen; **PT** - Pine tree; **PWAE** - "Prandy" wine Assiut, Egypt; **RCNK** - Ripe coffee berries, Nairobi, Kenya; **RMAE** - Rotten *Citrus reticulate* (mandarin) fruit, Assuit, Egypt; **ROAE** - Rotten *Citrus sinensis* (orange) fruit, Assuit, Egypt; **RSAE** - Rotten *Fragaria vesca* (strawberry) fruit, Assiut, Egypt; **S** - Soil; **SABG** - "Sakara" beer, Assiut, Egypt; **SAM** – Soil, Amazone; **SANK** - Soil, Arboretum, Nairobi, Kenya; **SVSA** - Soil, vineyard, Stellenbosch, South Africa; **SD** - Soil, Drakensberg, South Africa; **SBAE** - "Stela" beer,

Assiut, Egypt; **SCK** - Sorghum cernels, Kenya; **SESA** - Soil, St Lucia estuary, South Africa; **SGSA** - Surface of *Agave* sp. leaf, South Africa; **SI** - Saiccor; **SIMK** - Sea water, Indian Ocean, Mombasa, Kenya; **SKSA** - Soil, Knysna, South Africa; **SSLT** - Soil sample under lemon tree, Bloemfontein, South Africa; **SLK** - Seed of legume, Kenya; **SMSA** - Soil, Mauchsberg, South Africa; **SPOB** - Surface of oil painting, Oliewenhuis, Bloemfontein; **SPSA** - Soil, Pretoria, South Africa; **SSA** - soil, South Africa; **SE** - SASOL effluent, South Africa; **STMK** - Shell of giant tortoise, Mombasa, Kenya; **TBAE** - Tinned beef "Lanshon" Assiut, Egypt; **TGDF** - Tugela Gorge, Drakensberg, South Africa; **UFCC** - Unknown, From CSIR collection; **UK** - Unknown, Kenya; **UMSA** - Umgeni river mouth, South Africa; **WAGP** - Wet droppings of caged African Grey parrot, Bloemfontein, South Africa; **WDSA** - Water from dam, South Africa.

3.2 Biotechnological applications and importance of yeasts

Strains identified in this study represent species with potential biotechnological roles in the industrial and clinical aspects and to an extent, in environmental and agricultural aspects. For instance, *Pichia guilliermondii* (represented by a number of strains in this study, e.g. UOFS Y-1934) as well as *Candida famata* (teleomorph: *Debaryomyces hansenii*) are classified as “flavinogenic” yeasts because they are used as model organisms in the production of riboflavin (vitamin B_2) (Sibirny & Boretsky, 2009; Sibirny & Voronovsky, 2009). On the other hand, strains of *P. guilliermondii* are also implicated in the production of xylitol from xylose which in turn has some clinical implications in dental caries (Sampaio *et al*., 2006; Bader *et al*., 2010). Furthermore, strains of *Candida shehatae* (represented by strain UOFS Y-0264) were initially found to convert xylose to

ethanol during fermentation process by du Preez and van der Walt (1983) and du Preez and co-workers (1984). Xylose forms part of the lignocellulosic matrix which, after pretreatment steps, requires organisms such as *C. shehatae* and others such as *Pichia stipitis* and *Pachysolen tannophilus* to ferment monosaccharides produced during this processes (Sun & Tao, 2010).

Lipomyces species on the other hand are known for their potential to accumulate lipids when cultivated on synthetic medium. However, studies have exploited wide industrial and environmental applications for these species, especially strains of *L. starkeyi* (represented by strain UOFS Y-2823) (Nishimura *et al*., 2002). Some of the important applications exploited from these strains include possible production of biodiesel from lipids accumulated from sewage sludge and potato starch (Angerbauer *et al*., 2008; Wild *et al*., 2010).

Yeasts are also used to control post harvest diseases in agriculture. Antagonistic yeasts associated with flower and fruit habitats (e.g. *Candida oleophila*) have the potential to compete for nutrients and space with other pathogens (Pimenta *et al*., 2009). For instance, *Penicillium expansum* (blue mould) infection in apples is reduced by competition for compounds such as L-proline between this pathogen, *Candida membranifaciens* and *Cryptococcus laurentii*. Most fruit pathogens such as species of *Alternaria*, *Botrytis*, *Penicillium* and *Rhizopus* are antagonized by *Candida* and *Pichia species*. Furthermore, control of *Mucor* (e.g. *M. piriformis*) in fruits such as pears is managed by basidiomycetes such as *Cryptococcus laurentii*, *C. flavus* and *C. albidus*

(latter species is represented by strain UOFS Y-1843) (Pimenta *et al*., 2009). Yeasts are therefore largely used for pre- and post-harvest control of pathogens in the production of fruits. Yeasts such as *Williopsis mrakii* have been reported to produce mycocins which have the potential to reduce spoilage in crops and yoghurt (Lowes *et al*., 2000). However yeast-like ascomycetes have been reported to cause diseases in agricultural crops.

Molnar and co-workers (2004) described *Trichosporon mycotoxinivorans* (represented by strain UOFS Y-0689 in this study) which was implicated in the detoxification of food-contaminating toxins such as ochratoxin A, produced by *Aspergillus ochraceus* and *Penicillium verrucosum*. However, Hickey and co-workers (2009) recently reported that this species also plays a role as a respiratory pathogen in patients with cystic fibrosis. *Exophiala dermatitidis*, (represented by strains UOFS Y-2113, UOFS Y-2115 and UOFS Y-2116) is a black ascomycete yeast-like fungus which is reported to produce fatal infections in the brain (Sudhadham *et al*., 2008). This fungus has been recovered from air passages of individuals with non-cystic fibrosis bronchiectasis (bronchial dilation) and from patients with endocarditis (inflammation of the endocardium) (Mukaino *et al*., 2006; Ventin *et al*., 1987). *Exophiala* species or simply, the black yeasts (e.g. *E. jeanselmei* and *E. xenobiotica*), are mostly of clinical concern due to roles they play in fungal infections such as cutaneous and sub-cutaneous infections or mycetoma (Eumycetoma or Actinomycosis) (de Hoog *et al*., 2006).

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CHAPTER 3

Cryptococcus cyanovorans sp. nov., basidiomyceteous yeast isolated from cyanide contaminated soil

Chapter is submitted for publication in the International Journal of Systematic and Evolutionary Microbiology. The work presented here is written according to standards of this journal

Summary

Eighteen yeast strains were isolated and identified from cyanide contaminated soil in South Africa. According to sequence-based analyses using D1/D2 region of the large ribosomal subunit and ITS regions, three of these strains were identical and belonged to the genus *Cryptococcus*. These strains showed approximately 3% substitutions in these regions, suggesting that they were members of an undescribed species in this genus. They clustered with *Cryptococcus curvatus* in the combined data set of the D1/D2 and ITS regions, representing a defined clade in the order Trichosporonales. The name Curvatus is proposed for this clade. The three *Cryptococcus* strains are demarcated from *Cryptococcus curvatus* by standard physiological tests such as assimilation of lactose, xylitol, 5-keto-D-gluconate, succinate and citrate as well as growth on media containing 10% NaCl and 5 % glucose. In addition, it was established that these strains could utilize up to 10 mM NaCN as sole carbon source on solid media and as sole nitrogen source in liquid media. On the basis of these findings, it is suggested that the three strains represent a new species for which the name *Cryptococcus cyanovorans* sp. nov. is proposed.

1. Introduction

Isolation of yeasts from industrial waste waters and related habitats such as contaminated soils has become the subject of research due to economic and public impacts arising from these sites (Boening & Chew, 1999; Kjeldsen, 1999). Industrial processes such as gold and silver extraction through cyanidation contribute towards the production of a number of toxic cyanide compounds which affect living organisms, causing serious environmental and health concerns (Atkinson *et al*., 1998; Rachinger *et al*., 2002; Pham *et al*., 2007). Previous efforts to combat contamination by cyanides were based on the use of chemicals such as ferrous sulphate (Adams, 1992). Currently, the use of microorganisms to transform or mineralize toxic pollutants has gained prominence as a relevant substitute to reduce toxicity of polluting cyanides (Boopathy, 2000).

