AIX-MARSEILLE UNIVERSITE

FACULTE DE MEDECINE DE MARSEILLE

ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

Infections Parasitaires : Transmission, Physiopathologie et Thérapeutique UMR MD3

THÈSE

Présentée et publiquement soutenue devant

LA FACULTÉ DE MÉDECINE DE MARSEILLE
Le 26 mai 2016

Par

M. Mohammed Hashim Yasir AL-YASIRI

01 mars 1978- Thi-Qar / Iraq

Réservoirs environnementaux des champignons pathogènes humains : Effet de l'anthropisation sur les communautés fongiques chez *Larus michahellis*

Pour obtenir le grade de DOCTORAT d'AIX-MARSEILLE UNIVERSITÉ

SPECIALITÉ : MALADIE INFECTIEUSES

Membres du jury :

Jean-François Guegan	Institut de recherche pour le développement	Rapporteur
Jacques Guillot	Université de Paris-Est	Rapporteur
Gauthier Dobigny	Institut de recherche pour le développement	Examinateur
Renaud Piarroux	Université d'Aix-Marseille	Examinateur
Jean-François Mauffrey	Université d'Aix-Marseille	Co-directeur de thèse
Stéphane Ranque	Université d'Aix-Marseille	Directeur de thèse

Abstract

The yellow-legged gull (*Larus michahellis*) is endemic in the French Mediterranean area. It has been reported to carry antibiotic-resistant *Escherichia coli*. Although it was known that it might carry potential human pathogenic fungi, its gut mycobiota has never been studied. In particular, whether the biotopes of the breeding colonies influence these seabirds' gut mycobiota is unknown. This work aimed to better understand their role as potential reservoir and in the spreading of potentially human pathogenic fungi. This study's primary objective was to characterize the species composition of the yeast gut communities in the yellow-legged gull and to detect the carriage of azole-resistant yeasts. Therefore, we sampled and cultured 177 yellow-legged gull's faecal samples in five study sites along the Mediterranean littoral South of France. These sites were exposed to a variable anthropogenic impact ranging from building roofs in two cities, La Grande-Motte (GM) and Palavas-les-Flots (PF), to a natural reserve, the lagoon of Pierre-Blanche (PB), and including two suburban ecoclines, the Frioul (FR) and Riou (RI) archipelagos, the latter being exposed to sewers pollution. We identified seventeen yeast species; the most frequent were *Candida krusei* (53.5%), *Galactomyces geotrichum* (44.1%), *C. glabrata* (40.9%), *C. albicans* (20.5%) and *Saccharomyces cerevisiae* (18.1%). The yeast gut community structure in the gulls at PB and FR were characterized by a higher species richness and diversity than those at GM, PF and RI. Noteworthy, (i) the frequency of the anthropic yeast species *C. glabrata* and *C. albicans* increased with the synanthropy of the gull's colonies and (ii) antifungal resistance was found in each of the five most frequent yeast species.

We further analyzed the airborne filamentous fungi species isolated from the same sample cultures. We identified 35 filamentous fungi species in 16 genera. Our two main findings were that (i) both fungal diversity and abundance were low in urban area when compared to suburban ecocline or environments that were little affected by anthropogenic impact and (ii) particular fungal species were clearly associated with distinct environments. In particular, *Mucor circinelloides* was associated with a natural environment, distant from human settlements and little affected by anthropogenic impact, whereas *Scedosporium apiosperma* was associated with an ecocline polluted by sewage.

Finally, we analyzed the population genetic of the human pathogenic yeast *C. glabrata*, which is prone to develop fluconazole resistance and was one of the three main yeast species isolated from the yellow-legged gulls' mycobiota. Therefore, we typed 111 gulls' isolates and 79 isolates collected from patients in Nimes, Montpellier and Marseille

hospitals via multiple-locus variable number tandem repeat analysis. We found that the *C. glabrata* populations isolated from gulls or humans shared a similar genetic diversity and that there was no significant population structure between them. Moreover, antifungal-resistant *C. glabrata* isolates were evenly distributed in both gull and human populations.
These findings demonstrate that gulls are reservoir and facilitate the diffusion of *C. glabrata* and its indirect transmission to sympatric human or animal hosts via environmental contamination. This eco-epidemiological view, which can be applied to other species, broadens our perspective regarding the reservoirs and dissemination patterns of antifungal-resistant human pathogenic yeast.

