The Diversity of Culturable Yeasts in Soils of Cameroon and Saudi Arabia

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Lay Abstract

Yeasts are single-celled microorganisms that are widely dispersed in a wide variety of natural and human-related habitats such as plant leaves, flowers, fruits, water, and soil. Yeast are probably one of the earliest domesticated organisms; people have used yeast for fermentation and baking through much of the recorded human history. My project focused on studying yeast diversity in two geographically distinct countries, Cameroon and Saudi Arabia, in the context of environmental factors such as climate conditions and pH. I identified yeast present in soil samples morphologically, sequenced them at the ITS locus, and determined the genetic variation among isolates within individual yeast species using PCR fingerprinting. The analysis revealed differences in yeast species distribution and in yeast genotypic diversity among the analyzed geographic regions. These results suggest that Cameroon and Saudi Arabia contain significant novel yeast diversity.

Abstract

Yeasts are unicellular microorganisms that belong to the kingdom of fungi. Yeasts are found in a diverse range of habitats, including soil, sugar- rich surfaces on the fruit and flowers of plants, and on the surface of plants and animals. Yeast plays an important role in soil by participating in nutrient cycles and mineralization processes. In contrast to the various studies investigating soil yeasts in Western countries, there is a dearth of research investigating soil yeasts in Africa and the Middle East. In these projects, we collected 493 soil samples from nine different geographical locations in Cameroon, and 562 soil samples from six geographical locations in Saudi Arabia. I identified yeast isolates by using the culture-based method and the fungal barcode sequence at the ITS region. In addition, the yeast species were analyzed using PCR- fingerprinting to investigate the genetic variation among strains within the same species. In sum, we have isolated and identified a total of 110 yeast isolates from Cameroon as well as a total of 114 yeast isolates from Saudi Arabia. Our results showed that soil samples from different regions in Cameroon had different yeast colonization (0%-56%), with the highest found in northwest Cameroon. The Saudi Arabian yeast isolation was different from those in Cameroon, with less diversity; the isolation rate ranged from 0.7 to 40.2 %, with the highest found in western Saudi Arabia. PCR-fingerprinting results suggested that yeast migration was common among the geographic regions within each country. However, local climatic conditions can significantly impact yeast diversity at the local and continental levels.

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Abbreviations

ITS: Internal Transcribed Spacer regions of the nuclear ribosomal RNA gene cluster

PCR: Polymerase Chain Reaction

UV: Ultra-Violet

YEPD: Yeast Extract Peptone Dextrose medium

Chapter 1: Introduction

1.1 Yeast

Yeasts are microscopic, single-celled eukaryotic organisms classified as members of Kingdom Fungi. The 1,500-yeast species identified thus far only constitute ~1% of all described fungal species. Most yeasts fall under two main phyla: Ascomycota and Basidiomycota (Hoffman *et al.*, 2015). Yeasts inhabit a wide range of natural habitats including plant leaves, flowers, water, and soil. Most yeasts reproduce asexually by budding (e.g., *Saccharomyces spp.*) or through fission (e.g. *Schizosaccharomyces spp.*). Some yeasts may grow as simple filaments under certain conditions. However, during optimal environmental conditions, the haploid yeast cells can fuse, ultimately forming a diploid zygote, through sexual reproduction. For the past few decades, the yeast *Saccharomyces cerevisiae* has been a "model organism" for scientific investigations. Studies of yeasts provide insights into the basic biological processes in eukaryotes, including human diseases. Indeed, aside from being the first sequenced eukaryotic genome, *S. cerevisiae* replicates rapidly and a variety of tools that make it easy to manipulate genetically (Botstein *et al.*, 1997).

A number of molecular methods have been developed for characterizing fungal communities, with the main molecular method relying on using sequence variations at the internal transcribed spacer (ITS) regions of the ribosomal RNA gene (rDNA) cluster (Pham *et al.*, 2011). Yeasts have been a part of the human history for more than 8,000 years. For example, *S. cerevisiae* is a significant yeast economically and industrially worldwide. *Saccharomyces cerevisiae* and other related yeasts have long been utilized to ferment the sugars of rice, wheat, barley, and corn to produce alcoholic beverages. In addition, they are often taken as a vitamin supplement (Moyad *et al.*, 2008; Walker & Stewart, 2016).

1.2 Soil as an ecosystem

Soil is a rich ecosystem composed of both living and non-living matter; soil has an important role in all-natural ecological cycles such as carbon, nitrogen, and water. The diversity and abundance of microorganisms that exist within the soil are greater than in any other ecosystem (Buée *et al.*, 2007). In a soil ecosystem, soil organisms represent a large fraction of global terrestrial biodiversity. These microorganisms include bacteria, fungi, algae, protozoa, and viruses. They carry out a range of processes important for soil health and fertility of both natural ecosystems and agricultural systems. Soil fungi contribute to the microbial biomass and the genetic diversity of soil microorganisms. For example, mushrooms have underground roots (mycelium) that absorb nutrients and water in order to form mushroom fruiting bodies (Kredics *et al.*, 2010). Yeasts have been found in many types of soil samples, they not only influence nutrient cycling but also has a significant role in soil aggregate formation, with a consequent effect on soil structure that helps improve water infiltration and water-holding capacity (Botha, 2011).

Soils are the environment that link plant production, water, and microbial activities. Soil also is accountable for many functions of the structure of the natural system, including temperature regulation, water concentration, carbon, and nutrient cycling (Climate & Development, 2013). Moreover, biological and chemical processes in the soil may affect the balance in organic carbon compounds and other materials in the soil structure, which may directly or indirectly affect the microorganisms living in the soil, including fungi/ yeast (Torsvik & Øvreås,). In general, two types of habitats occur in the soil: one with low nutrient availability, and one called the rhizosphere that usually contains more. The microbial taxa present in these two habitats differ due to differences in their chemical composition (Vreulink *et al.*, 2007).

1.3 Environmental factors

It has long been recognized that under certain conditions, a microorganism such as a fungus regulates its metabolism in response to changes in environmental conditions such as temperature, pH, and nutrient concentrations. Therefore, the structure of soil yeast communities is a direct reflection of environmental factors.

Temperature is an important factor that affects many physical, chemical, and biological processes in the soil environment, such as regulating fungal growth and development. Fungi such as yeasts have the ability to grow in a minimum temperature of below 20°C and a maximum temperature of up to 50°C, with an optimal temperature range of 20°C to 30°C (Newsham *et al.*, 2015). In addition, temperature has a significant effect on the rate of growth and fermentation in yeast; the rate of growth and fermentation increases at a temperature range from 20°C to 40°C and may decrease at temperatures above 50°C. Indeed, there are many industrial alcohol productions that rely on the relationship between yeast activation and temperature regulation (Jones & Hough, 1970; Bässler *et al.*, 2010).

In addition, one of the most influential factors in yeast physiology is pH. In the soil, pH affects a number of factors that may influence biological activities such as solubility and ionization of organic materials; these will, in turn, affect soil enzyme activity and microbial activities in the soil habitat. pH also directly affects the microbial composition of the soil. Acidic soils with pH below 5.5 favor fungi including yeast. Due to the fact that yeasts are acid tolerant, they can grow in a pH environment lower than 4, suggesting that in forest soils with a pH below 4, the fungal growth rates are higher than those in soil with a high pH (Rousk *et al.*, 2009).

Moreover, the availability of key nutrients in the soil, such as sugars, amino acids, carbon, and nitrogen compounds, regulates the growth and behavior of yeast cells. Yeast usually grows well when these sources of energy are present at an optimal concentration. However, when yeast is present in an extreme concentration of nutrients, only a certain group of yeast can grow and survive. For example,

both *Debaryomyces hansenii* and *Zygosaccharomyces rouxii* are able to tolerate high salt concentrations and survive (Sánchez *et al.*, 2008). Industrially, the concentration of fermentable sugar plays a role in wine fermentation that can affect the quality, flavor, and final composition of wine. Among several yeast species, *S. cerevisiae* is the most important species present during the fermentation process of wine production (Arroyo-López *et al.*, 2009; Mestre *et al.*, 2014).

1.4 Pathogenesis of Yeast infection

In most natural environments, yeasts occur regularly as a member of the local microflora. Some of these yeasts are potentially pathogenic to humans, animals, and plants. The epidemiology of many yeast species in many regions around the world has not been fully understood. Recently, the rate of invasive fungal infection has significantly increased among hospital patients, particularly in those with an immunocompromised system; such fungal infections engender a high rate of morbidity and mortality.

A growing number of fungal species have been linked to human infectious diseases. Most of the patients with fungal diseases typically have underlying conditions. For instance, yeast infections are highly common in areas with a high prevalence of immunosuppressed patients, including patients with cancer, patients with AIDS, and elderly patients. In addition, there are major risk factors that play a role in increasing the severity of a yeast infection, such as poorly controlled diabetes, kidney failure, and chemotherapy (Kauffman, 2006). The epidemiology of fungal infection has previously been investigated in most areas of the world. However, many parts of Africa and the Middle East have not been studied extensively

Candida species, including *Candida albicans, Candida tropicalis*, and other non- *albicans Candida* are the most prevalent yeast pathogens. Infections caused by these yeasts have increased dramatically in the past decades (Ann Chai *et al.*, 2010). The epidemiology of candidemia, the bloodstream infection caused by Candida spp, has been extensively studied in developed countries, but rarely in the Middle East, with only a few local studies being conducted at a number of medical

institutes in the Gulf region (Ahmadinejad *et al.*, 2013; Al Thaqafi *et al.*, 2014; Taj-Aldeen *et al.*, 2014). In European countries, Candida spp. is a major cause of bloodstream infection and a major cause of morbidity and mortality. The annual incidence of bloodstream infection in Europe and the USA ranges from 3.0 to 26.2/100,000 inhabitants (Mascellino & Oliva, 2016). A meta-analysis study of the prevalence of an invasive Candida infection in Africa and the Middle East showed the prevalence of candida infection among HIV-infected patients (30.6%) was higher than in HIV-negative patients (15.07%) in Africa (Omrani *et al.*, 2014). The prevalence of Candida infections was highest at 31% in North African countries and lowest at 11.8% in the Arabian Peninsula (Omrani *et al.*, 2014).

In addition to Candida infection, Cryptococcus species have been a major cause of cryptococcosis that affects patients with an immune-compromised system, such as patients with AIDS, and patients with sarcoidosis or chronic lymphoproliferative diseases (Mitchell & Perfect, 1995). The prevalence rates of cryptococcosis have been reported in HIV-infected patients in several countries, including: Zaire, 19%; Mexico, 7%-11%; United States, 7%; Brazil, 5%; Ivory Coast, 5%; and Thailand, 2%. In Colombia, the mean annual incidence of cryptococcosis was found to be 2.4 cases per 1 million inhabitants in the general population and 3 cases per 1000 patients with AIDS (Meyer *et al.*, 2003; Pappalardo & Melhem, 2003; Leal *et al.*, 2008).

1.5. Objectives of this thesis

The aim of this thesis is to assess the diversity and community structure of soil yeast in two distinct countries in West Africa and middle East using a variety of field sampling and analytical methods. This work consists of four Chapters.

Chapter Two: **The Diversity of Culturable Yeasts in Cameroon Soils**

The objectives of this study were to characterize the diversity of yeasts in Cameroonian soil through culturing methods followed by sequencing at the ITS locus for species identification. Then, I investigated the genotypic diversities within individual species as well as other potential

characteristics. In this study, the soil samples were obtained from two climatic regions, namely the "Monsoon" climate and the "Tropical Savanna" climate. In the study site and calculate differences in diversity using a variety of measures, including traditional diversity tests and tree-based (or phylogenetic) measures. I preformed explicit statistical methods to test the hypothesis that yeast diversity was not differs among different geographical regions within Cameroon.

Chapter Three: **The Diversity of Soil Yeasts in Saudi Arabia**

The objectives of this study are to characterize the species diversity of soil yeasts and genetic diversity among strains within individual yeast species in Saudi Arabia using culturing methods followed by sequencing at the ITS locus for species identification. Based on the climate and soil conditions (Desert climate), our hypothesis was that yeast diversity in Saudi Arabia will be low, with slow decomposition of plant materials in the moisture-deprived soils. I preformed explicit statistical methods to test our hypothesis in this project.

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Chapter 2: The Diversity of Culturable Yeasts in Cameroon Soils

2.1 Preface

Although the diversity and distribution of yeast has been extensively studied in Europe and other developed countries around the world, little is known about the structure of soil yeast populations in West Africa. Here, we obtained and analyzed soil samples from three regions and nine locations in Cameroon, West Africa. Our results showed a significant difference in the diversity and distribution of yeasts between the collection regions. However, there was abundant evidence for gene flow in multiple yeast species among the regions within Cameroon.

I am the primary contributor of this work. Jianping Xu designed the experiments, with the majority of the experiments conducted by me. Tabi Ashu collected the soil samples and Himeshi Samarasinghe provided technical assistance. Analyses and writing of the manuscript were also predominantly completed by me and Jianping Xu. References in this chapter appear as they are in the original manuscript.

This chapter has been written as a manuscript, and it is being prepared for submitting to a journal of Scientific Reports.

Authors of this article are:

Renad Aljohani, Himeshi Samarasinghe, Tabi Ashu, Jianping Xu

2.2 Abstract

Yeasts are unicellular fungi; they are found in a diverse range of natural habitats, including soil, aquatic environments, the surface of plants, and the skin and mucosal surfaces of animal hosts. A variety of yeasts have been found in the soil environment. However, most studies of soil yeasts have come from developed countries, and there is a dearth of research on soil yeasts in Africa. In this study, we analyzed 493 soil samples from nine geographical locations in Cameroon for yeasts, using a culture - based method. The yeast isolates were identified to species level based on their sequences at the fungal barcode locus, the Internal Transcribed Spacer (ITS) regions of the nuclear ribosomal RNA gene cluster. In addition, the genetic variation among isolates within individual yeast species was analyzed using PCR fingerprinting. A total of 110 yeast isolates were obtained from the 493 soil samples. These isolates belonged to 16 yeast species, including 15 Ascomycetes and one Basidiomycetes. Significant differences in yeast species distribution and in yeast diversity were observed between the analyzed geographic regions. Our results suggest that Cameroon, and likely Africa in general, contain significant yeast diversity.

2.3 Introduction

The kingdom of fungi comprises diverse groups of organisms that include saprophytes, which are crucial players in nutrient cycling in natural ecosystems; symbiotic mutualists; as well as deadly pathogens of plants and animals. These fungi are often grouped based on their evolutionary relationships and/or morphological forms. Morphologically, fungi in their vegetative state can exist unicellularly such as yeasts or multicellularly such as filamentous hyphae and macroscopic mushrooms. Phylogenetically, yeasts are primarily found in Ascomycota and Basidiomycota, the two major phyla in fungi[1]. Ecologically, yeasts have been found in a diverse range of habitats, including soil, aquatic environments, plant surfaces, foods, and skin and mucosal surfaces of animal hosts. Due to soil remarkable heterogeneity across diverse climate zones, the soil environments represent a major ecological niche for different types of yeast[2].