Bioremediation of cyanides is mainly reported in studies focusing on the use of algae, fungi and bacteria (Dumestre *et al*., 1997; Barclay *et al*., 1998; Akcil, 2003; Ezzi & Lynch., 2005; Gurbuza *et al*., 2009). Although studies have reported yeast strains that can bioremediate heavy metals including chromium, copper, zinc and nickel (e.g. *Pichia guilliermondii* and *Saccharomyces cerevisiae*) (Machado *et al*., 2008) as well as organic cyanides such as benzonitrile (*Cryptococcus* sp. UFMSY-28) and phenylacetonitrile (e.g. *Exophiala oligosperma*) (Rezende *et al*., 2000; Rustler & Stolz, 2007), only one strain, *Cryptococcus humicola* MCN2 is reported to biodegrade inorganic cyanide compounds (Kwon *et al*., 2002).

During the study of unidentified yeast isolates deposited in the UNESCO-MIRCEN Biotechnological Yeast Culture Collection housed at the Department of Microbial, Biochemical and Food Biotechnology of the University of the Free State, a group of isolates obtained from cyanide contaminated soil from Sasolburg, South Africa were identified. Three of these strains were found to belonging to a previously undescribed species in the genus *Cryptococcus*. The name *Cryptococcus cyanovorans* sp. nov. is proposed.

2. Materials and methods

Isolation of DNA was performed on yeast cells grown for approximately 24 hours at 30 $^{\circ}$C in 50 ml yeast malt extract agar (3 g yeast extract, 3 g malt extract, 5 g peptone, and 10 g glucose dissolved in a litre of distilled water). Isolation of DNA was achieved either by genomic DNA extraction (Labuschagne & Albertyn, 2007) or by boiling cells suspended in 20 µl distilled water for 10 min at 96 ^{0}C. Polymerase chain reaction (PCR) was performed for the 26S rDNA D1/D2 and ITS regions using fungal primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5' GGTCCGTGTTTCAAGACGG-3'), ITS-4 (5'-TCCTCCGCTTATTGATATGS-3') and ITS-5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') respectively (White *et al*., 1990; Kurtzman & Robnett, 1998). Sequence pairs for both rDNA and ITS were obtained with the ABI BigDye Terminator Cycle sequencing kit (Applied Biosystems®) and resulting sequences were analysed using GenePro 4.8 (Drummond *et al*., 2009) and compared with sequences on GenBank (httpwww.ncbi.nlm.nih.gov). Sequences were aligned

using CLUSTAL_X version 2.0 (Larkin *et al*., 2007). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 4 (Tamura *et al*., 2007) using the neighbour-joining method with the Kimura two parameter distance measure. Confidence values were estimated from bootstrap analysis of 1000 replicates.

Morphological and physiological properties of strains were determined according to standard methods (van der Walt & Yarrow, 1984; Yarrow, 1998). The ability of these strains to grow in the presence of NaCN as sole nitrogen source was assessed as follows. Cells from strains were starved by cultivation in yeast nitrogen base without ammonium sulphate and amino acids (1.7g l^{-1} YNB and 10g l^{-1} glucose) following the procedure explained by Muller and co-workers (2007). After this period, cells were tested for nitrogen source assimilation using minimal media containing 10mM NaCN (1.72g K_2HPO4, 1.36g KH_2PO4 and 0.12g $MgCl_2$ in 100 ml distilled water). Cells were incubated for 120 hours at 30 ^{0}C. The ability of strains to assimilate NaCN as sole carbon source was assessed as follows. Pre-culture was inoculated in complete YNB media (6.7g l^{-1}) containing 10mM NaCN and incubated at 30 ^{0}C for 120 hours. Solid media was prepared by adding 16g l^{-1} of agar to complete media. The pre-cultured cells were streaked out onto this media and incubated at 30 ^{0}C for seven days.

3. Results and discussion

3.1 Identification of yeasts from cyanide contaminated soil

Eighteen previously unidentified strains, isolated from cyanide contaminated soil and held in the UNESCO-MIRCEN Biotechnological Yeast Culture Collection, University of the Free State, were identified during this study (Table 1). The ascomycetous yeasts identified were *Candida rugosa* and *Candida sorbophila*. The known basidiomycetous yeasts species were *Cryptococcus curvatus*, *Rhodosporidium toruloides* and *Trichosporon montevideense*. Three strains were found to represent a previously undescribed species in the genus *Cryptococcus*.

3.2 Phylogenetic placement and species description

The basidiomyceteous genus, *Cryptococcus* Vuillemin is polyphyletic and comprises more than 50 anamorphic species distributed in the class Hymenomycetes, orders Cystofilobasidiales, Filobasidiales, Tremellales and Trichosporonales (Fell *et al*., 2000; Scorzetti *et al*., 2002; Golubev *et al*., 2006). Because of their polyphyletic nature, proper phylogenetic positioning is often difficult and requires comprehensive and thorough analyses of sequence data (Nakase *et al*., 1993; Fell *et al*., 2000). In addition, phylogenetic groups within orders such as the Trichosporonales are frequently being re-described based on inclusion of previously unknown species that may represent unrecognized sub-groups. The latter order is described in literature based on six sub-groups i.e. Cutaneum, Formosensis, Gracile/Brassicae, Humicola, Ovoides and Porosum (Takashima *et al*., 2001; Scorzetti *et al*., 2002; Middlehoven *et al*., 2004,

Takashima *et al*., 2009; Pagnocca *et al*., 2010). The Humicola sub-group is reported the only group in the Trichosporonales comprising only members of *Cryptococcus* (i.e. *C. curvatus*, *C. humicola*, *C. longus*, *C. musci, C. ramirezgomezianus* and *C. pseudolongus*) while other members of this genus in this lineage, such as *Cryptococcus curvatus*, *Cryptococcus daszewskae* and *Cryptococcus fragicola*, are poorly supported (Takashima *et al*., 2001; Okoli *et al*., 2007; Takashima *et al*., 2009). The current paper proposes the description of *Cryptococcus cyanovorans* (Type strain CBS11948^T, additional strains 11949^T and CBS 1150^T) which represent another sub-group, comprising only members of *Cryptococcus*, in the order Trichosporonales. This sub-group includes *Cryptococcus curvatus* as the first representative thus we propose the name Curvatus for this sub-group. Curvatus is supported by a bootstrap value of 100% based on the combined data set of the 26S rDNA D1/D2 domain and the ITS sequences (Fig. 1).

Physiological and biochemical studies demonstrate that the three *Cryptococcus* species fit the current description of this genus as observed by the positive reactions for Diazonium Blue B salt and urease activity (Fell & Statzell-Tallman, 1998). In addition, these species are able to utilize D-glucuronate and also lack fermentative ability. Growth studies on cyanide revealed that *Cryptococcus cyanovorans* is able to assimilate up to 10 mM NaCN as sole carbon and nitrogen sources. This species is distinct from from *Cryptococcus curvatus* as indicated in Table 2.

3.3 Latin diagnosis of *Cryptococcus cyanovorans* Motaung, Albertyn, J. L. F. Kock et Pohl sp. nov.

In medio liquid extracto fermenti confecto post dies tres ad 30 °C, cellulae globosae ad cylindricae (1.2-7.2 µm x 1.2-8.8 µm). Cellulae polariter gemmantes, clusterae et binae coharentes. In agaro extracto fermenti et extracto malti confecto, post dies tres ad 25 °C, cultura glabra, cremea, margine integra ad fimbriata. In agaro Dalmau nec pseudohyphae nec hyphae formantur. Non fermentat. D-glucosum, D-galactosum, N-acetyl-glucosaminum, ribosum, xylosum, L-arabinosum (variabile), sucrosum, maltosum, α,α-trehalosum, α-methyl glucosidum, cellobiosum, salicinum, arbutinum, raffinosum (variable), melezitosum, glycerolum, erythritolum (variabile), ribitolum (variabile), glucitolum (variabile), *myo*-inositolum, gluconatum, D-glucuronatum, lactatum (variabile), malatum, propan-1,2-diolum (variabile), butan-2,3-diolum (variabile) et cyanidum assimulantur, neque sorbosum, rhamnosum, lactosum, inulinum, amylum solubile, xylitolum, arabitolum, mannitolum, dulcitolum, 2-keto-D-gluconatum, 5-keto-D-gluconatum, succinatum, citratum, ethanolum, methanolum, saccharatum, hexadecanum. Nitratum (variabile), nitritum (variabile), ethylaminum, L-lysinum (variabile), cadaverinum (variabile) imidazolum (variabile) et cyanidum assimulantur. Ureum finditur. Vitaminum externum crescentiae non necessarium. Materia amyloidea formatur (variabile). In medio 0.01% cyclohexamidum crescit (variabile). Crescit in medio 1% aceticum acidum et 10% NaCl/5% glucosum neque in medio 50% glucosum contente. Gelatinum non liquescit. Crescit ad 37 °C. Typus praeservatus in collectione zymotica Centraalbureau voor Schimmelcultures, Utrecht, Neerlandia (holotypus lyophilizatus CBS 11948^{T}) et in collectione zymotica Agricultural

Research Services United States Department of Agriculture, Peoris, Illinois (isotypus NRRL Y-48730[T]).