Keywords:

Yellow-legged gulls; yeast communities; *Candida krusei*; *Galactomyces geotrichum;* antifungal resistance; *Mucor circinelloides*; *Aspergillus fumigatus*; *Candida glabrata*; MLVA; population genetics.

Résumé

Le goéland leucophée (*Larus michahellis*) endémique dans la région méditerranéenne française est décrit comme porteur d'entérobactéries résistantes aux antibiotiques. Bien qu'il soit reconnu comme porteur de champignons potentiellement pathogènes pour l'homme, son mycobiote intestinal n'a jamais été étudié. En particulier, l'influence du biotope des colonies pendant la période de reproduction sur le mycobiote intestinal n'a jamais été étudiée. Ce travail visait à mieux comprendre le rôle de ces oiseaux de mer comme réservoir potentiel et agent de dissémination de champignons potentiellement pathogènes pour l'homme.

L'objectif principal de cette étude était de caractériser la composition des communautés de levures intestinales chez le goéland leucophée et de chercher un portage de levures résistantes aux azolés. Pour cela, nous avons collecté et cultivé des échantillons de guano de 177 goélands leucophées dans cinq sites d'étude sur le littoral méditerranéen français. Ces sites étaient exposés à un impact anthropique variable allant des toits d'immeubles dans deux villes, La Grande-Motte (GM) et de Palavas-les-Flots (PF) à une réserve naturelle, l'étang de Pierre-Blanche (PB), en passant par deux ecoclines suburbaines, les archipels du Frioul (FR) et de Riou (RI). Nous avons identifié dix-sept espèces de levure; les plus fréquentes étant *Candida krusei* (53,5%), *Galactomyces geotrichum* (44,1%), *C. glabrata* (40,9%), *C. albicans* (20,5%) et *Saccharomyces cerevisiæ* (18,1%). La structure des communautés de levures intestinales des goélands à PB et au FR se caractérisait par une plus grande richesse et diversité d'espèces que chez ceux de GM, PF et RI. On notait d'une part une augmentation de la fréquence des espèces anthropiques de levures *C. glabrata* et *C. albicans* avec l'anthropisation des biotopes des colonies de goélands et d'autre part la mise en évidence d'isolats résistants aux antifongiques dans chacune des cinq espèces les plus fréquentes de levure. Nous avons de plus analysé les communautés de champignons filamenteux aérocontaminants isolés à partir des mêmes échantillons. Nous avons identifié 35 espèces de champignons filamenteux appartenant à 16 genres. Nos deux principales observations ont été (i) la faible diversité et abondance de champignons filamenteux dans les zones urbaines par rapport aux écoclines suburbains ou à un environnement peu affecté par l'anthropisation et (ii) l'association claire entre certaines espèces fongiques et des environnements particuliers. Notamment, *Mucor circinelloides* était associé à un environnement naturel, éloigné de l'occupation humaine et peu affecté par l'impact anthropique, alors que *Scedosporium apiosperma* était associé à un écocline pollué par les égouts.

Enfin, nous avons analysé la génétique des populations de la levure *C. glabrata*. Cette espèce de levure pathogène pour l'homme est l'une des trois principales du mycobiote du goéland. Elle se caractérise par une propension à développer une résistance au fluconazole. Nous avons typé - par MLVA - 111 isolats de goélands et 79 isolats collectés chez des patients des hôpitaux de Nîmes, Montpellier et Marseille. Nous avons observé une diversité génétique similaire et une absence de structuration entre les populations de *C. glabrata* isolées chez le goéland ou chez l'homme. De plus, les isolats de *C. glabrata* résistants au fluconazole se distribuaient uniformément dans les deux populations.