Among currently known yeasts, the brewer's/baker's yeast *Saccharomyces cerevisiae* is probably the best-known both scientifically and industrially. This yeast is the model eukaryotic organism for genetics and molecular biological research and has been used in the food and alcoholic beverages industries for thousands of years. However, there are many other yeast species that are of environmental, economic, and/or medical significance. For example, yeasts (as well as filamentous fungi) play important roles in nutrient cycling and can help release nutrients for crops in agricultural fields [3,4]. Industrially, there have been significant efforts to isolate and study environmental yeast populations as a repository of strains that used to improve traditional strains [5]. On the other hand, some environmental yeasts are opportunistic pathogens of humans, and they pose serious threats to human health. For example, the human fungal pathogen *Cryptococcus neoformans* has been found in the soil in many parts of the world, including Africa. However, most studies of soil yeasts have come from developed western countries in Eurasia and North America, with a dearth of research on yeasts in Africa.

Both the culture-based method and the culture-independent method have been employed to study microbial diversity in nature, including yeast diversity. In culture-based studies, artificial media

and incubation conditions that favor yeast growth while limiting other microbes are typically used to enrich the yeast population, followed by isolation and purification. These yeasts are then identified based on their morphological, physiological, and/or molecular characteristics. In contrast, the culture-independent method directly analyzes the community DNA by means of extracting all DNA from the sample (e.g., soil), amplifying specific target DNA fragments using PCR, sequencing the amplified fragments, and comparing the sequences with those of reference strains in databases. In both approaches, where DNA sequencing is concerned, the target locus is often the Internal Transcribed Spacer (ITS) regions within the nuclear ribosomal RNA gene cluster. The ITS is chosen because this locus is highly conserved within most fungal species and there is an extensive collection of publicly available fungal ITS sequences from which to compare the experimental data[6]. Each of the two approaches (culture-based and culture-independent) has its advantages and disadvantages. For instance, the culture-independent method can reveal difficult-to-culture and uncultured microbes that the culture-based method would miss. A recent global meta-genomics study on natural fungal communities in soil found that a large number of soil fungal/yeast species remain unidentified[7]. In contrast, the culture-based method allows researchers to obtain pure strains that can be further studied to address other biological questions related to their phenotypic and genotypic characteristics.

In this study, we employed the culture-based approach to isolate and analyze yeasts from 493 soil samples obtained from nine geographical locations in three provinces in Cameroon. Cameroon is a Central African nation along the Atlantic Ocean and is bordered by several countries, including Nigeria, Chad, Gabon, and Republic of the Congo. Cameroon has all the major climates and vegetation zones of the African continent and hence is known as “Africa in miniature”. Specifically, Cameroon has high mountains in the southwest, a desert in the far north, and rain forests in the north central regions. With the African continent remaining as one of the least explored regions in terms of fungal diversity, we sought to characterize the diversity of yeasts in Cameroonian soil through culturing methods followed by sequencing at the ITS locus for species identification. While the culture-based method may not reveal all of the yeasts existing in our soil samples, the obtained yeasts can provide materials for more in-depth investigations on the genotypic diversities within individual species as well as other potential

characteristics. In this study, the soil samples were obtained from two climatic regions, namely the "Monsoon" climate and the "Tropical Savanna" climate. Our analyses revealed the presence of a variety of yeast species in Cameroon, with significant intra-specific genetic diversity.

2.4 Material and Methods

2.4.1 Sampling sites and sample collection

A total 493 soil samples were obtained from nine different geographical locations in three Cameroonian regions/provinces (Table 1), including four sites (Mbingo, Bambui, Njinkejum, and Babanki) in the Northwest (NW) Region, one site (Makepe) in the Littoral (LI) Region, and four sites (Eloundem, Mbalgong, Simbock, and Mbandoumou) in the Center (CE) Region. Sampling was conducted in June 2016; that is the warmest month and is the season with highest rainfall in the Northwest. At each location, the top layer of agricultural soils was sampled (approximately 1 gram per sample) at approximately 5 meters apart from each other in all four cardinal directions. Details of the study sites and of the vegetation found in each region's samples were described in previous studies (‘ Ngachie V, 1992.’,; Assi-kaudjhis, 2009). Once the soil samples were collected, they were shipped to McMaster University in Canada, stored at 4°C, and processed within 2 weeks after receiving. The soil pH was determined by adding 0.1 g of soil to 1ml sterilized water, allowing the soil to rehydrate for at least 1 h at 23°C, and then measuring the pH using a bench-top Orion pH meter.

2.4.2 Isolation of yeast

For each soil sample, we transferred ~0.2 g of soil into 1 ml of autoclaved Sabouraud Dextrose Broth (SDB) containing 0.035 mg/ml of the antibiotic chloramphenicol. The samples were then incubated for 48 hours at 30°C(Maganti *et al.*, 2012). After that, the samples were streaked onto Yeast Extract-Peptone-Dextrose agar (YEPD) medium and further incubated for 48 hours at 30°C. For each sample, a representative of each morphologically distinct yeast or yeast-like colony was sub-cultured onto YEPD agar and incubated for another 48 hours at 30°C. The purified fresh yeast cells were then harvested for DNA extraction. A portion of the fresh yeast cells of each isolate was stored in 30%

glycerol at -80°C. For soil samples that failed to yield yeasts in the first attempt, an additional attempt was made with another 0.2g of soil and an incubation time of 5 – 7 days.

2.4.3 Yeast species identification

To identify the yeast species, we first extracted genomic DNA following the protocol described in Xu et al. (2000). These DNA samples were then used as templates for PCR amplification of the ITS region using primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). The PCR protocol included an initial denaturation step of 95°C for 5 minutes, followed by 45 cycles of 30s at 95°C, 30s at 55°C, and 30s at 72°C, with a final extension of 7 minutes (Maganti et al., 2011). The ITS PCR products were checked via electrophoresis on 1% agarose gel with 1X TAE buffer for 30 minutes at 125 V. The gels were stained with ethidium bromide, photographed under ultraviolet light, and the respective PCR products were then diluted for sequencing based on the amount of DNA in the amplified sample. Sequencing was performed at the MOBIX Laboratory of McMaster University. The obtained ITS sequence from each isolate was compared to two databases, GenBank and UNITE, for yeast species identifications using the BLAST algorithm. In general, an isolate with ITS sequence greater than 97% sequence identity to the closest known yeast species was taken as an affirmative species identification. In contrast, those showing less than 97% sequence identity to any known species in the database were tentatively assigned to the closest species group or genus and they likely represent new species. We would like to note that even though the 97% sequence identity at the ITS locus is commonly used in metagenomics studies to separate sequences into different species, it is also well-known that a substantial number of known sister species pairs of yeasts show greater than 97% sequence identity. In the case that our strain shows similar and very close relationships (>97% sequence identity) to two or more such known yeast species, the degree of sequence divergence between the known species pairs is used as a guide to determine the taxonomic affiliation of our strain, including whether our strain represents a potential new species.

2.4.4 PCR fingerprinting

To investigate the potential genetic variation among isolates within individual yeast species that

we obtained in this study, a PCR fingerprinting method was performed using two separate primers: (1) the M13-core sequence (5'GTAAAACGACGGCCAGT-3'), and (2) the repeat sequence $(GACA)_4$ (5'GACAGACAGACAGACA-3'). These two primers have been commonly utilized to help differentiate strains within individual fungal species (Andrighetto *et al.*, 2000; Franklin *et al.*, 2010; Maganti *et al.*, 2012; Chang *et al.*, 2016). For each PCR fingerprinting reaction, a total volume of 10µl containing 5µl of 2x GO Taq Master mix (Promega), 2µl of H_2O, 1µl of 10µM primer and 2µl of template DNA was used. For both primers, a PCR included a denaturing step at 98°C for 2 min, followed by 45 cycles at 93°C for 20s, 50°C for 45s, 72°C for 20s, followed by a final extension step at 72°C for 6 min. The PCR products were electrophoresed on 1 % agarose gel for 2.5 hours at 80 V, and all bands were scored manually using the 100bp and 1kb ladders as references. The MEGA 7 software (version 7.0.25) was used to calculate the genetic relationships among yeast isolates based on the PCR fingerprint patterns [14].

2.4.5 Statistical analysis

The yeast isolation rates for soil samples from each of the nine locations and three regions were calculated. For each location/region, the relative abundance of each yeast species was determined by dividing the number of isolates within each species by the total number of the yeasts isolated in the locations/regions. The Simpson's species diversity index was calculated using the formula as $(1 - \sum p_i^2)\ N/(N-1)$; where p_i is the frequency of the *i*th species and N is the total number of yeast isolates. The diversity index value ranges from 0 to 1. The species diversity value of 0 means all isolates from a given site belong to the same specie; while the value of 1 means that every isolate in the population sample belongs to a different species.

Similarly, within each species, the genotypic diversity index was calculated using the same formula except that p_i represents the frequency of the *i*th genotype in the species and N is the total number of yeast isolates in that species. A genotypic diversity value of 0 means all isolates from the same species have the same genotype, while a value of 1 means a high genotype diversity where every isolate has a different genotype.

The statistical significance of yeast isolation rate differences and of species diversity differences between the locations were determined using a Chi-square test against the null hypothesis that there was no difference between different locations/regions. A Pearson correlation test was preformed between the isolation rate of yeasts obtained in this study and the isolation rate of *Aspergillus fumigatus* in the same locations [15] using Excel (software XLSTAT 365, 2017).

2.5 Results

2.5.1 Yeast isolation and identification

A total of 110 yeast isolates were obtained from the 493 soil samples collected from nine geographical locations in Cameroon (Table 1). According to their ITS sequences, the 110 isolates likely belonged to 16 different yeast species. Out of these 16 species, one was a basidiomycete (*Cryptococcus laurentii*), and the remaining 15 were ascomycetes. Nine of the 15 ascomycetes species belonged to known taxa: *Candida boidinii*, *Candida pseudolambica*, *Candida tropicalis*, *Cyberlindnera saturnus*, *Cyberlindnera subsufficiens*, *Debaryomyces nepalensis*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, and *Torulaspora globosa.* The remaining six species were putative new species as they either showed greater than 3% sequence divergence at the ITS locus from the six closest known yeast species or showed similar or greater divergence from the known species than the most closely related known sister species pairs. Among these six putative new species, two showed 98% and 99% ITS sequence identity to two sequences deposited in GenBank, but those two sequences were from strains not yet associated with any species name. Here, these six putative new species are tentatively called novel species #1 – 6; they consisted of nine of the 110 isolates. Novel species 1 (SP.NOV.1) belonged to the ascomycete genus *Cyberlindnera*, while novel species 2 (SP.NOV.2), 3 (SP.NOV.3), and 5 (SP.NOV.5) were identified as most closely related to *Candida* spp. Novel species 6 (SP.NOV.6) belonged to genus *Hanseniaspora*, while novel species 4 (SP.NOV.4) was found to be most closely related to genus *Saccharomyces* (Table 2 & Figure 1). Table 2 shows the accession numbers of the most closely related reference ITS sequences in the NCBI GenBank used to identify our yeast isolates, as well as the percent identity. Among these 16 species, eight were found in more than one location, while

the remaining eight species were each found in only one location each (Table 2).

Table 1. Sampling locations, sample sizes, and the yeast species diversity among soil samples from Cameroon

Region	Local site	Geographic coordinates	Total number of soil samples	No. of soil samples with yeasts (%)	Soil pH	Number of yeast species	Yeast species diversity index
Northwest	Mbingo	6.1640546 / 10.64054	51	29 (56.8%)	6.5	6	0.78
Northwest	Bambui	6.046586 / 10.232775	50	14 (28%)	6.3	8	0.9
Northwest	Njinikejum	6.254341 / 10.298138	50	19 (38%)	6.4	6	0.6
Northwest	Babanki	6.121895 / 10.266294	49	11 (22.4%)	6.9	5	0.7
Littoral	Makepe	4.146774 / 9.839220	47	25 (53.2%)	7.01	6	0.81
Center	Eloundem	5.403848 / 11.844950	98	11 (11.2%)	6.97	4	0.48
Center	Mbalgong	4.447906 / 11.902442	51	1 (1.9%)	7.07	1	NA*
Center	Simbock	4.639568 / 12.045264	52	0 (0%)	7.10	0	NA*
Center	Mbandoumou	3.779870 / 11.511865	45	0 (0%)	7.02	0	NA*
Total	-	-	493	110 (22.3%)	-	16	-

* **Due to their small sample sizes, these samples were excluded from the Simpson's Diversity Index calculations.**

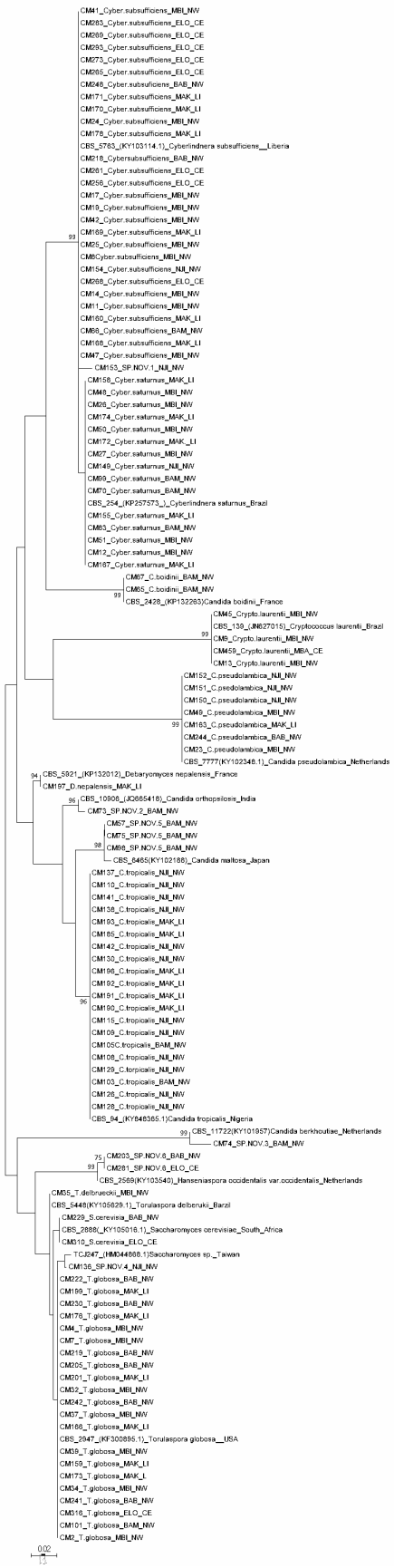
Table 2. Summary information about the yeast species identified from Cameroon soil. Included in the table are the GenBank accession numbers of the closest reference strains, the sites where each species was found in our samples, the percentage of sequence identity, and the number of yeast isolates in each species.

Species	Closest GenBank Accession Number	Sites	Percent sequence identity	Number of yeast isolates
Candida boidinii	KP132263	Bambui(NW)	99%	2
Candida pseudolambica	KM384611	Mbingo(NW) Njinikejum(NW) Makepe(LI) Babanki(NW)	98%	7
Candida tropicalis	KY102481	Bambui(NW) Njinikejum(NW) Makepe(LI)	99%	20
Cryptococcus laurentii	HM469461	Mbingo(NW) Mbalgong(CE)	99%	4
Cyberlindnera saturnus	KJ706378	Mbingo(NW) Bambui(NW) Njinikejum(NW) Makepe(LI)	99%	15
Cyberlindnera subsufficiens	KY103114	Mbingo(NW) Bambui(NW) Njinikejum(NW) Makepe(LI) Babanki(NW) Eloundem(CE)	99%	28
Debaryomyces nepalensis	KP132012	Makepe(LI)	100%	1
Torulaspora globosa	KY105654	Mbingo(NW) Bambui(NW) Makepe(LI) Babanki(NW) Eloundem(CE)	99%	21
Torulaspora delbrueckii	KP132792.1	Mbingo(NW)	99%	1
Saccharomyces cerevisiae	KY105016	Babanki(NW) Eloundem(CE)	99%	2
*SP.NOV.1	LC229712	Njinikejum(NW)	98%	1
*SP.NOV.2	FM178396	Bambui(NW)	90%	1
*SP.NOV.3	KY101957.1	Bambui(NW)	87%	1
*SP.NOV.4	HM044867	Njinikejum(NW)	99%	1
*SP.NOV.5	KY102188	Bambui(NW)	96%	3
*SP.NOV.6.	KU218502	Babanki(NW) Eloundem(CE)	96%	2

***Novel species and their closest matches to reference sequences in NCBI GenBank**

Figure 1 (next page). Phylogenetic relationships among the 110 isolates based on their ITS sequences. Their closest GenBank matches based on NCBI Blast and UNITE database searches are shown, along with the soil samples numbers, the strains references, and regions /local sites (Mbingo, Bambui,

Njinkejum, Babanki in the Northwest (NW) region, Makepe in the Littoral (LI) region, and Eloundem, Mbalgong, Simbock, and Mbandoumou in the Center (CE) region).