3.4 Description of *Cryptococcus cyanovorans* Motaung, Albertyn, J. L. F. Kock & Pohl sp. nov.

Cryptococcus cyanovorans (cy.a.no.vo.rans. N.L. adj. *cyanovorans* referring to the ability of this strain to assimilate cyanide)

Cryptococcus cyanovorans (cy.a'no.vo'rans. N.L. adj. *cyanovorans* referring to the ability of this strain to assimilate cyanide)

In yeast extract peptone medium after 3 days at 30 °C, cells are globose to elongate (1.2-7.2 µm x 1.2-8.8 µm). Mono- and bipolar budding is observed and cells occur in clusters or pairs (Fig. 2). In yeast malt extract (YM) agar after 3 days at 25 °C colonies are smooth, cream coloured with entire or fringed margins. In Dalmau plate cultures on cornmeal agar, pseudo or true mycelia are not formed. No fermentation of carbon compounds was observed. The carbon and nitrogen source assimilation profiles are given in Table 3. Urea is hydrolysed and vitamins are not required for growth. Starch production and growth in the presence of 0.01% cyclohexamide is variable. Growth is observed on 1% acetic acid and 10% NaCl plus 5% glucose but not on 50% glucose. Gelatine is not liquefied. Growth is observed at 37 °C. The type strain [CBS 11948[T] (=NRRL Y-48730[T])] as well as two additional strains [CBS 11949 (=NRRL Y-48728); CBS 11950 (=NRRL Y-48729), are deposited in the yeast culture collection of the

Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, with a copies at the yeast culture collection at the Agricultural Research Services United States Department of Agriculture, Peoria, Illinois.

4. Acknowledgements

Authors would like to acknowledge Mrs Andri van Wyk for providing the strains used in the study, Professor Peter van Wyk (Centre for Confocal and Electron Microscopy, University of the Free State) for his assistance during microscopic experiments, Ms Keneuwe Selefo for conducting cyanide experiments. This work is based upon research supported by the National Research Foundation and Department of Science and Technology under the SABI Programme. Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors and therefore the NRF and DST do not accept any liability in regard thereto.

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Table 1: Strains identified from cyanide contaminated soil.

Species	Strains
Ascomycetes	
Candida rugosa	UOFS Y-2229
Candida sorbophila	UOFS Y-2372
Basidiomycetes	
Cryptococcus curvatus	UOFS Y-2254
Cryptococcus curvatus	UOFS Y-2263
Cryptococcus cyanovorans sp. nov.	CBS 11948^T (NRRL Y-48730^T)
Cryptococcus cyanovorans sp. nov.	CBS 11949 (NRRL Y-48728)
Cryptococcus cyanovorans sp. nov.	CBS 11950 (NRRL Y-48729)
Rhodosporidium toruloides	UOFS Y-2233
Rhodosporidium toruloides	UOFS Y-2257
Rhodosporidium toruloides	UOFS Y-2265
Rhodosporidium toruloides	UOFS Y-2234
Rhodosporidium toruloides	UOFS Y-2373
Rhodosporidium toruloides	UOFS Y-2261
Rhodosporidium toruloides	UOFS Y-2247
Rhodosporidium toruloides	UOFS Y-2255
Rhodosporidium toruloides	UOFS Y-2231
Rhodosporidium toruloides	UOFS Y-2370
Trichosporon montevideense	UOFS Y-2266

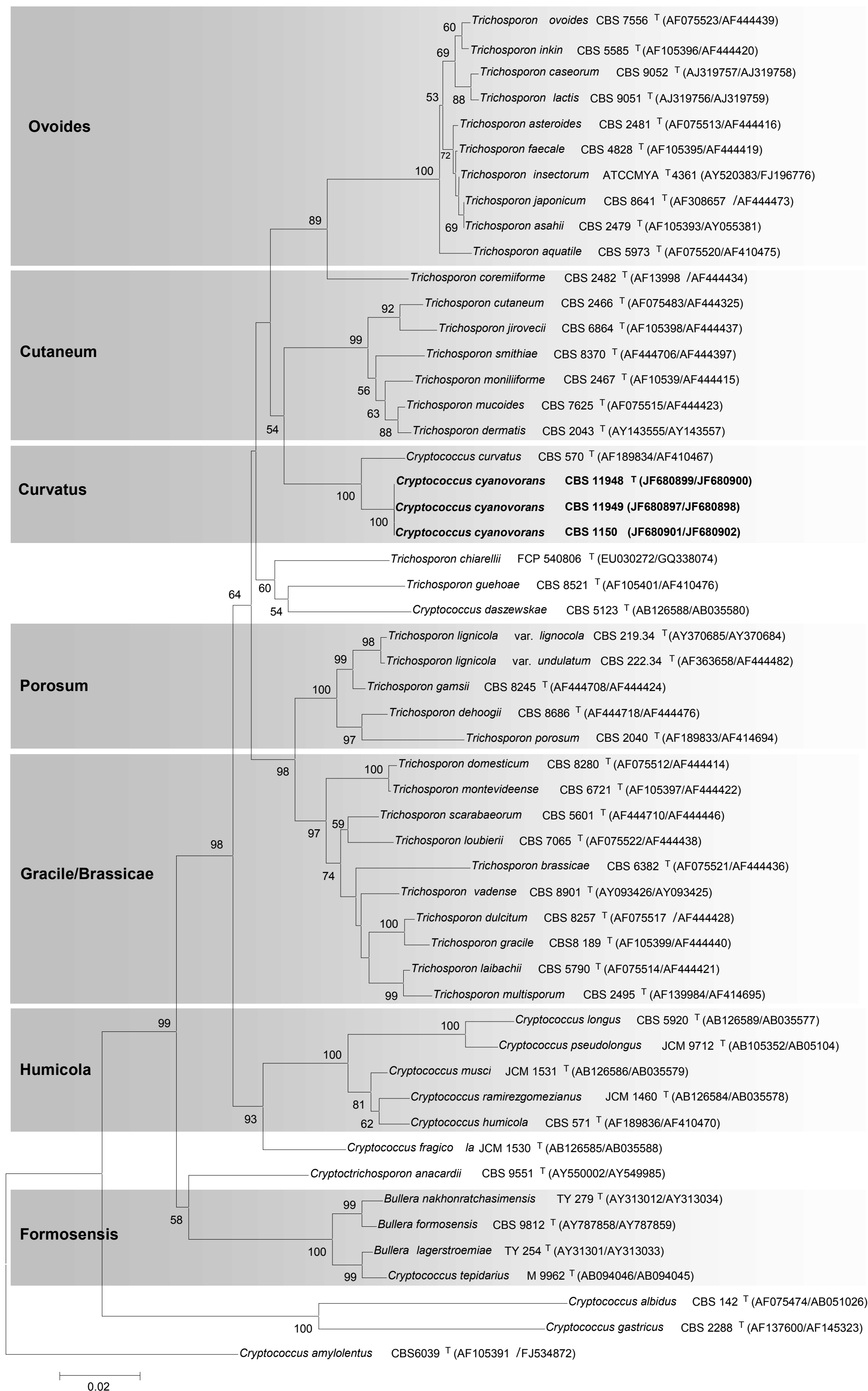
UOFS: Strains are maintained in the UNESCO-MIRCEN Biotechnology Yeast Culture Collection, University of the Free-State.