Nos résultats montrent que le goéland leucophée est un réservoir de *C. glabrata*. En contaminant l'environnement, il facilite la dissémination et la transmission indirecte de cette levure à des hôtes sympatriques, humains ou animaux. Cette perspective éco-épidémiologique élargit notre vision des réservoirs et des modes de diffusion des champignons pathogènes humains éventuellement résistants aux antifongiques. Elle ouvre la voie à des recherches élargies à d'autres espèces d'hôtes et de pathogènes fongiques dans une approche globale de la santé.

Mots clés :

Goéland leucophée ; communautés de levures ; *Candida krusei ; Galactomyces geotrichum* ; résistants aux antifongiques ; *Mucor circinelloides*; *Aspergillus fumigatus*; *Candida glabrata*; MLVA; génétique des populations

Acknowledgement

This work is the product of joint efforts by hands of the people who painted targets on the water surface of the Mediterranean Sea. These targets were transmitted by gulls to bring them between the arms of the population of Marseille city.

By modest words, I like to introduce gratefully thanks to everyone who supported me or made the efforts to improve this work.

I would like to thank my supervisors; **Dr Stéphane Ranque** and **Dr Jean-François Mauffrey** for their invaluable scientific advice and their generosity that overwhelms me, concerning our project. Many thanks also to **Professor Renaud Piarroux** for his precious scientific comments during my PhD project; I really appreciate these efforts as long as you devoted the time to improve the results of this work.

I would like to thank all the staff of laboratory of Parasitology and Mycology at La Timone hospital from **technicians and secretary personnel** that helped and supported me through my PhD project, particular, **Dr. Anne-Cécile Normand** for the great effort from the beginning of the collection of samples to the last point in this work.

I would like to thank **the ministry of higher education of Iraq** for funding me during my PhD thesis period.

I am so thankful for the help of **Mr. Laurent Argiro** (Ingenieur de Recherche / INSERM / Faculty of Medicine / Aix-Marseille University) in autoclave culture media.

I thank **Dr. Stanislas Rebaudet** (Hôpital de la Timone) and **Miss. Sandy Moore** (PhD, Aix-Marseille University) to participate in the improvement of this work.

I like to thank **Mr. Mohamed Belhocine** (PhD Bioinformatics and Genomics, Aix-Marseille University) to support me in biostatistics.

I gratefully thank my mother as long as she sent me the beautiful words to give me the confidence to complete my PhD project. I really thank my wife and my four kids to accompany me here in France during my PhD period. Again, I would like to thank everyone that supported me even in one word during my PhD project.

Finally, I thank the jury members as long as they have spent a long time to read and improve this work.

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List of abbreviations

PB	Pierre-Blanche
PF	Palavas-les-Flots
GM	La Grande-Motte
RI	Riou
FR	Frioul
MLVA	Multiple loci variable-number tandem repeat analysis
HA	Hemagglutinin
N	Neuramidase
LPAIVs	Low pathogenic avian influenza viruses
HPAI	Highly pathogenic influenza virus
WNV	West Nile Virus
CDC	Centers for Disease Control and Prevention
WHO	World Health Organization
NDV	Newcastle disease virus
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
MG	*Mycoplasma gallisepticum*
ETEC	Enterotoxinogenic *E. coli*
EPEC	Enteropathogenic *E. coli*
EAggEC	Enteroaggregative *E. coli*
EIEC	Enteroinvasive *E. coli*
STEC/VTEC	Shiga (Vero) Toxin-producing *E. coli*
AIDS	Acquired Immune Deficiency Syndrome
OIE	World Organization for Animal Health
DRBC	Dichloran Rose Bengal Chloramphenicol agar
RT	Room Temperature
µl	Milliliter
pH	potential Hydrogen
PCR	Polymerase Chain Reaction
Fwd	Forward
Rev	Reverse
Min	Minute
MLP	Microsatellite Length Polymorphisms
RPMI	Roswell Park Memorial Institute medium
VNTR	variable-number tandem repeat
MST	Minimum Spanning Tree
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