CM41_Cyber.subsufficiens_MBI_NW
CM283_Cyber.subsufficiens_ELO_CE
CM269_Cyber.subsufficiens_ELO_CE
CM293_Cyber.subsufficiens_ELO_CE
CM273_Cyber.subsufficiens_ELO_CE
CM265_Cyber.subsufficiens_ELO_CE
CM248_Cyber.subsufficiens_BAB_NW
CM171_Cyber.subsufficiens_MAK_LI
CM170_Cyber.subsufficiens_MAK_LI
CM24_Cyber.subsufficiens_MBI_NW
CM178_Cyber.subsufficiens_MAK_LI
CBS_5763_(KY103114.1)_Cyberlindnera subsufficiens__Liberia
CM218_Cybersubsufficiens_BAB_NW
CM261_Cyber.subsufficiens_ELO_CE
CM256_Cyber.subsufficiens_ELO_CE
CM17_Cyber.subsufficiens_MBI_NW
CM19_Cyber.subsufficiens_MBI_NW
CM42_Cyber.subsufficiens_MBI_NW
CM169_Cyber.subsufficiens_MAK_LI
99
CM25_Cyber.subsufficiens_MBI_NW
CM8Cyber.subsufficiens_MBI_NW
CM154_Cyber.subsufficiens_NUI_NW
CM268_Cyber.subsufficiens_ELO_CE
CM14_Cyber.subsufficiens_MBI_NW
CM11_Cyber.subsufficiens_MBI_NW
CM160_Cyber.subsufficiens_MAK_LI
CM66_Cyber.subsufficiens_BAM_NW
CM168_Cyber.subsufficiens_MAK_LI
CM47_Cyber.subsufficiens_MBI_NW
CM153_SP.NOV.1_NUI_NW
CM158_Cyber.saturnus_MAK_LI
CM48_Cyber.saturnus_MBI_NW
CM26_Cyber.saturnus_MBI_NW
CM174_Cyber.saturnus_MAK_LI
CM50_Cyber.saturnus_MBI_NW
CM172_Cyber.saturnus_MAK_LI
CM27_Cyber.saturnus_MBI_NW
CM149_Cyber.saturnus_NUI_NW
CM99_Cyber.saturnus_BAM_NW
CM70_Cyber.saturnus_BAM_NW
CBS_254_(KP257573.)_Cyberlindnera saturnus_Brazil
CM155_Cyber.saturnus_MAK_LI
CM83_Cyber.saturnus_BAM_NW
CM51_Cyber.saturnus_MBI_NW
CM12_Cyber.saturnus_MBI_NW
CM167_Cyber.saturnus_MAK_LI
CM67_C.boidinii_BAM_NW
CM65_C.boidinii_BAM_NW
99
CBS_2428_(KP132283)Candida boidinii_France
CM45_Crypto.laurentii_MBI_NW
CBS_139_(JN827015)_Cryptococcus laurentii_Brazil
99
CM8_Crypto.laurentii_MBI_NW
CM459_Crypto.laurentii_MBA_CE
CM13_Crypto.laurentii_MBI_NW
CM152_C.pseudolambica_NUI_NW
CM151_C.psedolambica_NUI_NW
CM150_C.pseudolambica_NUI_NW
CM49_C.pseudolambica_MBI_NW
99
CM163_C.pseudolambica_MAK_LI
CM244_C.pseudolambica_BAB_NW
CM23_C.pseudolambica_MBI_NW
CBS_7777(KY102346.1)_Candida pseudolambica_Netherlands
94
CBS_5921_(KP132012)_Debaryomyces nepalensis_France
CM197_D.nepalensis_MAK_LI
96
CBS_10906_(JQ365418)_Candida orthopsilosis_India
CM73_SP.NOV.2_BAM_NW
CM57_SP.NOV.5_BAM_NW
CM75_SP.NOV.5_BAM_NW
98
CM98_SP.NOV.5_BAM_NW
CBS_6465(KY102188)_Candida maltosa_Japan
CM137_C.tropicalis_NUI_NW
CM110_C.tropicalis_NUI_NW
CM141_C.tropicalis_NUI_NW
CM138_C.tropicalis_NUI_NW
CM193_C.tropicalis_MAK_LI
CM185_C.tropicalis_MAK_LI
CM142_C.tropicalis_NUI_NW
CM130_C.tropicalis_NUI_NW
CM196_C.tropicalis_MAK_LI
CM192_C.tropicalis_MAK_LI
96
CM191_C.tropicalis_MAK_LI
CM190_C.tropicalis_MAK_LI
CM115_C.tropicalis_NUI_NW
CM109_C.tropicalis_NUI_NW
CM105C.tropicalis_BAM_NW
CM108_C.tropicalis_NUI_NW
CM129_C.torpicalis_NUI_NW
CM103_C.tropicalis_BAM_NW
CM126_C.tropicalis_NUI_NW
CM128_C.tropicalis_NUI_NW
CBS_94_(KY848365.1)Candida tropicalis_Nigeria
99
CBS_11722(KY101957)Candida berkhoutiae_Netherlands
CM74_SP.NOV.3_BAM_NW
75
CM203_SP.NOV.8_BAB_NW
99
CM281_SP.NOV.8_ELO_CE
CBS_2569(KY103540)_Hanseniaspora occidentalis var.occidentalis_Netherlands
CM35_T.delbrueckii_MBI_NW
CBS_5448(KY105629.1)_Torulaspora delbrueckii_Barzil
CM229_S.cerevisia_BAB_NW
CBS_2888(_KY105016.1)_Saccharomyces cerevisiae_South_Africa
CM310_S.cerevisia_ELO_CE
TCJ247_(HM044868.1)Saccharomyces sp._Taiwan
CM136_SP.NOV.4_NUI_NW
CM222_T.globosa_BAB_NW
CM169_T.globosa_MAK_LI
CM230_T.globosa_BAB_NW
CM176_T.globosa_MAK_LI
CM4_T.globosa_MBI_NW
CM7_T.globosa_MBI_NW
CM219_T.globosa_BAB_NW
CM205_T.globosa_BAB_NW
CM201_T.globosa_MAK_LI
CM52_T.globosa_MBI_NW
CM242_T.globosa_BAB_NW
CM37_T.globosa_MBI_NW
CM166_T.globosa_MAK_LI
CBS_2947_(KF300895.1)_Torulaspora globosa__USA
CM39_T.globosa_MBI_NW
CM159_T.globosa_MAK_LI
CM173_T.globosa_MAK_L
CM34_T.globosa_MBI_NW
CM241_T.globosa_BAB_NW
CM316_T.globosa_ELO_CE
CM101_T.globosa_BAM_NW
CM2_T.globosa_MBI_NW
0.02

2.5.2 Yeast diversity and distribution

The yeast isolation rates differed significantly between different geographical regions. The four sampled sites in the Northwest Region yielded the most yeast isolates:

At Mbingo, 29 of the 51 soil samples contained yeasts (56.8%), with the most commonly found yeast species being *C. subsufficiens* (34.4%), followed by *T. globosa* (24.13%), *C. saturnus* (20.6%), *C. laurentii* (10.3%), *C. pseudolambica* (6.8%), and *T. delbrueckii* (3.4%).

At Njinikejum, we obtained yeasts from 19 of the 50 soil samples (38%). At this site, *C. tropicalis* was the most prevalent (63.1%), followed by three isolates of *C. pseudolambica* (15.7%), and one isolate (5.2%) each of *C. subsufficiens*, *C. saturnus*, SP.NOV.1, and SP.NOV.4.

At Bambui, we obtained yeasts from 14 of the 50 soil samples (28%), with species distribution as follows: three isolates (21.4%) each of *C. saturnus* and SP.NOV.5; two isolates (14.2%) each of *C. tropicalis* and *C. boidinii*, and one isolate (7.1%) each of *C. subsufficiens*, *T. globosa*, SP.NOV.2, and SP.NOV.3.

At Babanki, eleven yeast strains were obtained from 49 soil samples (22%); these included six isolates of *T. globosa* (54.5%), two isolates of *C. subsufficiens* (18.1%), and one isolate (9.0%) each of *C. pseudolambica*, SP.NOV.4, and SP. NOV.6 (Tables 2 & 3).

Table 3. The distribution of yeast species in nine locations within Cameroon. Our analysis rejected the null hypothesis of no difference in yeast species distribution among the geographic locations.

Species	Northwest				Littoral	Center			
	Mbingon (%)	Bambuin (%)	Njinikejum n (%)	Babanki n (%)	Makepen (%)	Eloundem n (%)	Mbalgong n (%)	Mbandoumoun (%)	Simbockn (%)
Candida boidinii	0(0%)	2(14.2%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
Candida pseudolambica	2(6.8%)	0(0%)	3(15.7%)	1(9.0%)	1(4%)	0(0%)	0(0%)	0(0%)	0(0%)

Candida tropicalis	0(0%)	2(14.2%)	12(63.1%)	0(0%)	6(24%)	0(0%)	0(0.0%)	0(0.0%)	0(0%)
Cryptococcus laurentii	3(10.3%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(100%)	0(0%)	0(0%)
Cyberlindnera saturnus	6(20.6%)	3(21.4%)	1(5.2%)	0(0%)	5(20%)	0(0%)	0(0%)	0(0%)	0(0%)
Cyberlindnera subsufficiens	10(34.4%)	1(7.1%)	1(5.2%)	2(18.1%)	6(24%)	8(72.7%)	0(0%)	0(0%)	0(0%)
Debaryomyces nepalensis	0(0%)	0(0%)	0(0%)	0(0%)	1(4%)	0(0%)	0(0%)	0(0%)	0(0%)
Torulaspora globosa	7(24.13%)	1(7.1%)	0(0%)	6(54.5%)	6(24%)	1(9.0%)	0(0%)	0(0%)	0(0%)
Torulaspora delbrueckii	1(3.4%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
Saccharomyces cerevisiae	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(9.0%)	0(0%)	0(0%)	0(0%)
**SP.NOV.1*	0(0%)	0(0%)	1(5.2%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
**SP.NOV.2*	0(0%)	1(7.1%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
**SP.NOV.3*	0(0%)	1(7.1%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
**SP.NOV.4*	0(0%)	0(0%)	1(5.2%)	1(9.0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
**SP.NOV.5*	0(0%)	3(21.4%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
**SP.NOV.6.*	0(0%)	0(0%)	0(0%)	1(9.0%)	0(0%)	1(9.0%)	0(0%)	0(0%)	0(0%)
Total	29(56.8%)	14(13%)	19(38%)	11(22.4%)	25(53.19%)	11(11.2%)	1(1.9%)	0(0%)	0(0%)

Compared to the Northwest Region, the yeast isolation rate was lower for soil samples from the Central Region, with only 12 of the 246 (8.05%) soil samples found to contain yeasts:

At Eloundem of the Center Region, we obtained yeasts from 11 of the 98 soil samples (11.2%). These included eight isolates of *C. subsufficiens* (72.7%), and one isolate (9.0%) each of *T. globosa, S. cerevisiae*, and SP. NOV. 6.

At Mbalgong of the Center Region, only one yeast isolate (*Cryptococcus laurentii*) was obtained from the 51 soil samples (1.9%).

Overall, the rate of yeast isolation from soil samples originating from the Northwest Region was statistically higher than that from the Center Region ($P = <0.00001$). The Littoral Region was excluded from the statistical comparison due to the fact that only one location was sampled in this region.

Among the obtained yeasts, two species, *C. subsufficiens* and *T. globose*, were commonly found among the geographic samples from both the Northwest and the Central Regions, corresponding to 25.4% and 19% of all the yeasts respectively in our total yeast population. In contrast, several species (SP.NOV.1, SP.NOV.2, SP.NOV.3, SP.NOV.4, SP.NOV.5, *C. boidinii*, *T. delbrueckii*) were each isolated from only one location from the Northwest Region, while *D. nepalensis* was isolated only from the Littoral Region (Makepe).

2.5.3 ITS phylogeny construction

Figure 1 depicts the phylogenetic tree constructed based on the ITS sequences of the 110 yeast isolates as well as their closely related GenBank sequences. Bootstrap support for individual branches was obtained using 1000 random replications (Figure 1). The analyses showed close relationships between the two species in the genus *Cyberlindnera, C. saturnus*, and *C. subsufficiens.*

2.5.4 PCR fingerprinting results

Based on the combined PCR fingerprinting results of the M13 and $(GACA)_4$ primers, we constructed a dendrogram among isolates to illustrate the genetic diversity within individual species in our samples. Below we briefly discuss the results.

In total, twenty PCR fingerprint genotypes were found among the 21 *T. globosa* isolates. The only shared genotype was between two isolates from Babanki of the Northwest Region. Of the remaining 19 isolates/genotypes; seven were from Mbingo (NW), six in Makepe (LI), five from Babanki (NW), and one each from Eloundem (CE) and Bambui (NW). Though some isolates from the same local or regional populations were more similar to each other, isolates from the three regions did not exclusively cluster based on their geographic locations but were dispersed among each other (Supplementary Figure S1). This result is consistent with some degree of gene flow among the local yeast populations in Cameroon.

Similar to that in *T. globosa*, a high genotypic diversity was observed in the *C. tropicalis* population. Specifically, each of the 20 isolates of *C. tropicalis* belonged to a different genotype. The

20 isolates were from two regions: the Northwest region and the Littoral region. The majority of the isolates/genotypes (12 of the 20) came from Njinikejum (NW), six from Makepe (LI), and the remaining two from Bambui (NW). The isolates from Njinikejum and Makepe were inter-dispersed with each other on the phylogram, consistent with some degree of gene flow among the regions for C. *tropical* (see Figure S2).

The twenty-eight isolates of *C. subsufficiens* belonged to 25 PCR fingerprinting genotypes (Figure S3). Thirteen of the 25 genotypes were from the Northwest, including nine genotypes from Mbingo, two from Babanki, and one each from Bambui and Njinikejum. Six of the 25 genotypes were from Makepe (LI) and eight were from Eloundem (CE). Four isolates from Makepe (LI) and Mbingo (NW) shared two genotypes, with each genotype shared by one isolate from each of the two locations.

The fifteen isolates of *C. saturnus* belonged to 15 different genotypes (Figure S4). Six of the genotypes were found in Mbingo (NW), three from Bambui (NW), five from Makepe (LI), and the remaining one was from Njinikejum (NW). There was no shared genotype within or among the different locations for this species. However, isolates from the NW and LI were interspersed among each other in the dendrogram (Figure S4), consistent with gene flow between the two regions for this species.