Table 2. Distinguishing characteristics of *Cryptococcus cyanovorans* and *Cryptococcus curvatus* (taken from Boekhout *et al.,* 2011)

	C. cyanovorans	***C. curvatus***
Assimilation test		
Carbon source		
Lactose	-	+
Xylitol	-	+
5-keto-D-Gluconate	-	+
Succinate	-	+
Citrate	-	+
Additional tests		
10% NaCl/ 5% D-Glucose	+	-

Table 3: Assimilation of carbon and nitrogen sources by *Cryptococcus cyanovorans.* Characteristic are scored as: + positive; - negative; V variable

Carbon source			
D-Glucose	+	Dulcitol	-
D-Galactose	+	*myo*-Inositol	+
Sorbose	-	2-keto-D-Gluconate	-
N-acetyl-Glucosamine	+	5-keto-D-Gluconate	-
Ribose	+	Gluconate	+
Xylose	+	D-Glucuronate	+
L-Arabinose	V	Lactate	V
Rhamnose	-	Succinate	-
Sucrose	+	Citrate	-
Maltose	+	Malate	+
α,α-Trehalose	+	Ethanol	-
α-methyl-Glucoside	+	Methanol	-
Cellobiose	+	Propane-1,2-diol	V
Salicin	+	Butane-2,3-diol	V
Arbutin	+	Saccharate	-
Lactose	-	Hexadecane	-
Raffinose	V	Cyanide	+
Melezitose	+		
Inulin	-	**Nitrogen source**	
Starch	-	Nitrate	V
Glycerol	+	Nitrite	V
Erythritol	V	Ethylamine	+
Ribitol	V	L-Lysine	V
Xylitol	-	Cadaverine	V
Arabitol	-	Creatinine	V
Glucitol	V	Imidazole	V
Mannitol	-	Cyanide	+



Ovoides
Trichosporon ovoides CBS 7556 T (AF075523/AF444439)
Trichosporon inkin CBS 5585 T (AF105396/AF444420)
Trichosporon caseorum CBS 9052 T (AJ319757/AJ319758)
Trichosporon lactis CBS 9051 T (AJ319756/AJ319759)
Trichosporon asteroides CBS 2481 T (AF075513/AF444416)
Trichosporon faecale CBS 4828 T (AF105395/AF444419)
Trichosporon insectorum ATCCMYA T4361 (AY520383/FJ196776)
Trichosporon japonicum CBS 8641 T (AF308657 /AF444473)
Trichosporon asahii CBS 2479 T (AF105393/AY055381)
Trichosporon aquatile CBS 5973 T (AF075520/AF410475)
Trichosporon coremiiforme CBS 2482 T (AF13998 /AF444434)
Cutaneum
Trichosporon cutaneum CBS 2466 T (AF075483/AF444325)
Trichosporon jirovecii CBS 6864 T (AF105398/AF444437)
Trichosporon smithiae CBS 8370 T (AF444706/AF444397)
Trichosporon moniliiforme CBS 2467 T (AF10539/AF444415)
Trichosporon mucoides CBS 7625 T (AF075515/AF444423)
Trichosporon dermatis CBS 2043 T (AY143555/AY143557)
Curvatus
Cryptococcus curvatus CBS 570 T (AF189834/AF410467)
Cryptococcus cyanovorans CBS 11948 T (JF680899/JF680900)
Cryptococcus cyanovorans CBS 11949 (JF680897/JF680898)
Cryptococcus cyanovorans CBS 1150 (JF680901/JF680902)
Trichosporon chiarellii FCP 540806 T (EU030272/GQ338074)
Trichosporon guehoae CBS 8521 T (AF105401/AF410476)
Cryptococcus daszewskae CBS 5123 T (AB126588/AB035580)
Porosum
Trichosporon lignicola var. lignocola CBS 219.34 T (AY370685/AY370684)
Trichosporon lignicola var. undulatum CBS 222.34 T (AF363658/AF444482)
Trichosporon gamsii CBS 8245 T (AF444708/AF444424)
Trichosporon dehoogii CBS 8686 T (AF444718/AF444476)
Trichosporon porosum CBS 2040 T (AF189833/AF414694)
Gracile/Brassicae
Trichosporon domesticum CBS 8280 T (AF075512/AF444414)
Trichosporon montevideense CBS 6721 T (AF105397/AF444422)
Trichosporon scarabaeorum CBS 5601 T (AF444710/AF444446)
Trichosporon loubierii CBS 7065 T (AF075522/AF444438)
Trichosporon brassicae CBS 6382 T (AF075521/AF444436)
Trichosporon vadense CBS 8901 T (AY093426/AY093425)
Trichosporon dulcitum CBS 8257 T (AF075517 /AF444428)
Trichosporon gracile CBS8 189 T (AF105399/AF444440)
Trichosporon laibachii CBS 5790 T (AF075514/AF444421)
Trichosporon multisporum CBS 2495 T (AF139984/AF414695)
Humicola
Cryptococcus longus CBS 5920 T (AB126589/AB035577)
Cryptococcus pseudolongus JCM 9712 T (AB105352/AB05104)
Cryptococcus musci JCM 1531 T (AB126586/AB035579)
Cryptococcus ramirezgomezianus JCM 1460 T (AB126584/AB035578)
Cryptococcus humicola CBS 571 T (AF189836/AF410470)
Cryptococcus fragico la JCM 1530 T (AB126585/AB035588)
Cryptoctrichosporon anacardii CBS 9551 T (AY550002/AY549985)
Formosensis
Bullera nakhonratchasimensis TY 279 T (AY313012/AY313034)
Bullera formosensis CBS 9812 T (AY787858/AY787859)
Bullera lagerstroemiae TY 254 T (AY31301/AY313033)
Cryptococcus tepidarius M 9962 T (AB094046/AB094045)
Cryptococcus albidus CBS 142 T (AF075474/AB051026)
Cryptococcus gastricus CBS 2288 T (AF137600/AF145323)
Cryptococcus amylolentus CBS6039 T (AF105391 /FJ534872)
0.02

Figure 1: Phylogenetic placement of *Cryptococcus cyanovorans* in the Curvatus sub-group using the NJ method (K2P) based on concatenated sequences of D1/D2 and ITS regions. Bar, Kimura's distance measure. The tree was obtained by 1000 bootstrap replications. *Cryptococcus amylolentus* was used as the out group species in the analysis. Bootstrap values less than 50% were omitted. Sequences were retrieved from the GenBank/EMBL/DDBJ database using the accession numbers indicated on the tree.

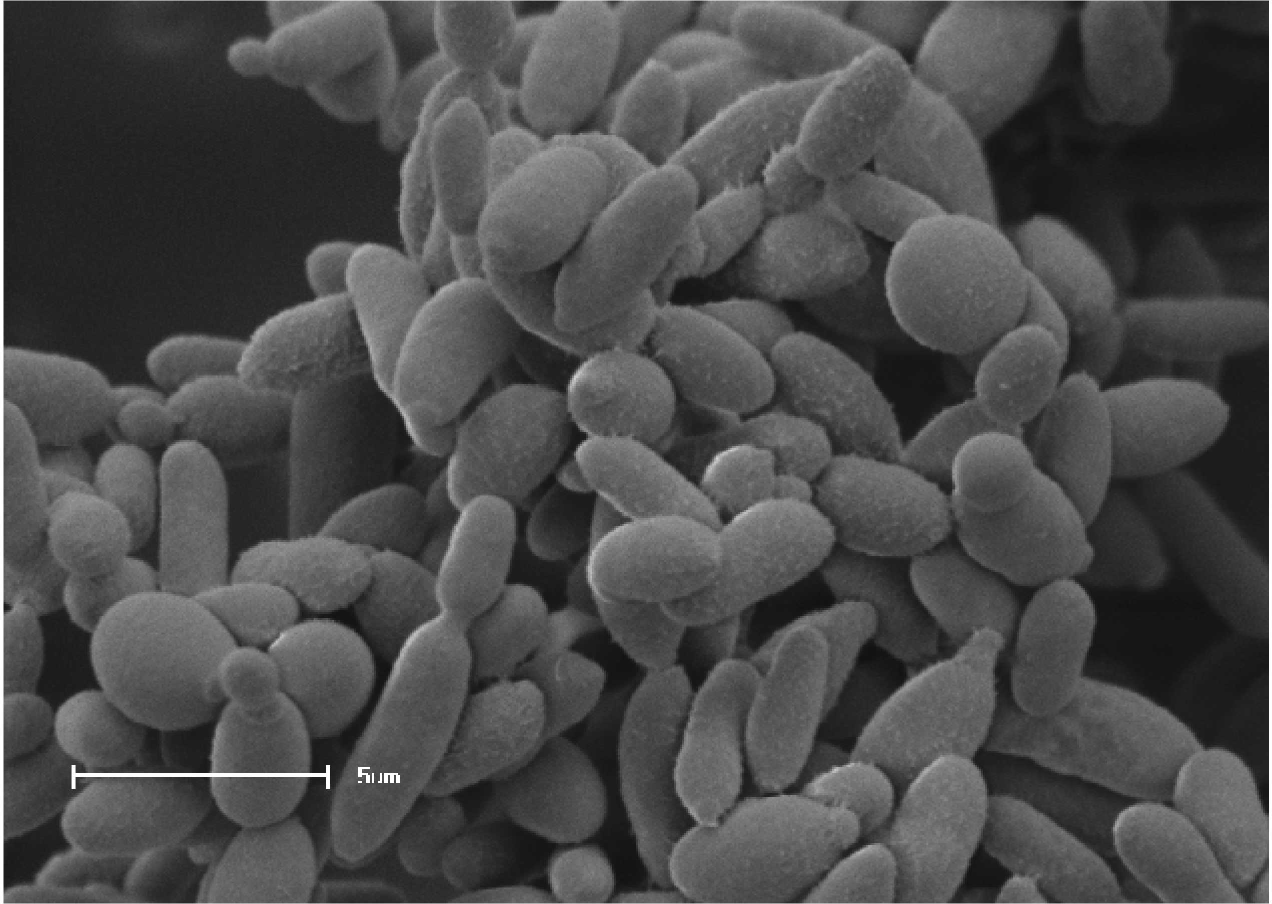


Figure 2: Globose to elongate budding cells of *Cryptococcus cyanovorans* sp. nov. after 3 days at 25 ^{o}C on YM agar. Scale bar represents 5 µm.