Chapter 1: General Introduction with literature review

1.1 Introduction

The number of fungi species on the earth was estimated 3.5 to 5.1 million, according to analysis environmental DNA samples from a soil community (Blackwell, 2011), 600 species are known to be responsible of human diseases (Brown, Denning, and Levitz, 2012). These range from the infections of the skin and nails caused primarily by dermatophytes, to invasive or systemic infections caused by e.g., *Candida* species, *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Histoplasma capsulatum* (Brown, Denning, Gow, *et al.*, 2012). Fungi can also cause zoonotic infection via pathogens transmission from animals to humans or from humans to animals, being called in the latter case reverse zoonotic infections (Messenger *et al.*, 2014). The World Health Organization Expert Committee on zoonoses (WHOEC) classified zoonoses infections based on the life cycle of pathogens into five categories as following: **Direct zoonoses**, when pathogens are transmitted without propagative changes from infected vertebrate host to susceptible vertebrate host via direct contact; **Cyclo-zoonoses**, when pathogens requires more than one vertebrate host species to complete their life cycle; **Meta-zoonoses**, when pathogens are transmitted biologically (multiply and develop) via invertebrate vectors; **Sapro-zoonoses**, when pathogens are transmitted from food, soil or plants (inanimate developmental sites) to susceptible hosts; and **Anthroponoses**, when pathogens are transmitted from lower vertebrate animals (such as mice or rats) to humans (Jaffry *et al.*, 2009). However, zoonotic infections can develop a new population of pathogens followed by the spreading from their reservoirs to new hosts in the presence of certain factors, such as host movement within geographical areas, which enables to spread pathogens via increasing the interactions between different host populations (Cutler *et al.*, 2010). Here the term reservoir is defined as: "one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population" (Haydon *et al.*, 2002). In other words, increasing the interactions between both human and animal populations living in the same ecosystem, increases pathogens-transmission dynamics within sympatric populations. The transmission of pathogens might occur either via direct contact with the vector or reservoir, as the contact between pets and their owners (Damborg *et al.*, 2015), or via indirect contact such as food-borne and water-borne pathogens (Yugo and Meng, 2013). In addition, the pattern of interaction between populations, depending on the population

density and individual behavior, is considered the major factor that contributes to increase the risk of exposure to an endemic pathogen (Lindahl and Grace, 2015).

Among animals, birds are probably those with the highest zoonotic potential because most of them can fly long distances, during migration period or even in search for food. Many studies reported that pathogens are carried via cloacae or feces of birds, which disseminate the pathogens in the ecosystem (Cafarchia *et al.*, 2006; Georgopoulou and Tsiouris, 2008; Foti *et al.*, 2011; Hernandez *et al.*, 2013). Furthermore, birds can be reservoirs and transmit pathogens including fungi to other domestic animals and wild birds (Georgopoulou and Tsiouris, 2008). Fungal pathogens transmitted by birds may be resistant to several antifungal drugs and can cause human infections (Mattsson *et al.*, 1999; Lord *et al.*, 2010). Several studies demonstrated that the majority of migratory and wild birds as well as domestic birds play a role in the spreading of fungal pathogens in various environments, and, in particular, in the vicinity of human (Sidrim *et al.*, 2010; Chryssanthou *et al.*, 2011; Tokarzewski *et al.*, 2012; Mendes *et al.*, 2014). The yeast carried in the birds gastrointestinal tract exhibited antifungal drugs-resistance as well as phospholipase activity, which is one of several virulence factors that enable yeast to switch from commensal to pathogenic state (Sidrim *et al.*, 2010).