The seven isolates of *C. pseudolambica* belonged to six genotypes (Figure S5). Six of the seven isolates were from the Northwest with each of the six having a different genotype, including two genotypes from Mbingo (NW) and three genotypes from Njinikejum (NW). The only isolates from outside of the NW region [i.e., from Makepe (LI)] shared the genotype with one isolate from Babanki (NW).

The four isolates of *C. laurentii* belonged to three genotypes (Figure S6). Of the three genotypes, two were from Mbingo (NW), with one genotype containing two isolates from this region. The remaining genotype was represented by one isolate from Mbalgong.

Of the remaining 10 (putative) species, each was represented by three or fewer isolates, and our PCR fingerprinting results showed that they all had different PCR fingerprinting genotypes. For species

represented by only one isolate each, their Simpson's genotype diversity index was not calculated (Table 4).

Table 4: Strain genotype, sample size, and the genetic diversity index of the common yeast species in Cameroon.

Species	No. isolates	No. genotypes	Genetic Diversity Index	Geographic location (Region, number of isolates)
Candida boidinii	2	2	1	Bambui (NW, 2)
Candida pseudolambica	7	6	0.9	Mbingo (NW, 2) Njinikejum (NW, 3) Makepe (LI, 1) Babanki (NW, 1)
Candida tropicalis	20	20	1	Bambui (NW, 2) Njinikejum (NW, 12) Makepe (LI, 6)
Cryptococcus laurentii	4	3	0.8	Mbingo (NW, 3) Mbalgong (CE, 1)
Cyberlindnera saturnus	15	15	1	Mbingo (NW, 6) Bambui (NW) #3 Njinikejum (NW, 1) Makepe (LI, 5)
Cyberlindnera subsufficiens	28	27	0.9	Mbingo (NW, 9) Bambui (NW, 2) Njinikejum (NW, 1) Makepe (LI, 6) Babanki (NW, 2) Eloundem (CE, 8)
Torulaspora globosa	21	20	0.9	Mbingo (NW, 7) Bambui (NW, 1) Makepe (LI, 6) Babanki (NW, 6) Eloundem (CE, 1)
SP.NOV.5	3	3	1	Bambui (NW, 3)
SP.NOV.6	2	2	1	Babanki (NW, 1) Eloundem (CE, 1)

2.5.6 Yeast species diversity based on geographical sites

The yeast species richness and Simpson's species diversity index for different sites within each of the three regions in Cameroon were calculated. The highest yeast species richness was found in Bambui (NW); eight species were found among the 14 yeast isolates with a species diversity index of 0.89. Three local populations from Mbingo, Makepe, and Njinikejum contained six species each, from

among 29 (diversity = 0.78), 25 (diversity =0.81), and 19 (diversity=0.58) isolates, respectively. Of the remaining locations, five species were found from the 11 isolates in Babanki, (diversity=0.7); four species among 11 isolates in Eloundem (diversity=0.48), and only one isolate (species) in Mbalgong.

2.5.7 Relationship between yeast species diversity and other factors

The pH values in our soil samples ranged from pH 6.4 to 7.10, with the highest value found in Mbandoumou. The sites of the Center Region generally had lower pH values than other sites. However, we found no apparent correlation between soil pH and yeast biodiversity (Pearson correlation coefficient = -0.18, $p < 0.6$) (Detailed calculations not shown). Interestingly, however, when we compared soil yeast isolation rates with those of *Aspergillus fumigatus* from the same soil samples, a statistically significant negative correlation was found (Pearson correlation coefficient = -0.67, $p<0.04$). Specifically, the presence of *A. fumigatus* in the soil was more likely to be associated with no yeast than those without *A. fumigatus* (see Figures S7 & S8).

2.6 Discussion

One hundred and ten yeast isolates belonging to six genera and 16 (putative) species were isolated from 493 soil samples. Table 2 provides a list of isolated species (15 ascomycetes and one basidiomycete) and their rates in Cameroonian regions/sites representing two climatic zones (Tropical Monsoon and Tropical Savanna climates). Our study yielded several yeast species that have been frequently reported from soil samples in other parts of the world. These included *C. tropicalis*, *C. saturnus*, *C. subsufficiens*, and *T. globosa* (Nomoto *et al.*, 1984; Salgado Vital *et al.*, 2002; Kothavade *et al.*, 2010; Rosa *et al.*, 2010). These four species were found at more than one site in our samples (Figure 1 and Table 2) and they represented about 25% of the total yeast isolates obtained in our soil samples. In addition, PCR fingerprinting analyses showed that some genotypes were shared between regions/ sites and that isolates were not clustered exclusively based on geographic locations; these results are consistent with some levels of gene flow between sites and regions in Cameroon. Below we focus on the relevance of the four common yeast species in discussing the implications of the results.

Cyberlindnera subsufficiens and *C. saturnus* are two of the 22 yeast species in the genus *Cyberlindnera* [20]. Species in this genus are commonly found in soils and plant surfaces collected in several countries/regions such as the Himalayas, Austria, and Liberia (Naumova *et al.*, 2004). Interestingly, isolates of *C. saturnus* as well as several other yeast species from natural environments have been reported to be capable of producing killer toxins (mycocines) with a wide spectrum of activities against competing fungal (including yeast) species (Rosini, 1983; Nomoto *et al.*, 1984; Marquina *et al.*, 2002). Whether the isolates of *C. saturnus* and *C. subsufficiens* obtained here can also produce toxins and kill other fungi remains to be analyzed. However, based on the significant negative correlation between the isolation rates of yeasts and the filamentous fungus *A. fumigatus* described recently in the same Cameroonian soils, it is possible that there were antagonistic interactions between yeasts and *A. fumigatus* in their natural environments. Further studies are needed in order to identify the nature of this potential antagonistic interaction and their potential application for biocontrol of fungal infectious diseases. Aside from fungal toxins, the isolates obtained here may have other applied importance. For example, a recent study reported that a strain of *C. saturnus* from a tropical mangrove wetland in India showed the ability to assimilate xylose and produce xylitol, an industrially important compound [24]. The large number of strains of *C. saturnus* as well as other yeasts isolated here represent a potential resource from which to screen for industrially, medically, and agriculturally important activities.

Given the monsoon and tropical climates in our study sites, the high rate of isolation of *C. tropicalis* was not surprising. In our study, *C. tropicalis* was most prevalent in soil samples from Njinikejum in the Northwest Region (63.1%, Table 2). Our finding has significant medical implications for this region. *Candida tropicalis* is among the most common opportunistic yeast pathogens in humans and is especially prevalent in tropical regions, responsible for up to 66% of cases of candidemia (Ann Chai *et al.*, 2010; Kothavade *et al.*, 2010). A recent meta-analysis on candida species responsible for oral candidiasis in HIV and AIDS–infected African patients revealed that *C. tropicalis* was the second most common yeast species causing invasive fungal infections, accounting for 22% of all cases in

immunocompromised patients[26]. Furthermore, a recent study showed that *C. tropicalis* might be prone to become resistant to a variety of antifungal drugs[27]. Our results suggest that local public health in Cameroon should be aware of the epidemiology of fungal infections in this region.

The fourth species we would like to discuss here is *T. globosa.* Similar to the other three species described above, *T. globosa* is broadly distributed in the soil environments, especially in tropical regions. In our samples, *T. globosa* represents 19.1% of the 73 yeasts isolated from the Northwest (Tropical Monsoon Climate) and 8.3 % of the 12 yeasts from the Tropical Savanna Climate (Table 2). Several studies have reported positive effects of strains of *T. globosa* in the biocontrol of phytopathogens. For example, *T. globosa* was reported to be effective at controlling the sugarcane fungal pathogen *Colletotrichum sublineolum* [19]. Its abundance in the rhizosphere as reported previously is also consistent with its ecological roles in the soil ecosystems (El-Tarabily & Sivasithamparam, 2006; Rosa-Magri *et al.*, 2011).

Of the 110 yeast isolates obtained from the Cameroon soil samples, nine had ITS sequences that exhibited significant sequence divergence with all the deposited sequences in the current NCBI GenBank database and the UNITE database, greater than those that have been shown for their closely related species pairs. These nine isolates belong to six groups within which the strains shared >97% ITS sequence identity among each other. Of these six groups, four showed ITS sequence identity of less than 97% to any known sequence while two showed 98% and 99% of sequence identities to two known sequences. However, there was no formal species name attached to these two sequences. Based on the most commonly used criteria for operational taxonomic unit (OTU) identification of fungi, these six groups likely represent six novel yeast species. The closest relatives of these six species were distributed in several genera including *Cyberlindnera* (closest species were *C. subsufficiens* and *C. saturnus*), *Hanseniaspora* (closest being *H. occidentalis)*, *Candida* (closest species include *C. orthopsilosis*, *C. maltosa*, *C. berkhoutiea*), and *Saccharomyces* (Figure 1). The findings in the present study are consistent with significant novel species diversity revealed in an earlier study for soil samples from Africa based on metagenome analyses[30].

We noticed that there was very limited sequence difference at the ITS locus between *C. subsufficiens* and *C. saturnus*. Indeed, only one nucleotide site showed a difference between these two species: at site 173 of ITS1, *C. subsufficiens* contains a gap, whereas *C. saturnus* contains *C* at that position. Similar results have also been reported in many other yeast species pairs at the ITS region[6]. Despite the low level of sequence divergence between the two species, they can be distinguished using the diagnostic nucleotide at this particular position. However, this difference is too small to meet the general barcode gap requirement. Indeed, sequences at secondary or even tertiary barcodes are needed for further confirmation[31].

The two main regions sampled here, the Northwest and the Center Regions, showed different yeast isolation rates and yeast species distributions. At present, the reasons for the observed differences are not known. However, several factors could have contributed. One of these factors is climatic conditions. The Northwest Region was the coolest of the three regions and had the highest rain fall during the month of June when the samples were collected in 2016. Previous studies have found that a relatively average temperature and the availability of water favor the growth of fungi, including yeasts. Another reason lies in the difference in soil factors such as soil pH, nutrient levels, and other soil microorganisms. Earlier studies demonstrated that acidic pH favors the growth of fungi, including yeasts, while alkaline pH favors other microorganisms such as bacteria [32]. In the present study, however, there was no observed correlation between yeast colonization and soil pH ($p < 0.6$). On the other hand, we found a statistically significant negative correlation between *Aspergillus fumigatus* presence and yeast presence in these soil samples, suggesting that competition among fungi might be an important factor determining yeast distribution. Indeed, several studies have reported the influence of vegetation properties and soil type on soil yeast diversity. The study suggest that agriculture soil may reduce the species diversity in the system more than natural soil with diverse plant species and less interfered from human[33].

A high genotypic diversity was found for all yeast species recovered from the Cameroon soil samples. This result is consistent with the hypothesis that these species have likely existed in Cameroon for a long time and are likely native residents of the soil environments there. Furthermore, though

relatively limited, evidence for DNA fingerprint genotype sharing was found in a few cases, consistent with some level of gene flow within and between the locations. Human activities such as travelling and the exchange of agricultural and commercial goods could have contributed to some of the genotype sharing.

In summary, we obtained 110 yeast isolates from 493 soil samples from Cameroon. These yeasts were identified as belonging to 16 (putative) species, with ten having already been described previously and the remaining six likely represent new species. Eight of 16 species were present in more than one location. Soil samples from different regions in Cameroon had different yeast isolation rates ranging between 0%-56%. Furthermore, the geographic populations of yeasts had different yeast species compositions, and strains from the same species were genetically highly diverse, with some evidence of clonal dispersal and gene flow. Our comparative analyses suggested that climate conditions and other components of the soil microbiota have likely contributed to the differences in both yeast isolation rate and species distribution patterns. We briefly discussed the implications of these results to human health and to the biocontrol of plant fungal pathogens.

2.7 Acknowledgments

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2.8 Author contributions statement

J.X. designed the study; T.A. collected the soil samples; R.A. and H.S. performed the experiments; R.A. and J.X. analyzed the data; R.A. and J.X. drafted the manuscript.

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Supplementary figures

FigureS1: Genotype diversity based on PCR-fingerprinting of *Transpire globosa*

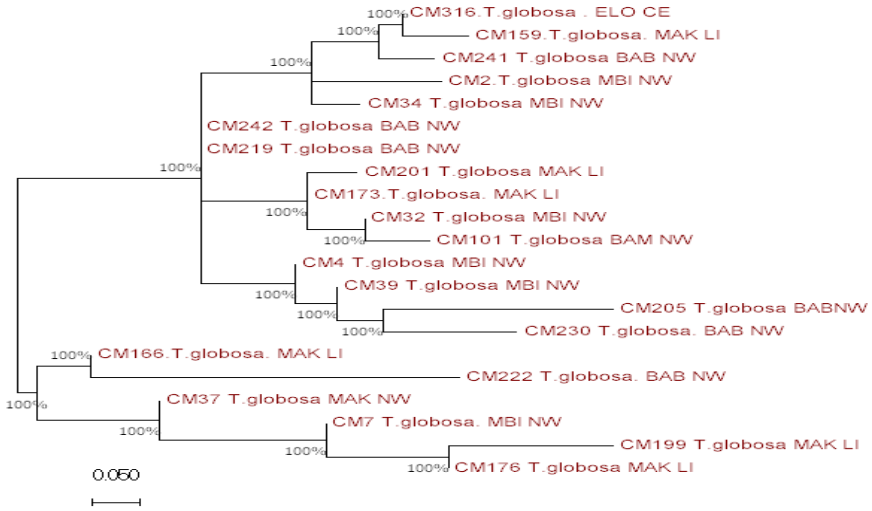


Figure S2: Genotype diversity based on PCR-fingerprinting of *Candida tropicalis*

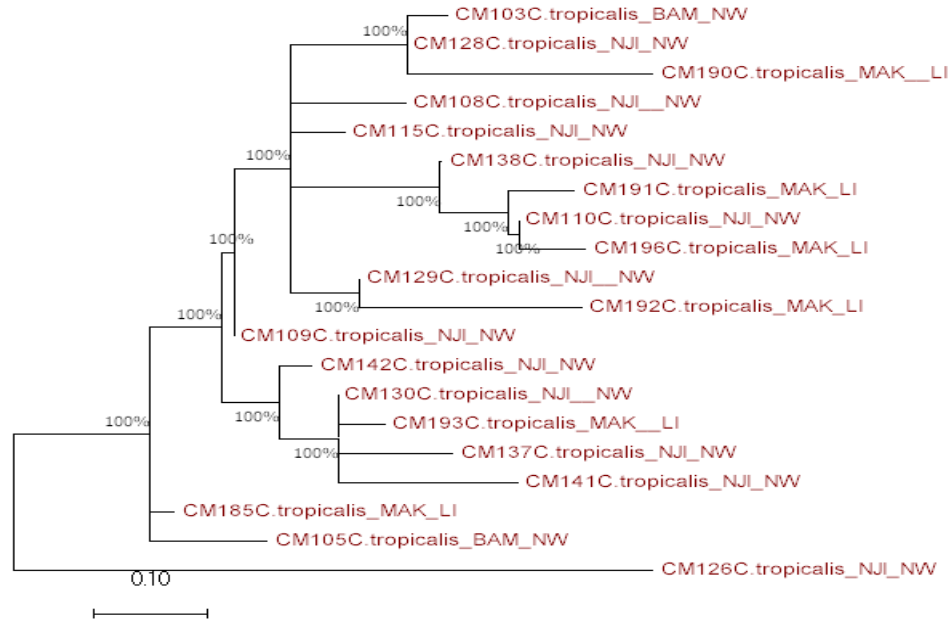


Figure S3: Genotype diversity based on PCR-fingerprinting of Cyberlindnera subsufficiens