CHAPTER 4

Description of *Trichosporon africanensis* sp. nov., an anamorphic basidiomyceteous yeast isolated from South African soil

Chapter is submitted for publication in the International Journal of Systematic and Evolutionary Microbiology. The work presented here is written according to standards of this journal

Summary

During a survey of unidentified yeast isolates, 11 strains were identified from uncharacterized soil in South Africa. Sequence search using BLAST algorithm on GenBank/DDBJ revealed one strain, UOFS Y-1920, having 99-100% sequence identity with five undescribed basidiomycetous yeasts in the D1/D2 rDNA and only two undescribed basidiomycetous yeasts in the ITS region. Phylogenetic analysis using sequences of these regions indicated that this strain forms a well supported sub-clade in the order Trichosporonales with these basidiomycetous yeasts and this may suggest that they are species of similar generic group. In addition, strain UOFS Y-1920 exhibited 2-3% substitutions and 95% sequence identity with *Cryptococcus fragicola* and *Trichosporon montevideense* in both the D1/D2 and ITS region and this has further suggested that it is undescribed and represents a new basidiomycetous yeast species. According to microscopic studies performed on this strain using different media, vegetative reproduction by budding rarely occurred and the vegetative state was mainly dominated by formation of arthroconidia and fusiform giant cells as well as mycelia, characters of species assigned to the genus *Trichosporon*. In addition strain UOFS Y-1920 has the ability to liquefy gelatine and can grow in the presence of 0.01% cyclohexamide. Based on these findings, the current paper reports on the description of a new basidiomycetous yeast belonging to the genus *Trichosporon* [type strain, CBS 12124^{T} (=NRRL Y-48732^{T})], for which the name *Trichosporon africanensis* sp. nov. is proposed.

1. Introduction

The order Trichosporonales Boekhout & Fell is dominated by species belonging to the genus *Trichosporon* Behrend which are distributed throughout the clusters Cutaneum, Gracile/Brassicae, Ovoides and Porosum (Middlehoven *et al*., 2004; Takashima *et al*., 2001, 2009; Pagnocca *et al*., 2010). Taxonomic characters used to describe species of this genus include colonies with elated, lateral and clavate arthroconidia, presence of fusiform giant cells as well as presence of xylose in whole-cell hydrolyzates (Kreger-van Rij, 1984; Gueho *et al*., 1998; Sugita & Nakase, 1998). *Trichosporon* species are isolated from leaf surfaces, rotten wood, waste water, air and soil (Sugita *et al*., 2000). However, species with pathogenic activities have also been observed, particularly members of the Ovoides clade which are characterized by growth at temperatures of 37 °C or more and the presence of coenzyme Q-9 (Takashima *et al*., 2001; Middlehoven, 2004). *Trichosporon* is a monophyletic genus with the only polyphyletic species having been *Trichosporon pollulans* which clusters with members of the family Cystofilobasidiaceae (Scorzetti *et al*., 2002). This is one of the reasons why *T. pollulans* is currently reassigned to the genus *Guehomyces* (renamed *G. pullulans*) and has been accepted as a member in the order Cystofilobasidiales (Fell & Scorzetti, 2004). Recently described species of the genus *Trichosporon* include *T. mycotoxinovorans* (Gracile/Brassicae sub-clade) (Molnar *et al*., 2004), *T. insectorum* (Ovoides sub-clade) (Fuentefria *et al*., 2008), *T. chiarellii* (undefined sub-clade) (Pagnocca *et al*., 2010) and *T. xylopini* (Porosum sub-clade) (Gujjari *et al*., 2010).

In the current study, we investigated 11 yeast strains associated with soil in South Africa. Nine strains were identified as ascomycetous yeasts and two as basidiomycetous yeasts, using sequence analysis of the D1/D2 rDNA. One of the basidiomycetous yeast strains (UOFS Y-1920) represented an undescribed species with 0-1 base pair difference with five other undescribed strains, suggesting that they were of the same generic group. However, ITS sequences of three of these strains were not available on GenBank/DDBJ (http://www.ncbi.nlm.nih.gov/ or http://www.ddbj.nig.ac.jp/) and thus comparisons in this region were limited to a reduced number of these strains.

2. Materials and methods

Isolation of DNA was performed on yeast cells grown for approximately 24 hours at 30 ^{o}C in 50 ml yeast malt extract agar (3 g yeast extract, 3 g malt extract, 5 g peptone, and 10 g glucose per litre of distilled water). Isolation of DNA was achieved either by genomic DNA extraction (Labuschagne & Albertyn, 2007) or by boiling cells suspended in 20 µl distilled water for 10 min at 96 ^{0}C. Polymerase chain reaction (PCR) was performed for the 26S rDNA D1/D2 and ITS regions using fungal primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5' GGTCCGTGTTTCAAGACGG-3'), ITS-4 (5'-TCCTCCGCTTATTGATATGS-3') and ITS-5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') respectively (White *et al*., 1990; Kurtzman & Robnett, 1998). Sequence pairs for both rDNA and ITS were obtained with the ABI BigDye Terminator Cycle sequencing kit (Applied Biosystems®) and resulting

sequences were analysed using GenePro 4.8 (Drummond *et al*., 2009) and compared with sequences on GenBank (httpwww.ncbi.nlm.nih.gov). Sequences were aligned using CLUSTAL_X version 2.0 (Larkin *et al*., 2007). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 5 (Tamura *et al*., 2011) using the neighbour-joining method with the Kimura two parameter distance measure. Confidence values were estimated from bootstrap analysis of 1000 replicates. Morphological and physiological properties were determined according to standard methods of yeast identification (van der Walt & Yarrow, 1984; Yarrow, 1998).

3. Results and discussion

Among the eleven strains identifiedin from soil habitats of South Africa using D1/D2 rDNA sequence comparison, one strain displayed 99-100% sequence identity with basidiomycetous yeasts of unknown generic affinity. As a result the name *Trichosporon africanensis* was proposed for this species after analysis under light microscopy. Generic nature of this species was suggested by formation of lateral mycelial structures that articulate to form arthroconidia on 4% glucose peptone yeast extract (GPY) and Sabouraud's glucose agar (van der Walt & Yarrow, 1984). In addition, fusiform giant cells similar to those of *Trichosporon loubieri* CBS 7065^{T} and white to cream colored colonies with filamentous margins were also observed (Gueho *et al*., 1998). Furthermore this species was able to grow in the presence of 0.01% cyclohexamide as well as to ferment D-glucose and maltose. The current paper provides the description of

the type strain of this species and also proposes that other undescribed strains of this sub-clade belong to the species *Trichosporon africanensis.*

The D1/D2 rDNA phylogenetic tree was constructed and included five undescribed basidiomycetous yeast sequences (data not provided). *Trichosporon africanensis* showed 99 and 100% sequence identity with two and three of these species respectively (Table 1) and resulted in a supported sub-clade in the order Trichosporonales. This grouping was also detected in the ITS tree although as reduced since sequences of three of the undescribed basidiomycetous yeasts were not available on GenBank/DDBJ (data not provided). The sequence data of D1/D2 and ITS was combined and strengthened the branch of the *T. africanensis* sub-clade (Fig. 1). According to this analysis, *T. africanensis*, *Cryptococcus* sp. EN17S11 and *Cyptococcus* sp. GY10S03 represent strains of a species and this may also be the case with other strains only dected with D1/D2 rDNA sequences. However, when the tree was drawn based on the analysis of Gujjari and co-workers (2010), the three strains appeared to be grouping as as separate species (Fig. 2).

3.1 Latin diagnosis of *Trichosporon africanensis* Motaung, Albertyn, Kock J. L. F., Pohl CH sp. nov.

In medio liquid extracto fermenti confecto, post dies tres ad 30 °C, cellulae ovodiae, ad cylindricae (1.2-13.7 μm x 1.2-10.1 μm), singulae. Arthroconidia formantur. In agaro extracto fermenti et extracto malti confecto, post dies septem ad 25 °C, cultura glabra vel rugosa, cremea, magrine fimbriata. Post hebdomades tres cellulae gigantea, fusiformiae formantus. In agaro Dalmau pseudohyphae et hyphae formantur. D-Glucosum fermentat. D-Glucosum, D-galactosum, L-sorbosum, D-ribosum, D-xylosum, L-arabinosum, D-arabinosum, rhamnosum, sucrosum, maltosum, trehalosum, a-methyl-glucosidum, cellobiosum, salicinum, lactosum, melezitosum, arbutinum, N-acetyl-glucosaminum, glycerolum, xylitolum (exigue), L-arabitolum (exigue), inositolum, dulcitolum, propane-1,2-diolum, D-glucuronatum, lactatum, acidum malatum et acidum succinatum (exigue) assimulantur. Melibiosum, inulinum, ethanolum, methanolum, amylum solibile, erithrytolum, sorbitolum, 2-keto-D-gluconatum, 5-keto-D-gluconatum, citratum et hexadecanum non assimulantur. Nitratum, nitritum (exigue), L-lysinum, creatininum nec ethylaminum, cadaverinum et imidazolum assimulantur. Ureum finditur. Materia amyloidea formatur. Vitaminum externum crescentiae non necessarium. In medio 0.01% cycloheximidum vel 10% NaCl et 5% D-glucosum continente crescit. Crescit ad 37 °C. Typus strainus, CBS 12124^T, is praeservatus in collection zymotica Centraalbureau voor Schimmelcultures, Utrecht, Neerlandia et in collectione zymotica Agricultural Research Services United States Department of Agriculture, Peoris, Illinois (NRRL Y-48732^T).

3.2 Description of *Trichosporon africanensis* Motaung, Albertyn, Kock J.L.F. & Pohl CH sp. nov.

Trichosporon africanensis (a.fri.can.en'sis., africanensis referring to South Africa, where the type strain was isolated)

In yeast extract peptone medium after 3 days at 30 °C, ovoidal, ellipsoidal or elongated cells (1.2-13.7 μm x 1.2-10.1 μm) as well as arthrospores and septate hyphae are formed (Fig 4). Budding rarely occurs. In glucose peptone yeast extract agar and yeast malt extract (YM) agar, after 7 days at 25 °C, colonies are smooth to rough, cream coloured with fimbriate margins. After 3 weeks on YM agar, fusiform giant cells are observed. In Dalmau plate cultures on cornmeal agar, pseudo or true mycelia are formed. Only D-glucose is fermented after 3 weeks. Urea is hydrolized and extracellular starch is formed. Growth is observed at 37 °C. Utilization of carbon and nitrogen as well as other compounds is given in Table 2. The type strain, CBS 12124^T, is currently maintained in the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, with a copy (NRRL Y-48732^T) at the yeast culture collection at the Agricultural Research Services United States Department of Agriculture, Peoria, Illinois.

4. Acknowledgements

Authors would like to acknowledge Mrs Andri van Wyk for providing the strains used in the study and Professor Peter van Wyk (Centre for Confocal and Electron Microscopy, University of the Free State) for his assistance during microscopic experiments. This work is based upon research supported by the National Research Foundation and

Department of Science and Technology under the SABI Programme. Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors and therefore the NRF and DST do not accept any liability in regard thereto.

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Table 1: Species identified from soil in South Africa using D1/D2 rDNA sequences.

Species	Strains	*E*-values	Percentage identity
Ascomycetes			
Candida aaseri	UOFS Y-2121	0.0	100%
Candida parapsilosis	UOFS Y-0645	0.0	100%
Candida silvae	UOFS Y-549	0.0	99%
Candida silvae	UOFS Y-1855	0.0	99%
Candida silvae	UOFS Y-1854	0.0	99%
Exophiala dermatitidis	UOFS Y-2116	0.0	100%
Meyerozyma guilliermondii	UOFS Y-1936	0.0	99%
Meyerozyma guilliermondii	UOFS Y-1934	0.0	100%
Meyerozyma guilliermondii	UOFS Y-1930	0.0	99%
Basidiomycetes			
Trichosporon africanensis sp. nov.	UOFS Y-1920 (CBS 12124^{T}, NRRL Y-48732^{T})	0.0	100%
Ustilago triodiae	UOFSY-0117	0.0	98%

UOFS: Strains are maintained in the UNESCO-MIRCEN Biotechnology Yeast Culture Collection, University of the Free-State.

Table 2: Characterization of *Trichosporon africanensis* sp. nov. CBS 12124[T] based on fermentation and assimilation patterns as well as additional tests. Reactions are scored as: + positive; - negative; W weak.

Test compound	Result	Test compound	Result
Fermentation			
D-Glucose	+	Sorbitol	-
Maltose	+	Dulcitol	+
		Butane-2,3-diol	-
Carbon source		Propane-1,2-diol	+
D-Glucose	+	D-Glucuronate	+
D-Galactose	+	DL-Lactate	+
L-Sorbose	+	Citrate	-
D-Ribose	+	Mallic acid	+
D-Xylose	+	Succinic acid	+, W
L-Arabinose	+		
D-Arabinose	+	**Nitrogen source**	
L-Rhamnose	+	Nitrate	+
D-Sucrose	+	Nitrite	+, W
Maltose	+	Ethylamine	-
α,α-Trehalose	+	L- Lysine	+
α-methyl-glucoside	+	Cadaverine	-
Cellobiose	+	Creatinine	+
Salicin	+	Imidazole	-
Melibiose	-		
Lactose	+	**Additional tests**	
Melizitose	+	2-Keto-D-gluconate	-
Inulin	-	5-Keto-D-gluconate	-
Arbutin	+	Vitamin free medium	+
Ethanol	-	Diazonium blue B	+

Test compound	Result	Test compound	Result
Methanol	-	Amyloid compounds	+
N-Acetyl-Glucosamine	+	Growth in:	
Starch	-	50% D-Glucose	-
Glycerol	+	10% NaCl plus 5% D-glucose	+
Erythritol	-	0.01% Cycloheximide	+
Xylitol	+, W	0.1% Cycloheximide	-
L-Arabitol	+, W	Hexadecane	-
myo-Inositol	+		