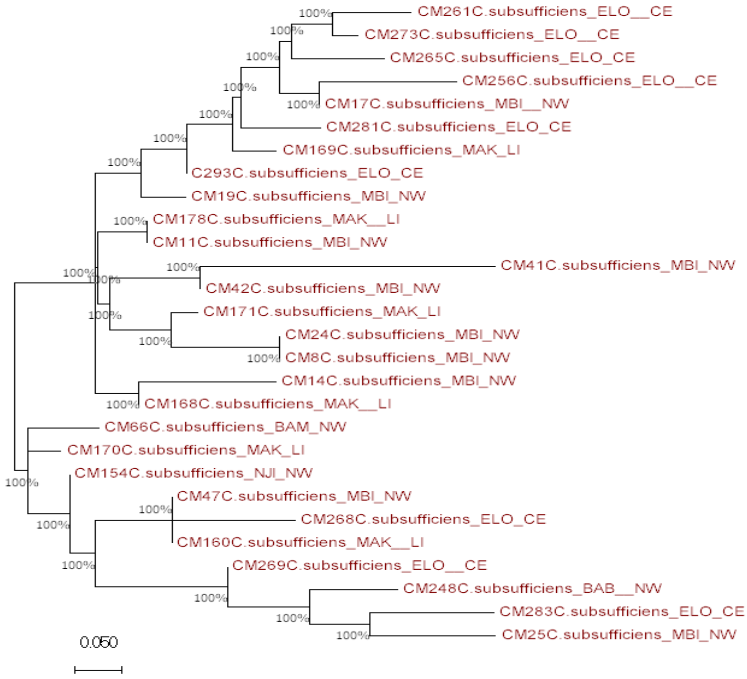


Figure S4: Genotype diversity based on PCR-fingerprinting of *Cyberlindnera saturnus*

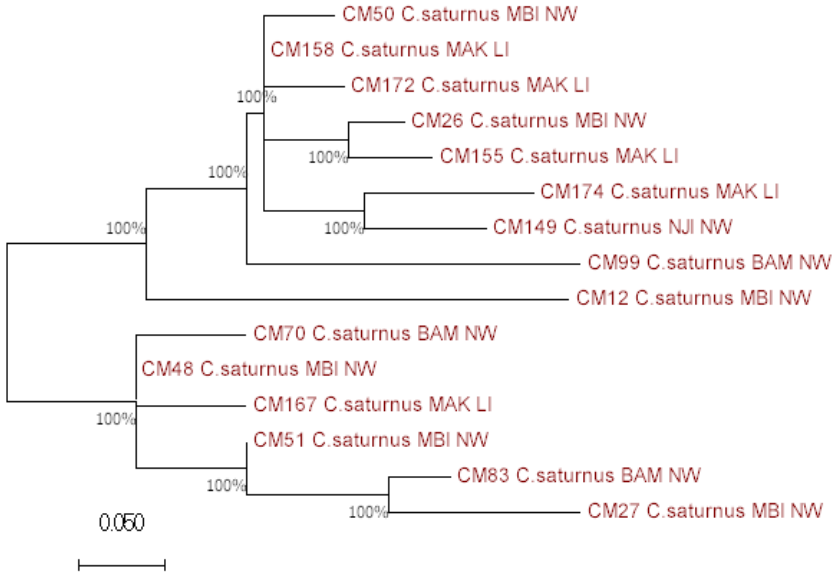


Figure S5: Genotype diversity based on PCR-fingerprinting of *Candida pseudolambiac*

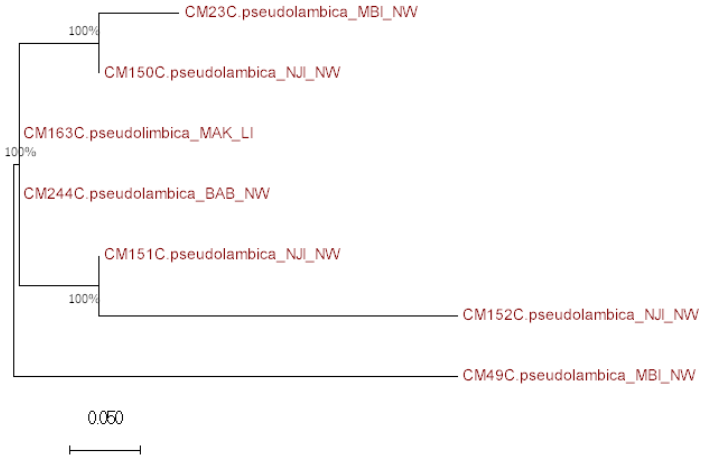
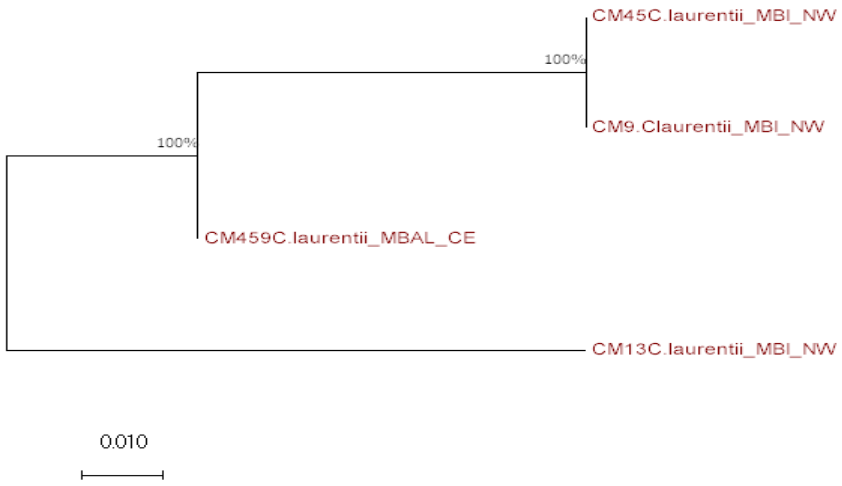


Figure S6: Genotype diversity based on PCR-fingerprinting of *Cryptococcus laurentii*



Chapter 3: The Diversity of Soil Yeasts in Saudi Arabia

3.1 Preface

The occurrence of yeasts in soil has been studied in various parts of the world. However, there are no available studies on the distribution and diversity of soil yeasts in the Middle East, particularly in Saudi Arabia. Here we obtained and analyzed 562 soil samples from six sites in Saudi Arabia. Our results showed a high rate of *Cryptococcus neoformans* isolation in the western region of Saudi Arabia and significantly different distributions of yeast diversity among the analyzed regions in Saudi Arabia.

I am the primary contributor of this work. Jianping Xu designed the experiments, and I conducted the majority of the experiments. Analyses and writing of the manuscript were also predominantly completed by myself and Jianping Xu.

This chapter has been written as a draft manuscript; it is being prepared for submitting to the Canadian Journal of Microbiology.

Authors of this article are: Renad Aljohani, Jianping Xu.

Abstract

A soil environment consists of a variety of physical, biological, and chemical factors that can directly or/and indirectly affect the diversity of yeasts found in the soil. Yeasts fulfill a range of important ecological functions, yet current understanding of the yeast biodiversity in Saudi Arabian soil is limited. The aim of this study is to investigate the diversity of soil yeast and its genetic distribution in Saudi Arabia. In the present study, we analyzed 562 soil samples from six sites and four different geographical regions in Saudi Arabia. A total of 114 yeast isolates were identified based on the culture-based method. The identified yeasts were characterized to the species level based on sequences at their internal transcribed spacer (ITS) regions within the nuclear ribosomal RNA gene cluster. Their genotype profiles were also assessed based on DNA-PCR fingerprinting. The results revealed an abundant distribution of *Cryptococcus neoformans* in two sites in the west coast regions. Overall, there were significant differences in yeast species richness and diversity between the analyzed geographical regions in Saudi Arabia.

Introduction

Soil is the environment with links between plant production, water concentration, and microbial activities. Soil also is accountable for many functions of natural ecosystems, such as regulations of temperature, carbon dioxide, and nutrient contents (Arroyo-López *et al.*, 2009). The soil ecosystems are diverse and complex and is one of the richest reservoirs of microorganisms. Estimates are that 1 gram of agricultural soil may contain several billion colony forming units of microorganisms belonging to thousands of different species (Neale & Philipps, 2002). In addition, soil microbes significantly influence a wide range of soil properties. Yeasts are unicellular fungi that participate in a wide range of important ecological functions. The ubiquity of yeasts in natural ecosystems is due to their ability to inhabit a wide range of environments such as soil, water (marine and freshwater), and animals (De Clercq & Inzé, 2006; Date *et al.*, 2017; Glushakova *et al.*, 2017). Yeasts are common soil inhabitants and have been isolated from nearly every type of soil. Soil yeasts are vital for plant growth, nutrient cycling, decomposition, and soil quality. For instance, soil yeasts are important decomposers which ultimately can result in increased nutrient availability for plants, including crops (Botha, 2011).

Many studies have reported significant influences of soil structure, soil moisture, and soil temperature on fungal diversity and community structure (Mccalla, 1950). The concentration of water within soils plays a crucial role in governing fungal / yeast growth and activities (Climate & Development, 2013). Water in the soil ecosystem serves as a carrier of nutrients and hydrogen / oxygen to the fungal / yeast population. Different types of yeasts require different amounts of water concentration to reproduce and grow. A majority of yeast populations require a minimum water activity of 0.85 or a minimum relative humidity of 88% (Corpetti,). For example, the initial growth rate of *Cryptococcus albidus* in sterile sandy loam soil is significantly lower in soil with 1% or 2% less water than in soil with higher percentages of added water (Vishniac, 1995). Similarly, soil temperature can have direct effects on yeast community structure and their activities in ecosystems. Yeasts are active in a very broad temperature range of 20°C to 45°C, with an optimal temperature range of 20°C to 30°C. Many studies have shown that the synthesis of enzymes is affected by the growth temperature

(Newsham *et al.*, 2015). For example, seasonal changes in soil temperature significantly affect the growth of yeast populations and their fermentation process (Meyer *et al.*, 1993; Evans *et al.*, 2004). Thus, changes in temperature and moisture may significantly affect the diversity and distribution of yeast communities (Hood, 2006). Because yeasts are important decomposers and also a food source for other organisms such as nematodes and insects, factors that influence their activities and decomposition rate can further impact the functions of natural ecosystems as a whole (Talbot *et al.*, 2008).

The soil environments contain many fungal species that can cause human infections. Pathogenic fungi pose a serious threat to human health through skin contact and inhalation of dust particles. If the soil environments contain rich organic compounds such as manure and rotten fruits, these pathogenic fungi can become more prevalent (Talbot *et al.*, 2008; Leite *et al.*, 2012; Kemoi *et al.*, 2013). For example, the existence of a large number of free-living wild birds (e.g., pigeons), which are found in major cities and frequently live close to humans, creates organic-rich microenvironments that favor the growth of many fungi such as Cryptococcus spp., Candida spp., Malassezia spp., and Rhodotorula spp. and they pose threats to public health (Radimersky et al., 2010). These fungi are opportunistic pathogens, commonly found as responsible for infections in immunocompromised patients. There is a significant burden of these infections in the Middle East (Leal *et al.*, 2008; Richardson & Lass-Flörl, 2008; Zarrin & Mahmoudabadi, 2010; Kauffman *et al.*, 2011; Al Thaqafi *et al.*, 2014; Imran & Ali, 2014; Moghnieh *et al.*, 2017). Due to the importance of yeast infections in humans and the prevalence of yeasts in natural environments, understanding the structure of the environmental populations of yeasts can help us develop better control and prevention strategies.

In this study, we obtained 562 soil samples from six geographical sites within four regions in Saudi Arabia. Saudi Arabia represents almost 80% of the Arabian Peninsula, and it is bordered by several countries: Jordon, Iraq, Kuwait, Qatar, Bahrain, the United Arab Emirates, Oman, and Yemen. Saudi Arabia has a desert climate, and most of its terrain consists of arid desert and a series of mountains that extend through the Southwestern region and the western region (Blihid et al., 1975; Congress, 2006). Except the southwestern region, most of Saudi Arabia has a high annual-temperature and a low precipitation. A desert climate with high-temperature conditions increases

evaporation and reduces moisture in soil, resulting in salt precipitation. Indeed, salt concentrations in the subsoil of high-temperature climates of Saudi Arabia are invariably high. Ground water in some places is found to have a salt content as high as that found in sea water (Soc, 2002). At present, there is no published information on fungal diversity in Saudi Arabian soils. Based on the climate and soil conditions, we expect that fungal diversity in Saudi Arabia will be low, with slow decomposition of plant materials in the moisture-deprived soils. The objectives of this study are to characterize the species diversity of soil yeasts and genetic diversity among strains within individual yeast species in Saudi Arabia.

Material and methods

Soil sample collection

A total of 562 soil samples were collected from six sites in four Saudi Arabian regions: Yanbu (Yan), Jeddah (JED), and Al-medina (ALM) in the Western region, Umluj (UM) in the Northwest, Alqunfudah (ALQ) in the Southwest, and Dammam (DAM) in the East. The soil type and including potential vegetation were different among the regions including, agriculture soil , deserts, clay and sands). The samples were taken during the month of December (coolest, dry season) in 2016. Each sample was approximately 1 gram and was collected at a depth of approximately 5 – 10 cm below the surface; geographic coordinates are shown in Table 1. For all sampling procedures, the soil samples were deposited independently in 2/ml sterile tubes and transferred to the McMaster University laboratory, where they were stored at 4 °C and processed within 72- 120 hours after arrival. The soil pH measurements were determined by adding 0.1 g of soil to 1ml sterilized water, vortexing and allowing the soil to rehydrate for at least 1 h at 23°C, then measuring using a bench-top Orion pH meter and confirming by pH strips. Ten soil samples were measured for their soil pH at each site (Table 1).

Isolation of yeast

In order to isolate yeast, 0.1 g of the soil was transferred into 1 ml of autoclaved Sabouraud Dextrose Broth (SDB) containing 0.035 mg/ml of the antibiotic chloramphenicol. The samples were then vortexed for 10 seconds and incubated at 30°C for 2 days. Samples then were sub-cultured onto Yeast Extract-Peptone-Dextrose agar (YEPD) and grown for 2 days at 30°C. Samples with morphologically potential yeast or a yeast- like colony were streaked onto YEPD medium and incubated for anther 48 h at 30°C for purification of yeast cells. The purified yeast colonies were collected for DNA extraction, and the yeast cells were sorted in YEPD broth with 30% glycerol at 80°C. For soil samples that failed to yield yeasts in the first attempt, an additional attempt with another 0.5g of soil was transferred into 1 ml of autoclaved SDB containing 0.035 mg/ml of the antibiotics chloramphenicol and penicillin sodium salt, to eliminate possible competition for nutrients in the soil.

DNA extraction and Identification of yeast species

Total genomic DNA extraction was performed using a previously described protocol (Xu *et al.*, 2000). All isolated strains were subjected to sequencing of the internal transcribed spacer (ITS) region. DNA was amplified using the primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). PCR protocols have been described by Aljohani et al. (2018). The ITS region was sequenced by the MOBIX Laboratory of McMaster University and was compared to sequences reported in the GenBank database by using the BLAST algorithm. Phylogenetic analysis was performed using the molecular evolutionary genetics analysis (MEGA) version 7 using neighbor-joining analysis. Bootstrap analysis (1000 replicates) was used to investigate the relationship between the identified species (Kumar *et al.*, 2016).