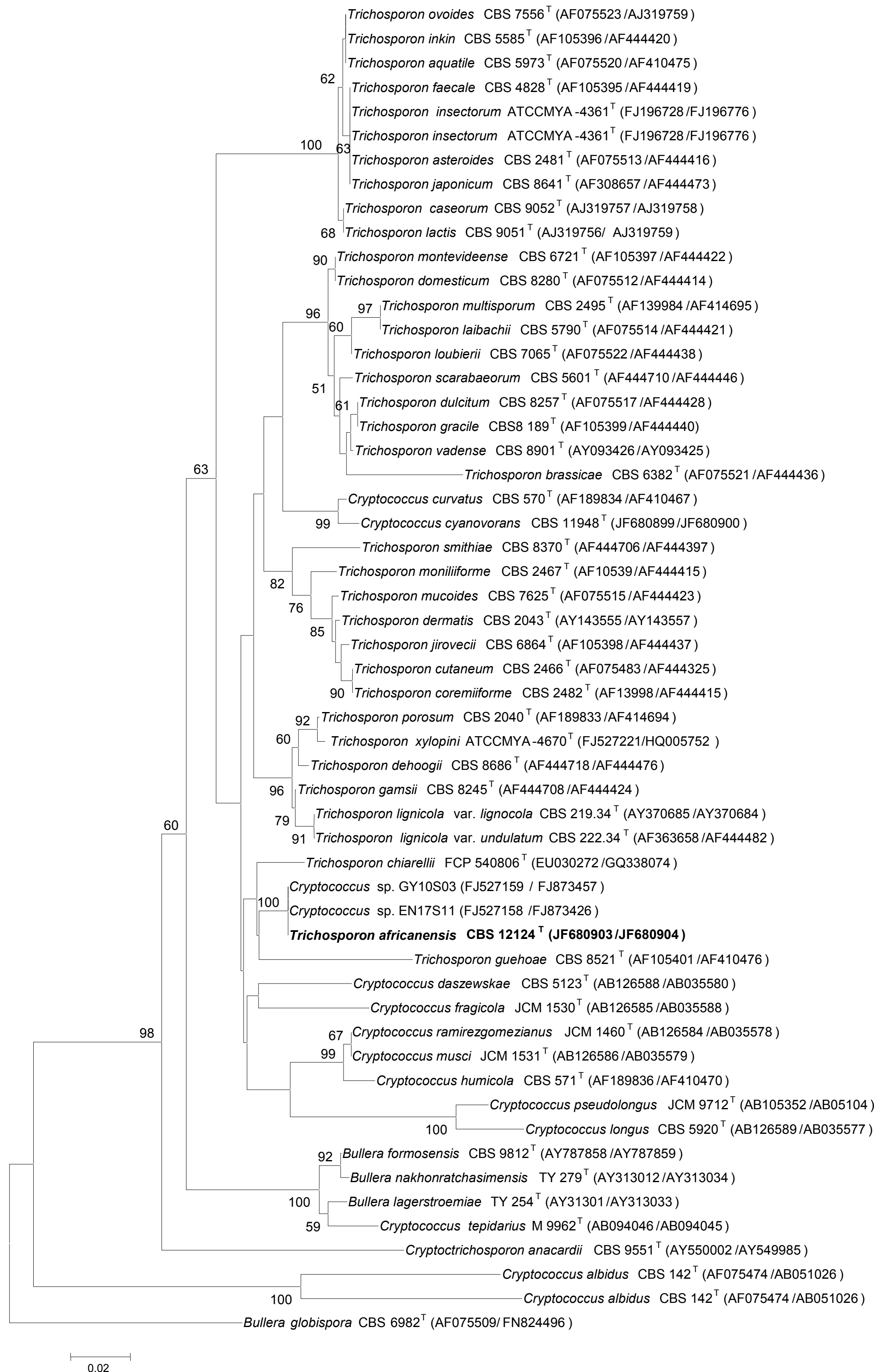

Figure 1: Phylogenetic placement of *T. africanensis* using the NJ method (K2P) based on sequences of D1/D2 and ITS regions. Bar represents Kimura's distance measure. The tree was obtained by 1000 bootstrap replications. Bootstrap values < 50% are omitted. Sequences were retrieved from the GenBank/EMBL/DDBJ database using the accession numbers indicated on the tree. *Bullera globispora* was used as the out group in the analysis.

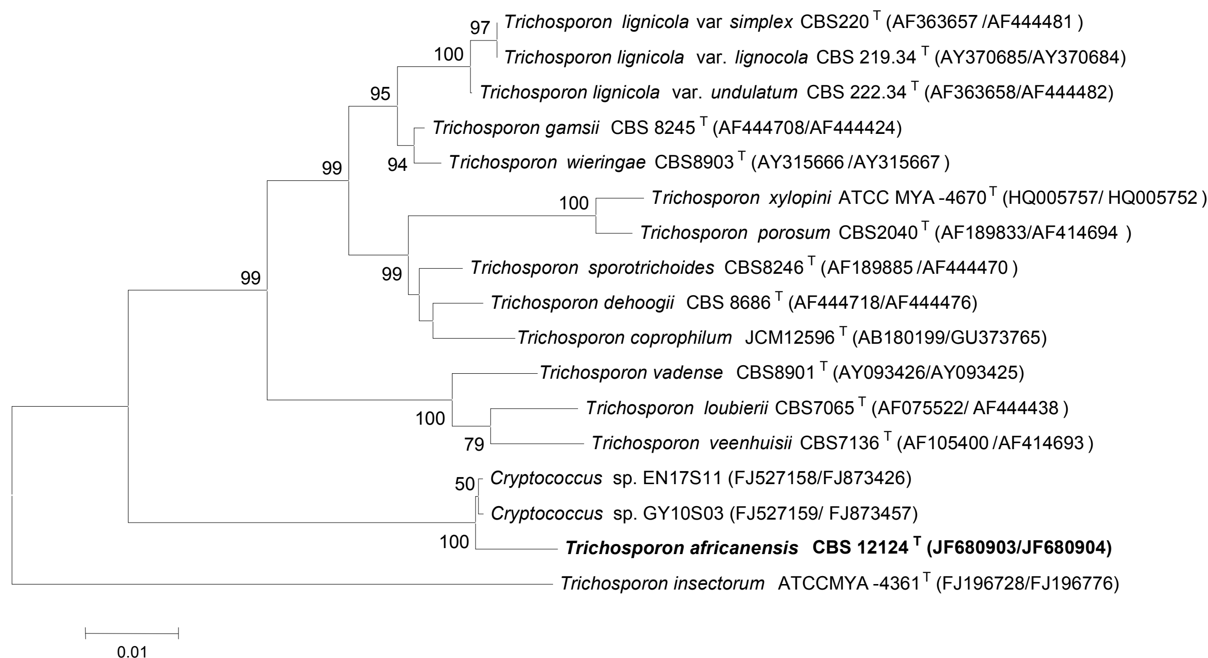

Figure 2: Phylogenetic placement of *Trichosporon africanensis* sp. nov. using the NJ method (K2P) based on concatenated sequences of D1/D2 and ITS regions respectively. Bar, Kimura's distance measure. The tree was obtained by 1000 bootstrap replications. Bootstrap values < 50% are omitted. Sequences were retrieved from the GenBank/EMBL/DDBJ database using the accession numbers as indicated on the tree. The tree was constructed according to Gujjari and co-workers (2010).

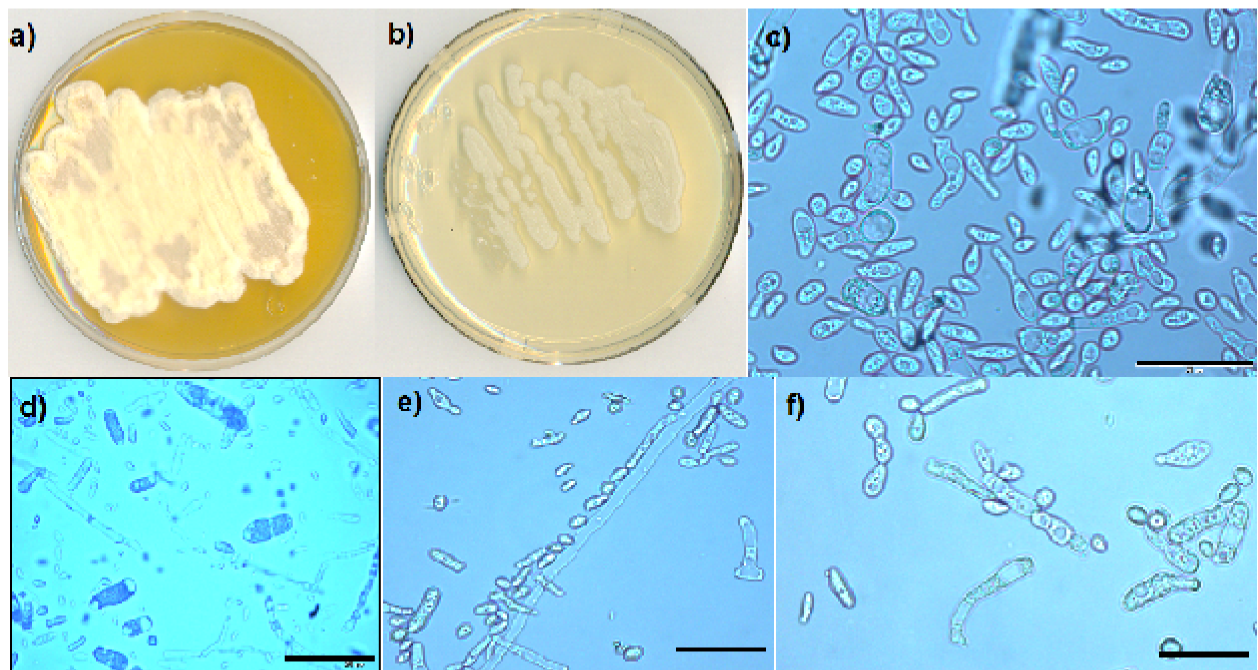

Figure 3: *Trichosporon africanensis* sp. nov., a)-b) Colonies with fimbriate edges cultivated on GPY and YM agar respectively, c) ovoidal, ellipsoidal and elongated non-budding cells, d) septate hyphae and fusiform giant cells, e) disarticulating arthroconidia and f) hyphae with lateral, clavate conidia. Scale bar represents 20 μm.