PCR fingerprinting pattern

DNA fingerprinting was performed to estimate the genetic variation between the identified yeast strains within species that we obtained in the study. PCR fingerprinting with microsatellite-specific oligonucleotides was derived from two separate primers: (i) the M13-core sequence (5'-

GTAAAACGACGGCCAGT-3'), and (ii) the repeat sequence $(GACA)_4$ (5'-GACAGACAGACAGACA-3'). These markers have been used to determine the potential genetic variations among strains within many fungal species (Pulvirenti *et al.*, 2001). For both primers, the PCR included a denaturing step at 98°C for 2 min, followed by 45 cycles at 93°C for 20s, 50°C for 45s, 72°C for 20s, followed by a final extension step at 72°C for 6 min. The amplification products were analyzed by electrophoresis in 2 % agarose gel with 1 X TBE buffer for 3 hours at 80 V. The gels were stained with ethidium bromide and photographed under ultraviolet light. Band sizes were estimated by comparison with 1kb and 100bp ladders as references. The genetic relationships among the yeast strains were determined using the MEGA 7 software.

Serotyping and mating type profile

Serotyping and mating type profiles were analyzed of the *Cryptococcus neoformans* strains that were isolated in this study. Serotyping and mating types were determined by PCR amplification of the PRT1 region using PRT1F (5'-TGGTGGAACGGACGCAATGG-3') and PRT1R (5'-TCTCTCTGCGACGTT GGGCG-3'). The PCR protocol included an initial denaturation step of 95°C for 4 minutes, followed by 40 cycles of 45s at 95°C, 45s at 62°C, and 1minute and 15s at 72°C, with a final extension of 7 minutes. This was followed by double digestion with the restriction enzymes HhaI and DdeI in a total of volume of 20 µl that contained 8 µl of PCR products, 2µl of 10X CutSmart Buffer, 0.2 µl of HhaI enzyme (one unit), 0.4 µl of DdeI enzyme (one unit), and 9.4 µl Nuclease-free Water. The samples were incubated at 37°C for 3.5 hours. The digested products were electrophoresed on 1.5 % agarose gel with 1X TAE buffer for 1 hour at 100 V. The patterns were assigned via visual comparison with patterns obtained for standard strains of the major molecular types of JEC20, JEC21, KN99a, and Kn99α as reference strains (NEB, 2013). In addition, we typed the URA5 gene to determine the genetic relationship among selective *C. neoformans* strains obtained in the present study with sequence data from the GenBank, using the URA5F primer (5'- ATGTCCTCCCAAGCCCTCGAC-3') and URA5R (5'-TTAAGACCTCTGAACACCGTACTC-3'). A dendrogram tree based on sequences at the URA5 gene region was constructed using software MEGA7.

Statistical data analyses

We quantified the percentage of yeast isolated from each of the six sites and the four regions in Saud Arabia as described in Aljohani et. al. (2018). Yeast species diversity was measured in terms of the richness and evenness given by Simpson's index, using the formula as $(1 - \sum p_i^2)\ N/(N-1)$. Similarly, the genetic diversity was quantified for each strain within the same species using the same formula. The value of Simpson's diversity ranges between 0 and 1; index value 1 represents high diversity, and 0 represents no diversity. Statistical differences of yeast species diversity between regions were determined by a Chi-square test using the formula $X^2 = \sum (O^i - E^i)^2 / E^i$, where O^i is the total number of yeast species in each site and E^i is the sum of the species isolated in the study divided by the number of sites.

The statistical significance of yeast isolation rate differences and yeast species composition between Cameroon and Saudi Arabia was determined using a T-test with the null hypothesis that there is no difference between different locations/regions within the two countries. For all statistical tests, differences were considered significant at $P < 0.05$.

Results

Identification and community composition of yeasts

Soil at six sites in four regions within Saudi Arabia were sampled in December 2016 to isolate yeasts (Table 1). In all, 114 yeast strains were obtained. They were identified by their sequences at the ITS regions using the UNITE and GenBank databases. Their accession numbers and percent sequence identities to their closest related match at the ITS regions are shown in Table 2. To ensure a clear identification, only the hits with a similarity > 97% were retained to the species level. The ITS sequences revealed that the 114 yeast strains can be assigned to 10 species (belonging to both ascomycetous and basidiomycetous genera): *Aureobasidium pullulans var. pullulans, Cryptococcus neoformans, Cryptococcus diffluens, Candida parapsilosis, Candida tropicalis, Debaryomyces hansenii,*

Rhodotorula glutinis, Rhodotorula mucilaginosa, and *Rhodotorula toruloides*. One isolate from Jeddah showed a sequence similarity of ~90% to the closest match in GenBank, that of *Cystofilobasidium sp*. (Table 2, Figure 1*)*. Among these species, three were found in more than one sites, while the rest were each found in only one site. Except *Cryptococcus diffluens* that was isolated in both the Western and Southwest regions, none of the four collection regions shared any of the species isolated (Tables 2 and 3).

Table 1: Summary information sites, soil samples for each site, and the diversity of yeast species in Saudi Arabia.

Region	Local site	Geographic coordinates	Total no. of soil samples	No. of soil sample with yeasts (%)	Soil pH	No. of yeast species	Yeast species diversity
West	Yanbu	24.0232° N, 38.1900° E	197	79(40.1 %)	6.8	6	0.1
West	Jeddah	21.2854° N, 39.2376° E	100	29(29%)	6.8	4	0.5
Northwest	Umluj	25.0500° N, 37.2651° E	129	1(0.77%)	6.4	1	NA*
West	Medina	24.5247° N, 39.5692° E	50	2(4%)	6.4	2	1
East	Dammam	26.3927° N, 49.9777° E	56	2(3.5%)	7.00	1	NA*
Southwest	Alqunfudah	19.1281° N, 41.0787° E	32	1(3.1%)	7.00	1	NA*
Total	-	-	562	114(20.2%)	-	10	-

***This site was excluded from the Simpson's species diversity statistical calculation due to the fact that only one species was isolated from this site in Saudi Arabia.**

Table 2: Identification of yeast isolated from each site on the basis of ITS sequences, identity percentage, and their ID and accession number with GenBank submissions.

Species	Closest GenBank Accession Number	Sites	Percent sequence identity	Number of yeast isolates
Aureobasidium pullulans var. pullulans	KX893321	Al medina(WE)	100%	1
Cryptococcus neoformans	KY102911	Yanbu(WE) Jeddah(WE)	100%	91
Cryptococcus diffluens	KY104326	Yanbu(WE) Jeddah(WE) Al-medina(WE) Alqunfudah(SW)	99%	5
Candida parapsilosis	KY694996	Umluj(NW)	99%	1
Candida tropicalis	KY848365	Yanbu(WE)	100%	3
Cystofilobasidium sp.	KM275211	Jeddah(WE)	90%*	1
Debaryomyces hansenii	KP132005	Yanbu(WE)	99%	1
Rhodotorula glutinis	KY104781	Yanbu(WE)	99%	2
Rhodotorula mucilaginosa	KY611824	Dammam(EA)	100%	2
Rhodotorula toruloides	KY104918	Yanbu (WE) Jeddah (WE)	99%	7

- **Potential new species and its closest match with reference species in NCBI GenBank**

Yeast diversity and distribution

Basidomycetous species constituted six of the 10 yeast species isolated from Saudi Arabian soils. Based on their ITS sequence data, a tree was constructed to illustrate the relationship of yeast cultures isolated in this study. The species represented in this study are dominated by Basidomycetous yeasts which represented 108 strains (60%) (Tables 2 and 3). The percent of yeast isolated from each genus was as follows:

- *Aureobasidium pullulans var. pullulans* species that belong to *Aureobasidium* genus was found in only one site Al medina (WE) (0.8%).
- *Cryptococcus neoformans,* belonging to the genus *Cryptococcus,* was isolated in only two sites, Yanbu and Jeddah, in the western coast regions (79.1%).

- *Cryptococcus diffluens,* belonging to the genus *Cryptococcus,* was isolated in more than one site: Yanbu(WE), Jeddah(WE), Al-medina(WE), and Alqunfudah (SW) with 4.3%.
- *Candida parapsilosis and Candida tropicalis,* belonging to *Candida* genus, were found in two sites, Al-medina and Yanbu, with a prevalence of 0.8% and 2.6%, respectively
- *Debaryomyces hansenii,* belonging to *Debaryomyces* genus, was only found in Yanbu (0.8%).
- *Rhodotorula glutinis, Rhodotorula mucilaginosa,* and *Rhodotorula toruloides,* all belonging to the genus *Rhodotorula*, were found in more than one site: two of each were isolated in Yanbu and Dammam (1.7%), one in Yanbu, and six in Jeddah (6.0%).

There were significant differences in the yeast isolation rate and yeast composition among different geographical sites within Saudi Arabia. The sampled sites in the Western region yielded the most yeast isolation with 96.4 % out of all the yeast isolated in the study, compared to the other regions in Saudi Arabia where the isolation rate was lower with only 4 (3.5%) isolates total out of 129 soil samples from Umluj, 56 soil samples from Dammam, and 32 soil samples from Alqunfudah. The soil samples in these regions contained one isolate of *Candida parapsilosis* from Umluj(NW), two isolates of *Rhodotorula mucilaginosa* from Dammam(EA), and one isolate of *Cryptococcus diffluens* from Alqunfudah (SW). In sum, the result of yeast isolation rate from the soil samples obtained from the six sites was significantly higher in the sites from the Western region than the sites from the Southwest, Northwest, and East regions ($P = <0.00001$).

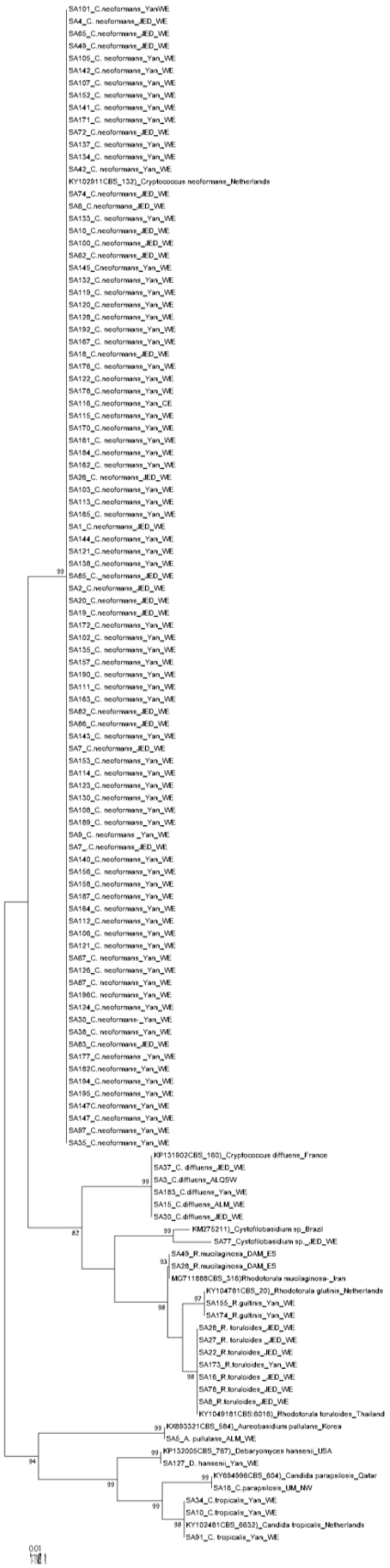
The Simpson's species diversity index was calculated to compare the richness and evenness between different geographical regions/sites in Saudi Arabia with respect to regional geographic differences. The highest species diversity was measured in Yanbu with six species out of 79 isolates, with a species diversity index of 0.1. This was followed by Jeddah with four species out of 29 isolates (diversity = 0.5), Medina with two species out of two isolates (diversity = 1), and three population sites from Umluj, Dammam, and Alqunfudah that only contained one isolate (species) of each (Table 1). Umluj, Dammam, and Alqunfudah were excluded from the Simpson's species diversity statistical calculation due to the fact that only one species was isolated from each of these sites.

Table 3: The distribution of species in each region/site in Saudi Arabia.

Species	West			Northwest	Southwest	East
	Yanbu	Jeddah	Al-medina	Umluj	Alqunfudah	Dammam
Aureobasidium pullulans var. pullulans	(0%)	(0%)	1(50%)	(0%)	(0%)	(0%)
Cryptococcus neoformans	71(89.8%)	20(68.9%)	(0%)	(0%)	(0%)	(0%)
Cryptococcus diffluens	1(1.2%)	2(6.8%)	1(50%)	(0%)	1(100%)	(0%)
Candida parapsilosis	(0%)	(0%)	(0%)	1(100%)	(0%)	(0%)
Candida tropicalis	3(3.7%)	(0%)	(0%)	(0%)	(0%)	(0%)
Cystofilobasidium sp.*	(0%)	1(3.4%)	(0%)	(0%)	(0%)	(0%)
Debaryomyces hansenii	1(1.2%)	(0%)	(0%)	(0%)	(0%)	(0%)
Rhodotorula glutinis	2(2.5%)	(0%)	(0%)	(0%)	(0%)	(0%)
Rhodotorula mucilaginosa	(0%)	(0%)	(0%)	(0%)	(0%)	2(100%)
Rhodotorula toruloides	1(1.2%)	6(20.6%)	(0%)	(0%)	(0%)	(0%)

ITS phylogeny construction

Figure 1 shows the phylogenetic tree depicting the relationship of yeast cultures isolated in this study to ITS GenBank sequences of published species. Bootstrap support for individual branches was obtained using 1000 random replications. The strains were isolated from six sites in four Saudi Arabian regions: Yanbu (Yan), Jeddah (JED), and Al-medina (ALM) in the Western region, Umluj (UM) in the Northwest, Alqunfudah (ALQ) in the Southwest, and Dammam (DAM) in the East.