Conclusion

Conclusion

The availability of a database comprising sequences of basidiomycete, ascomycete and yeast-like taxa was utilized during this study. Guidelines for yeast identification have been applied and aided in the demarcation of species using number of base substitutions between sequences of strains and species pairs (Kurtzman & Robnett, 1998; Fell *et al*., 2000; Scorzetti *et al*., 2002; Kurtzman & Fell, 2006). As a result identification of unidentified yeast strains in the UNESCO-MIRCEN biotechnological yeast culture collection was predominantly based on D1/D2 rDNA sequencing followed by BLAST searches on NCBI (www.ncbi.nlm.nih.gov). As a result 197 strains were identified. These strains represented 19 ascomycete and five basidiomycete genera (including the yeast-like taxa). Among these, three strains isolated from cyanide contaminated soils are proposed to represent a new species in the genus *Cryptococcus* and this species is described in this study using standard techniques (Yarrow, 1998). The name *Cryptococcus cyanovorans* CBS 11948^T (=NRRL Y-48730) is proposed for this species [with additional strains, CBS 11949 (=NRRL Y-48728) and CBS 11950 (=NRRL Y-48729)]. This species belong to a cluster Curvatus, order Trichosporonales and class Hymenomycetes. Verification of new and undescribed species was performed using the internal transcribed spacers (ITS) sequences. It is also acknowledged in this study that although molecular techniques identified a strain (UOFS Y-1920) which was related to undescribed basidiomycetes, the generic nature of this strain was resolved by morphological techniques and therefore the importance of these techniques are also highlited here. Since this strain came from a group of isolates obtained from soil in South Africa, the name *Trichosporon africanensis* CBS 12124^T (=NRRL Y-48732^T) is

proposed. Phylogenetic analysis indicated that this strain forms a sub-clade with undescribed basidiomycetes in the order, Trichosporonales. Therefore strain UOFS Y-1920 represents the type strain of *T. africanensis*. This species is characterized by formation of giant cells, abundant hyphae and lateral conidia or arthrospores on glucose agar and 2% glucose-YNB after 3 days at 25 ^{0}C. Five undescribed basidiomycetes were found to be close relatives of *T. Africanensis* and should be described and demarcated from the type strain in the *T. africanensis* sub-clade in follow up studies.

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Summary

Summary

As the initiative for establishing a yeast culture collection began in the early 1980's in the department of Microbial, Biochemical and Food Biotechnology, research pertaining to the identification of isolates became the main focus. Yeasts such as *Candida shehatae* and *Pichia stipitis* claimed potential applications in the fermentation industry and received the most research attention. This research area and others reflected the potential of the culture collection as one of the sources of microbial diversity. As a result, this granted this culture collection a Microbial Resources Centre by the United Nations Educational, Scientific and Cultural Organization (UNESCO) in 1996. Isolation, preservation, identification and determination of taxanomic affinities of yeasts are some of the important aspects of this culture collection in addition to others such as bioprospecting and elucidation of biotechnological applications of strains. As a general practice, these isolates must be identified reliably and rapidly using techniques such as polymerase chain reaction (PCR) and sequencing of the ribosomal DNA.

Polymerase chain reaction (PCR) and sequenceing of the ribosomal DNA was mainly used in this study to identify isolates present in the UNESCO-MIRCEN Biotechnological Yeast Culture Collection. Ribosomal DNA was amplified and sequenced, followed by analysis of sequence data that was searched against available sequences on National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). In the case of new species, verification of novelty was performed by sequencing the internal transcribed spacers and doing phylogenetic analysis based on multigenic approach. New species

were assigned to their phylogenetic groups and described using standardized traditional techniques.

It was apparent from this study that using molecular identification methods, the number of identified ascomycetes and basidiomycetes as well as yeast-like taxa, with possible applications in the industrial, clinical, pharmaceutical, environmental and agricultural settings can be rapidly identified in great numbers. In this study, three basidiomycete strains representing new species in the genus *Cryptococcus*, with potential applications in cyanide bioremediation, were identified and described. These strains represent a new species which form part of a small group of yeasts (including *Cryptococcus humicolus* strain) with the ability to utilize inorganic cyanide as sole carbon and nitrogen source. Some of the applications and importance of strains representing known ascomycetes, basidiomycetes and yeast-like taxa are also highlighted here. Not only was the identification of isolates achieved in this study, but also full description of new yeasts present in the UNESCO-MIRCEN Biotechnological Yeast Culture Collection with possible applications in bioremediation. However, the applications of species such as *Trichosporon africanensis* sp. nov. will be elucidated elsewhere.

Keywords

Yeast identification • UNESCO-MIRCEN Biotechnological Yeast Culture Collection • D1/D2 rDNA • ITS region • Sequence analysis • *Cryptococcus* •

Trichosporon • Description of species • Cyanide bioremediation • Biotechnological applications

Opsomming

Opsomming

Die inisiatief vir die stigting van 'n giskultuurversameling in die Departement Mikrobiese, Biochemiese en Voedselbiotegnologie het begin in die vroeë 1980's. Navorsing met betrekking tot die identifisering van isolate was die hooffokus. Vir giste soos *Candida shehatae* en *Pichia stipitis* is moontlike toepassings in die fermentasie industrie bewys en hulle het dus die meeste navorsingsaandag ontvang. Hierdie en ander navorsing weerspieël die potensiaal van die kultuurversameling as een van die bronne van mikrobiese diversiteit. As gevolg hiervan, ontvang hierdie kultuur versameling 'Microbial Resources Centre' status in 1996, toegeken deur die Verenigde Nasies se Opvoedkundige, Wetenskaplike en Kulturele Organisasie (UNESCO). Isolasie, identifikasie en bepaling van taksonomiese verwantskappe van giste is sommige van die belangrike funksies van die kultuurversameling, tesame met ander soos bioprospektering en bepaling van biotegnologiese toepassings van isolate. As deel van algemene praktyke moet die isolate betroubaar en vinnig geïdentifiseer ward d.m.v. tegnieke soos polimerase kettingreaksie (PKR) en bepaling van basispaaropeenvolging van die ribosomale DNS.

Polimerase kettingreaksie (PKR) en basispaar-opeenvolgingbepaling van die ribosomale DNA is hoofsaaklik gebruik in hierdie studie om isolate te identifiseer wat teenwoordig is in die UNESCO-MIRCEN biotegnologiese kultuurversameling. Die basispaar-opeenvolging van die ribosomale DNA is bepaal, gevolg deur die analise van data deur veregelykende studies met beskikbare data vanaf National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Potensiële nuwe spesies is

bevestig deur die volgordebepaling van die ITS gebied asook filogenetiese analise. Nuwe spesies is toegeken aan hulle filogenetiese groepe en beskryf deur gebruik te maak van tradisionele tegnieke.

Dit was duidelik uit hierdie studie dat met molekulêre identifikasie metodes die getal van geïdentifiseerde askomisete en basidiomisete sowel as gis-agtige taksa, met moontlike toepassings in die nywerheid, kliniese, farmaseutiese, omgewings- en landbou gebiede, verder verhoog kan word. Drie basidiomiseet isolate wat ‘n nuwe spesie in die genus *Cryptococcus*, met moontlike toepassing in bioremediëring, verteenwoording, is geïdentifiseer en beskryf in hierdie studie. Hierdie isolate is deel van 'n klein groepie van giste (insluitend *Cryptococcus humicolus*) wat oor die vermoë beskik om anorganiese sianied as enigste koolstof- en stikstofbron te benut. Sommige van die toepassings van bekende askomisete, basidiomisete en gis-agtige taksa word ook uitgelig in hierdie studie. Nie net was die identifisering van verskeie isolate bereik in hierdie studie nie, maar ook die volledige beskrywing van die nuwe giste teenwoordig in die UNESCO-MIRCEN biotegnologiese gis versameling met moontlike toepassings in bioremediëring. Die toepassing van spesies soos *Trichosporon africanensis* sp. nov sal elders aangespreek word.

Sleutelwoorde

Gis identifikasie • UNESCO-MIRCEN biotegnologiese gis kultuurversameling • D1/D2 rDNA • ITS gebied • Basispaar analise •

Cryptococcus • *Trichosporon* • Beskrywing van spesies • Sianied bioremediëring • Biotegnologiese toepassings