SA101_C.neoformans_YanWE
SA4_C. neoformans_JED_WE
SA65_C.neoformans_JED_WE
SA49_C.neoformans_JED_WE
SA105_C. neoformans_Yan_WE
SA142_C.neoformans_Yan_WE
SA107_C. neoformans_Yan_WE
SA152_C. neoformans_Yan_WE
SA141_C. neoformans_Yan_WE
SA171_C. neoformans_Yan_WE
SA72_C.neoformans_JED_WE
SA137_C. neoformans_Yan_WE
SA134_C. neoformans_Yan_WE
SA42_C. neoformans_Yan_WE
KY102911CBS_132)_Cryptococcus neoformans_Netherlands
SA74_C.neoformans_JED_WE
SA6_C.neoformans_JED_WE
SA133_C. neoformans_Yan_WE
SA10_C.neoformans_JED_WE
SA100_C.neoformans_JED_WE
SA62_C.neoformans_JED_WE
SA145_Cneoformans_Yan_WE
SA132_C.neoformans_Yan_WE
SA119_C. neoformans_Yan_WE
SA120_C.neoformans_Yan_WE
SA128_C.neoformans_Yan_WE
SA192_C. neoformans_Yan_WE
SA167_C. neoformans_Yan_WE
SA18_C.neoformans_JED_WE
SA176_C. neoformans_Yan_WE
SA122_C.neoformans_Yan_WE
SA178_C.neoformans_Yan_WE
SA116_C.neoformans_Yan_CE
SA115_C.neoformans_Yan_WE
SA170_C.neoformans_Yan_WE
SA161_C. neoformans_Yan_WE
SA184_C.neoformans_Yan_WE
SA162_C. neoformans_Yan_WE
SA26_C. neoformans_JED_WE
SA103_C.neoformans_Yan_WE
SA113_C.neoformans_Yan_WE
SA165_C. neoformans_Yan_WE
SA1_C.neoformans_JED_WE
SA144_C.neoformans_Yan_WE
SA121_C.neoformans_Yan_WE
SA138_C.neoformans_Yan_WE
SA85_C. neoformans_JED_WE
SA2_C.neoformans_JED_WE
SA20_C.neoformans_JED_WE
SA19_C.neoformans_JED_WE
SA172_C.neoformans_Yan_WE
SA102_C. neoformans_Yan_WE
SA135_C. neoformans_Yan_WE
SA157_C.neoformans_Yan_WE
SA190_C. neoformans_Yan_WE
SA111_C. neoformans_Yan_WE
SA183_C. neoformans_Yan_WE
SA82_C.neoformans_JED_WE
SA66_C.neoformans_JED_WE
SA143_C. neoformans_Yan_WE
SA7_C.neoformans_JED_WE
SA153_C.neoformans_Yan_WE
SA114_C. neoformans_Yan_WE
SA123_C.neoformans_Yan_WE
SA130_C.neoformans_Yan_WE
SA108_C. neoformans_Yan_WE
SA189_C. neoformans_Yan_WE
SA9_C. neoformans _Yan_WE
SA7_C.neoformans_JED_WE
SA140_C.neoformans_Yan_WE
SA156_C. neoformans_Yan_WE
SA158_C.neoformans_Yan_WE
SA187_C.neoformans_Yan_WE
SA164_C. neoformans_Yan_WE
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SA126_C. neoformans_Yan_WE
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SA186C. neoformans_Yan_WE
SA124_C.neoformans_Yan_WE
SA30_C.neoformans-_Yan_WE
SA38_C. neoformans_Yan_WE
SA83_C.neoformans_JED_WE
SA177_C.neoformans _Yan_WE
SA182C.neoformans_Yan_WE
SA194_C.neoformans_Yan_WE
SA195_C.neoformans_Yan_WE
SA147C.neoformans_Yan_WE
SA147_C.neoformans_Yan_WE
SA97_C.neoformans_Yan_WE
SA35_C.neoformans_Yan_WE
KP131902CBS_160)_Cryptococcus diffluens_France
SA37_C. diffluens_JED_WE
SA3_C.diffluens_ALQSW
SA183_C.diffluens_Yan_WE
SA15_C.diffluens_ALM_WE
SA30_C.diffluens_JED_WE
KM275211)_Cystofilobasidium sp_Brazil
SA77_Cystofilobasidium sp._JED_WE
SA49_R.mucilaginosa_DAM_ES
SA28_R.mucilaginosa_DAM_ES
MG711868CBS_316)Rhodotorula mucilaginosa-_Iran
KY104781CBS_20)_Rhodotorula glutinis_Netherlands
SA155_R.guitinis_Yan_WE
SA174_R.guitinis_Yan_WE
SA26_R. toruloides_JED_WE
SA27_R. toruloides _JED_WE
SA22_R.toruloides_JED_WE
SA173_R.toruloides_Yan_WE
SA16_R.toruloides _JED_WE
SA76_R.toruloides_JED_WE
SA8_R.toruloides_JED_WE
KY1049181CBS6016)_Rhodotorula toruloides_Thailand
KX893321CBS_684)_Aureobasidium pallulans_Korea
SA5_A. pallulans_ALM_WE
KP132005CBS_767)_Debaryomyces hanseniii_USA
SA127_D. hanseniii_Yan_WE
KY694996CBS_604)_Candida parapsilosis_Qatar
SA16_C.parapsilosis_UM_NW
SA34_C.tropicalis_Yan_WE
SA10_C.tropicalis_Yan_WE
KY102461CBS_6633)_Candida tropicalis_Netherlands
SA91_C. tropicalis_Yan_WE

DNA-Fingerprinting pattern analysis

Both primers (GACA)4 and the M13 core sequence generated polymorphic bands among the 114 yeast strains of the identified 10- species. The patterns generated with each of these primers were distinctive and highly reproducible (Figure 1). The number of bands obtained in the PCR fingerprint pattern depended on the primer that was used. With DNA obtained from these yeasts, primer M13 produced the fewer bands compared to (GACA)4, which yielded more bands. The PCR fingerprinting produced by these two primers' results were combined and used to distinguish the variation between strains within the same species at the genetic level. The results also revealed a high genetic diversity among strains within individual species, as described below.

In total, we found that the ninety-one isolates of *Cryptococcus neoformans* species belonged to 51 PCR fingerprinting genotypes. 29 of the 51 genotypes were from Jeddah (WS), and the remaining 22 genotypes were from Yanbu (WE). Only one strain from Jeddah shared one genotype from Yanbu. However, the strains from Yanbu were highly diverse and shared 12 genotypes out of the total 22 genotypes. The isolates from these two sites were interspersed among each other in the dendrogram (Figure S1).

The five isolates of *Cryptococcus diffluens* belonged to five genotypes (Figure S2). Four of the five isolates were from the Western region, with each having a different genotype: one genotype from Yanbu(WE), two genotypes from Jeddah (WE), and one genotype from Medina(WE). The only isolate from the Southwest region was from Alqunfudah (SW). There were no shared genotype among the different sites for this species. (Figure S2)

The seven isolates of *Rhodotorula toruloides* belonged to seven genotypes (Figure S3). Out of the seven genotypes, one was from Yanbu(WE) and contained one isolate. The remaining six genotypes were from Jeddah(WE), with each of the six having a different genotype.

The remaining seven species were represented by one to three isolates. The results also showed that they all have different genotypes. There were no shared species/ genotypes within or among all different

sites for any of the species. The species with only one isolate each were excluded from the statistical calculations shown in Table 4.

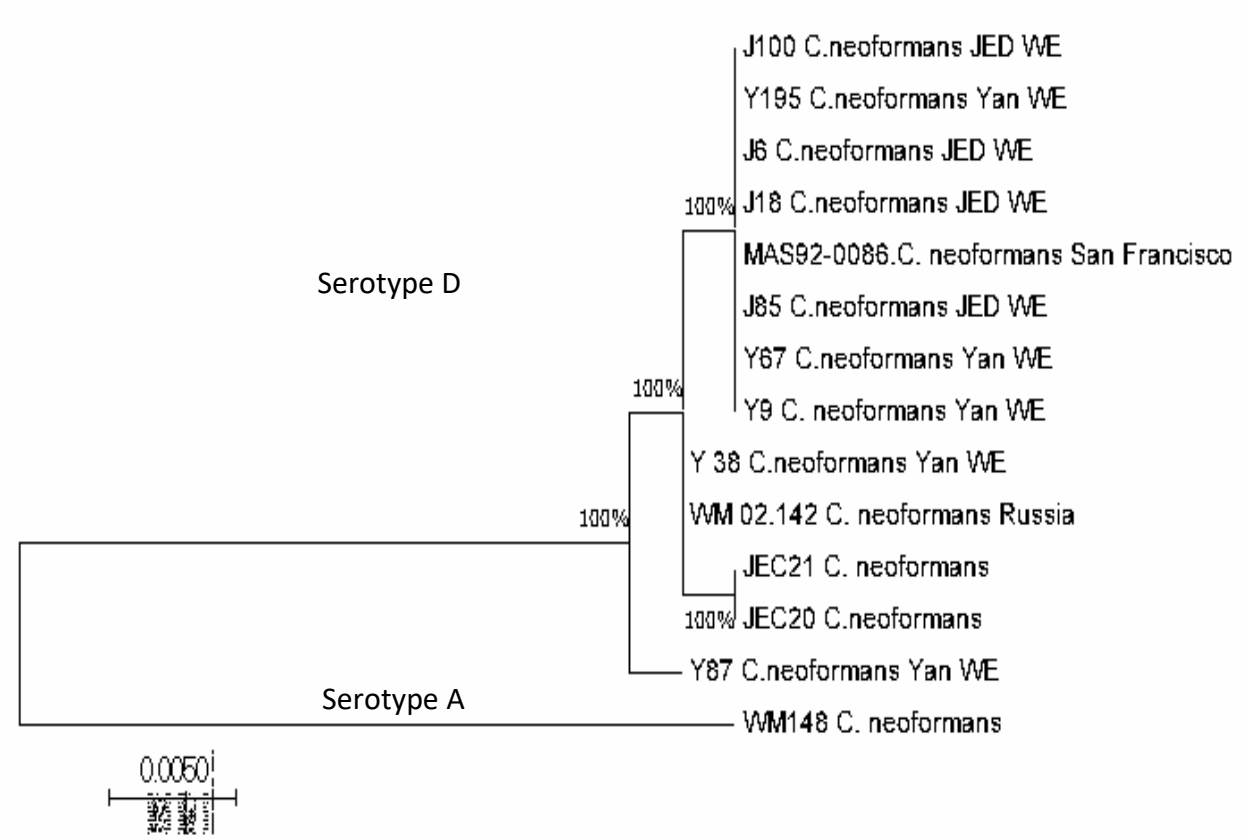
Table 4. The genetic diversity of the species isolated from each site in Saudi Arabia.

Species	No. isolates	No. genotypes	Genotype diversity	Geographic location (Region; # of isolates)
Cryptococcus neoformans	91	51	0.6	Yanbu (WE, #71) Jeddah (WE, #20)
Cryptococcus diffluens	5	5	1	Yanbu (WE, #1) Jeddah (WE, #2) Medina (WE, #1) Alqunfudah (SW, #1)
Candida tropicalis	3	3	1	Yanbu (WE, #3)
Rhodotorula glutinis	2	2	1	Yanbu (WE, #2)
Rhodotorula mucilaginosa	2	2	1	Dammam (EA, #2)
Rhodotorula toruloides	7	7	1	Yanbu (WE, #1) Jeddah (WE, # 6)

Serotyping and mating type profile

Further genetic investigation on the serotype and mating type of the *Cryptococcus neoformans* isolates showed that 90 of the 91 strains were serotype D, mating type alpha. Only one isolate (from Yanbu) was serotype D mating type a. Based on their PCR fingerprinting, this isolate also had a unique genotype. Then we sequenced the URA 5 gene of selected strains to allow comparison with known strains of *C. neoformans*. Our analyses showed that seven out of ten sequenced strains had identical URA5 sequences with each other and with a strain from San Francisco (MAS92-0086). Interestingly, the only Da strain shared an identical sequence with a strain from Brazil (WM 02.142). All the ten sequenced strains were different from the model strains in the lab, JEC20 and JEC21 (Figure 2).

Figure 2. The relationship between selected strains of *Cryptococcus neoformans* and reference strains from GenBank based on URA5 gene sequences.

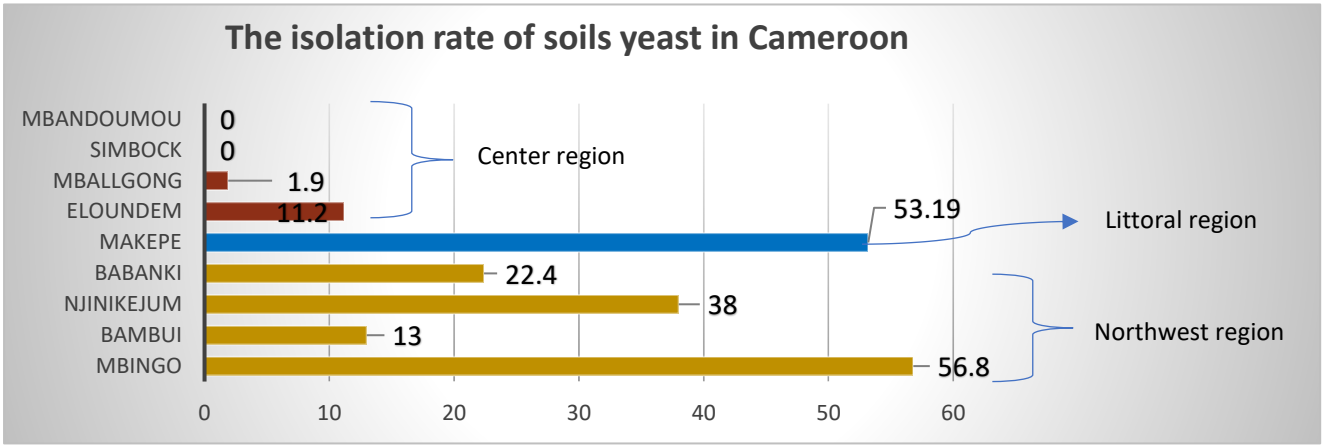


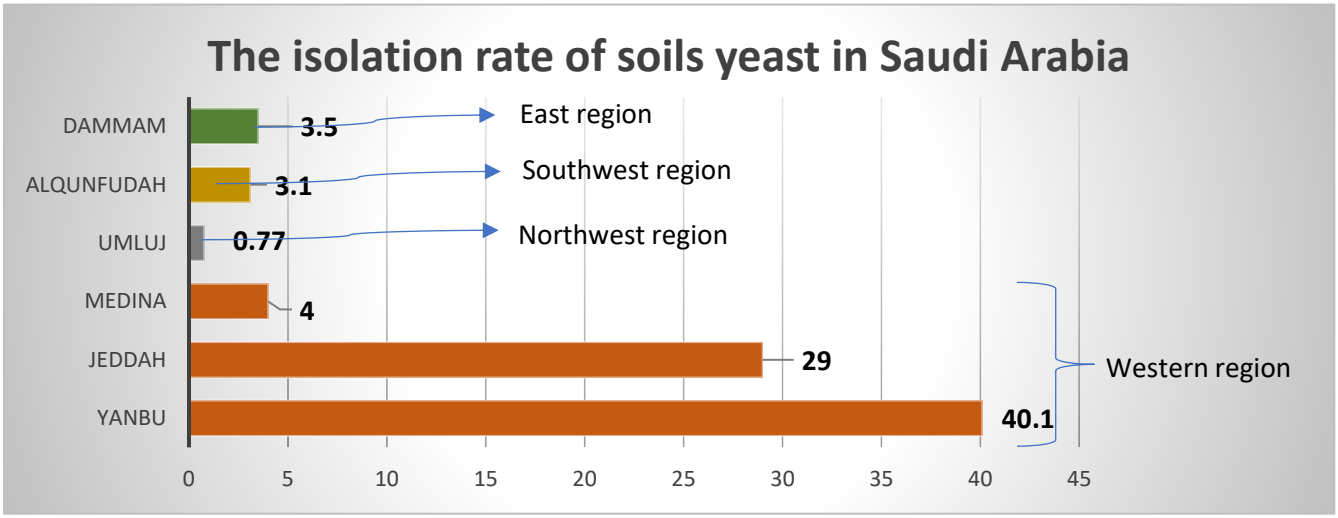
Comparison of yeast isolation between Cameroon and Saudi Arabia

The previous analysis of the diversity of culturable soil yeast from Cameroon is shown in Figure 3 (Aljohani *et al.*, 2018). A comparison was made of the difference in the number of diversity, compositions, and in the colonization rate of soil yeasts between the two ecologically distinct countries, Cameroon in center West Africa and Saudi Arabia in Southwest Asia. The two countries had very different climate conditions. For Cameroon, soils were all the top layer of agricultural field soils, while Saudi Arabian soils were from different types. The percent total yeast colonization at Cameroon sites were not significantly different from the colonization rate of Saudi Arabia sites (Table 1): 22.3 % and 20.2%, respectively (P value = 0.4). However, differences in yeast composition and species distribution

were observed among the analyzed regions within and between these two countries. In Cameroon, some of the species were represented in more than one region and were abundant in all three regions. In Saudi Arabia, all of the species were found only in one region, except *Cryptococcus diffluens* which was isolated from two regions. However, yeast species that were common in both countries included only one species, *Candida tropicalis*. In addition, yeast diversity was higher in Cameroon than in Saudi Arabia. It's likely that the divergent climates between Cameroon and Saudi Arabia contributed significantly to the yeast diversity and species distribution between the two countries.

Figure 3. Comparison of soil yeast isolation rate between collection sites within (a) Cameroon and (b) Saudi Arabia.





Discussion

One hundred and fifteen culturable yeast strains were isolated from 562 soil samples obtained from six local sites/ four regions in Saudi Arabia: three sites in the Western region, one site in the Southwest region, one site in the Northwest region, and one site in the East region. Except the geographic distances, all regions belonged to one climate (desert climate) with only a slight difference in the average temperature and total precipitation of rainfall (300mm between October and March) (Table 1 and Figure 1). The composition of identified species, their isolation rate, and distribution patterns in the Saudi Arabian soils were presented in Tables 2 and 3. All strains in this study were identified based on their ITS sequences analysis. These analyses showed that 114 isolate strains belonged to 10 species in 6 genera. These species have been isolated from soil in other parts of the world. Specifically, the following yeast species have been isolated at a high frequency worldwide: *Cryptococcus neoformans, Cryptococcus diffluens, Rhodotorula glutinis, Rhodotorula mucilaginosa,* and *Rhodotorula toruloides* (di Menna, 1955, 1966; Fracchia *et al.*, 2003; Gadanho *et al.*, 2006; Silva *et al.*, 2012; Cogliati *et al.*, 2017) (Figure 1 and Tables 2 &3). In addition, PCR fingerprinting analysis showed that isolates were clustered predominantly based on their geographic sites. The analysis of serotyping and mating types profiles of *Cryptococcus neoformans,* the dominant yeast in our samples, showed that they all belong to serotype D, with one being aD and the remaining 90 as αD (Figure 2).

Species of the Cryptococcus genus are commonly found in soil (Salgado Vital *et al.*, 2002). C. *neoformans* is classified into two varieties, three serotypes and five molecular types. C. *neoformans* and C. *gattii* are two sibling yeast species responsible for cryptococcosis, which is a life- threatening disease mainly associated with 62% of AIDS/HIV patients in the countries where HIV infection is high, such as sub-Saharan Africa and in Southeast Asia (Park *et al.*, 2009). However, it has been estimated that 10%- 40% of HIV-negative with cryptococcosis have no apparent underlying disease or immune deficiency (Kuroki *et al.*, 2004; Al-Tawfiq & Ghandour, 2007; Saeed *et al.*, 2015). Studies have reported that the major environmental source of C. *neoformans* throughout the world has been bird excreta. Indeed, pigeons are commonly found along the west coast of Saudi Arabia. The large number

of strains of C. *neoformans* as well as other opportunistic pathogenic yeasts found here suggest that the soil environment in western Saudi Arabia could be a significant source of human infectious diseases.

The importance of pigeons in C. neoformans colonization is shown both in this study and in previous studies. Here, the two sites with an extreme high isolation rate of C. *neoformans* (>79) are located in the Western region, which is the pigeon protection area. Abulreesh et al., (2015) first reported the environmental prevalence of Cryptococcus *neoformans* (>50%) and other Basidomycetous yeast in the same region. Indeed, birds and their droppings have been reported worldwide as a major source of the spread of yeast infection; they also can carry over 60 other diseases, and many of those diseases are airborne and can be transferred to humans just by being around droppings (Experientia, 1976). Pigeon droppings could especially be a potential carrier in the spread of pathogenic yeasts into an environment such as soil by providing a suitable breeding ground for a range of yeasts that can lead to certain diseases such as that caused by C. *neoformans* and manifested as meningoencephalitis, mainly in immunocompromised patients (Blanco-Peña *et al.*, 2017). It is very likely that pigeon droppings significantly contributed to the high distribution of this species in the area. Further studies are needed to determine the mechanisms of this contribution and where they were originally introduced to the soil environment in this region.

In addition, we also isolated another species of *Cryptococcus* genus from different sites in the Western and Southwest regions. C. *diffluens* is a basidiomycetous yeast that is widely distributed in soil environments. This also has been extensively reported as an opportunistic yeast pathogen in humans, and it has a major role in skin infection associated with 42% of atopic dermatitis (AD) patients and 20% of healthy individuals (Sugita *et al.*, 2003; Kantarcioğlu *et al.*, 2007; Kato *et al.*, 2007; Zhang *et al.*, 2011).

The second genus we would like to discuss here is *Rhodotorula* which included three species isolated in this study: *Rhodotorula glutinis, Rhodotorula mucilaginosa,* and *Rhodotorula toruloides.* These species were all isolated from more than one site of the analyzed regions. Similar to the other two species described above, *Rhodotorula* species are pigmented yeasts that are part of the Basidiomycota phylum, occurring naturally in air, lakes, ocean water, and soil (Salgado Vital *et al.*,

2002; Fracchia *et al.*, 2003). The genus contains 37 species total, including 34 non- pathogenic and three pathogenic ones: *R. glutinis, R. mucilaginosa,* and *R. toruloides.* The majority of the yeasts included in the species are mesophilic, although some of them thrive under lower temperatures, and aerobic organisms. The most common infection by these three species is fungemia, which accounts for 73% of all *Rhodotorula* infections ((Biswas *et al.,* 2001); Ments, 2001). Aside from soil environments as shown in our study, *R. mucilaginosa* is commonly found in fresh fruits, fruit juice, and cheese (Tournas *et al.*, 2006) and it's the third most commonly isolated yeast in sea water (Ekendahl *et al.,* 2003). In a study conducted between 1966 and 2006, fungemia infection of 66 patients was associated with catheters. *R. mucilaginosa* was responsible for most of the cases, followed by *R. glutinis* (Zarrin & Mahmoudabadi, 2010). Indeed, *Rhodotorula* species is considered an emerging opportunistic pathogen (Almeida *et al.*, 2018).

In our ITS sequence analysis of the 114 stains isolated from Saudi Arabia, one isolate revealed a significant divergent sequence with the closest related matches of all submitted sequences in the NCBI GenBank database. This isolate belongs to one basidiomycota group within which the strains shared greater than > 90% of ITS sequence identity among each other. The closest relative to this species was in the genera of *Cystofilobasidium sp.*

We noticed that there was a significant difference in yeast diversity, yeast composition, and isolation rate among the six sampled sites: Yanbu (Western), Jeddah (Western), Medina (Western), Umluj (Northwest), Alqunfudah (Southwest), Dammam (East). At present, the reasons for the differences are not known. However, a number of environmental characteristics may directly and/or indirectly have contributed to the yeast diversity and distribution patterns. One of these reasons is the climatic conditions and its effects on the soil structure and soil properties. A meteorological study analysis of the climatic conditions' effects on soil structure indicated that the temperature and moisture patterns are Aridic and hyperthermic (Resources & Service, 1999). The desert climate in Saudi Arabia would increase salt precipitation and reduce moisture in the soil, which directly affects soil yeast diversity and distribution (Hood, 2006; Arroyo-López *et al.*, 2009; Vigo & Building, 2009).

Another reason was raised in this study; we observed a high isolation rate of chloramphenicol-resistant bacteria in four sites out of six: Medina (WE), Umluj (NW), Alqunfudah (SW), and Dammam(EA) (data not shown). Such bacteria could reduce yeast growth in our enrichment protocol due to the rapid growth of bacteria and the associated rapid decline in nutrient availability to yeasts. Several studies have determined the prevalence of these resistance bacteria (< 48.6%) in the same areas (Yasir *et al.*, 2015; Alaidarous *et al.*, 2017; Alam *et al.*, 2017). The presence of bacteria suggests that competition among bacteria and yeast might be an important factor determining yeast distribution. In the Western region, the spread of Cryptococcus *neoformans* isolates in the area through pigeons and their dropping may result in overgrowth of this species over the rest of the isolations as discussed above and may be an important factor that affects the species distribution.

Our results of the genotypic diversity of all the strains from the same species was in clusters based on the geographic sites. However, PCR fingerprinting showed that the populations of yeasts belonging to the same species were highly heterogeneous. The high prevalence of mating type alpha strains of Cryptococcus *neoformans* in the Western region is consistent with previous studies of this species complex in most parts of the world (Steenbergen, 2000; Cogliati, 2013). The URA5 sequence analysis provided evidence for both genetic variability among strains within Saudi Arabia and similarities between the Saudi Arabian strains with those from other geographic regions.

To our knowledge, our study is the first to describe the isolation rate of culturable soil yeasts from in Saudi Arabia. We obtained and analyzed 114 yeast isolates from 562 soil types and different regions in Saudi Arabia. The yeast strains belonged to 10 species, and one isolate likely belonged to a new species. One species, C. *diffluens,* was represented in two regions: The Western and Southwest regions. The remaining species were each inclusively clustered in one region. The most common species was Cryptococcus *neoformans* (79%), a common opportunistic human fungal pathogen. Within each of the species with multiple isolates, we found extensive genetic variation among isolates. Our analysis suggests that climate as well as other environmental conditions likely contributed to the yeast species and genotype diversity and distribution pattern. Because the human

fungal infections in these regions in Saudi Arabia are not known, the medical implications of our results remain to be investigated.

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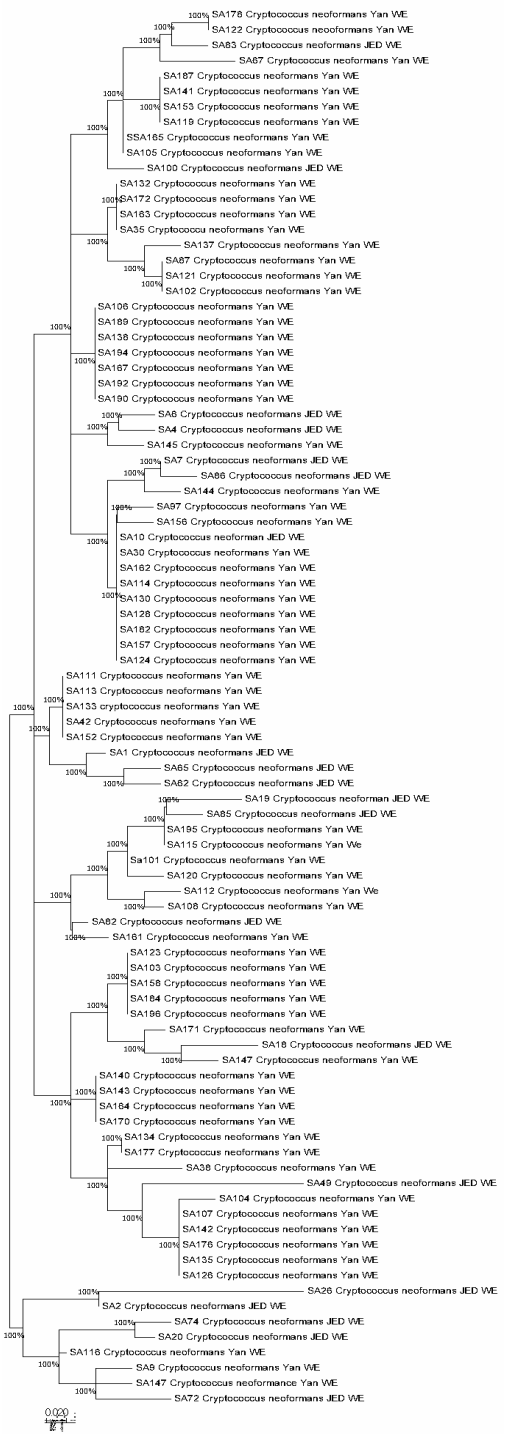
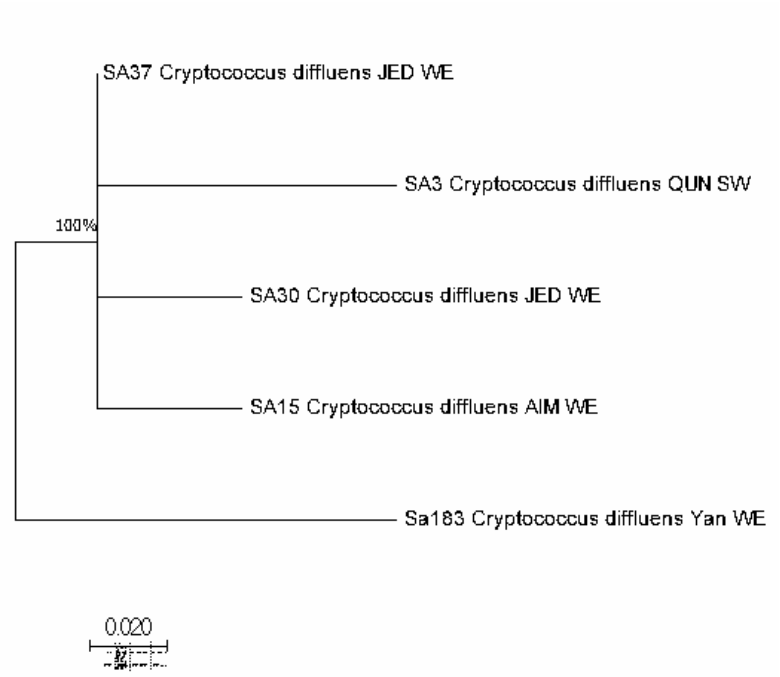
Supplementary figures

FigureS1: Genotype diversity based on PCR-fingerprinting of Cryptococcus neoformans



FigureS2: Genotype diversity based on PCR-fingerprinting of Cryptococcus diffluens



Chapter 4: General Conclusion

Overall, my thesis expands the current understanding of the diversity and population structure of culturable yeasts at diverse geographic scales. In Chapter 2, we found that soil samples from different geographic regions in Cameroon had different yeast compositions. The low isolation rate found in the soil in the center region of Cameroon is consistent with a previous study conducted in the same region (Stringini *et al.*, 2008). We reported evidence for some gene flow among geographic populations of the analyzed regions in Cameroon. Our results suggested that climatic conditions, temperature, above-ground and below-ground vegetation have likely contributed to the differences of the yeast diversity and species distribution pattern.

In Chapter 3, we investigated soil samples from different Saudi Arabian sites/regions and found that they differed in yeast abundance. We found a large number of *Cryptococcus neoformans* isolates in the Western region. Similar to that in Cameroon, our molecular-type analysis showed high diversity of strains and genotypes within the same species. However, significant differences in yeast diversity between the two countries were also observed. For example, aside from *C. tropicalis*, none of the other yeast species found in these two countries were shared. In addition, the number of potentially novel species from these two countries also differed.

While we have identified some very interesting patterns of yeast species and genotype distributions in both countries, the specific mechanisms for such patterns and the implications of our findings remain to be investigated. For example, in Chapter 3, with the frequent recovery of C. neoformans from Saudi Arabia, it would be highly desirable to obtain the multilocus sequence type at the seven consensus loci so as to infer the relationships among strains isolated from Saudi Arabia with those from other parts of the world.

4.1 References

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