In our study, we aimed to investigate the role of yellow-legged gulls as reservoir and in the dispersion of pathogenic fungi. Several studies have focused on the feeding behavior of the yellow-legged gull along the Mediterranean coast. This species has been able to colonize coastal cities looking for refuse areas that became its predominant source of food rather than sea fish (Duhem *et al.*, 2003; Ramos *et al.*, 2010). The increasing availability of city dump helped to boost dramatically the number of gulls, which now built their nests on the house or building roof tops. Some searchers investigated the role of yellow-legged gulls in the carriage of drug-resistant bacterial pathogens, such as *Escherichia coli* (Bonnedahl *et al.*, 2009). Therefore, our objectives were: i) to study the diversity of the gut yeast communities in yellow-legged gulls from various breeding sites; ii) analyze the population genetic of the major yeast species isolated from gulls or from humans; iii) to describe the diversity of the airborne filamentous fungi communities cultured from gulls feces collected in biotopes exposed to various anthropogenic influence.

1.2 Birds-associated zoonoses

First, we reviewed the studies that investigated the role of birds in transmission various pathogens, which can spread from animals to humans or vice versa, this phenomenon is known as zoonoses. Some pathogens, involved in zoonotic diseases, can infect both humans and animals. However, zoonotic infections can be acquired directly from animals, or through intermediate-contaminated host such as food and water. These infectious agents can be bacteria, fungi, viruses or protists. The most well-known human pathogen transmitted by birds is the Influenza virus. The natural host for Influenza virus is aquatic birds, which eventually transmit the virus to other environment or hosts. Then, we analyzed the studies that addressed the transmission of west Nile virus by birds. We found that birds act as reservoir to transmit this virus to mosquito, which transmits the virus to other environments or human only by mosquito biting. Paramyxovirus virus is another virus that infects bird, in particular, poultry. Human can be infected with this virus; mostly, poultry workers and laboratory workers are at risk. The great party from our review focuses on bird-transmitted bacterial pathogens. We found that the number of bacterial pathogens was higher than viral pathogens, as following: *Chlamydophila psittaci*, *Mycoplasma*, *Campylobacter*, *Salmonella*, *Pasteurella multocida*, *Clostridium* spp, *Mycobacterium* spp. and *Escherichia coli*.

Concerning fungal diseases, studies have shown that five important genera of fungal pathogens are associated with birds: *Histoplasma*, *Aspergillus*, *Candida*, *Cryptococcus* and *Microsporidia*. These fungal pathogens are transmitted by wild, aquatic or domestic birds. Finally, birds also carry and transmit pathogenic protists to other hosts or environments. We mainly focus on three parasites, *Cryptosporidium* spp., *Giardia* spp. and *Toxoplasma* spp., which can be transmitted by bird to other hosts including humans.

Birds-associated zoonoses

Mohammed Hashim Al-Yasiri[1], Jean-François Mauffrey[2], Anne-Cécile Normand[3], Renaud Piarroux[1, 3], Stéphane Ranque[1, 3]

1 Aix-Marseille University, IP-TPT UMR MD3, 13885 Marseille, France

2 Aix-Marseille University – IRD, Laboratoire Population Environnement UMR-151, 13331 Marseille

3 Parasitology & Mycology, APHM CHU Timone, 13005 Marseille, France

Review status: writing in progress

Abstract

Birds are a great population distributed throughout the world as domestic, wild and pet birds. Our objectives in this review were to investigate the role of birds as carrier or infectious-animal transporter. Therefore, we mainly reviewed the important pathogen agents of viruses, bacteria, fungi and parasites transmitted via birds. How bird received these pathogens and how bird transport them.

Introduction

Men meet birds almost every day, down town with huge populations of sparrows, starlings or feral pigeons, at home when birds are pets (parrots) or in a plate as chicken, are considered the most abundant population of birds in the world counting as many as 54 billion individuals. Any bird can carry diverse microorganisms that are potential human pathogens. Transmission of these pathogens from bird to human may be either direct contact between bird and human, as is in some cases of avian influenza virus and enteric bacteria, or indirect transmission by intermediate hosts (as west Nile virus is transmitted by mosquitoes) or water and food contaminated with birds faeces (Abulreesh et al., 2007). Birds may act only as mechanical vector of viral, bacterial, fungal or parasitic pathogens that occasionally spread to human but may also be infected by those parasite and act as a biological host (Hubálek, 2004). If direct contact is not so frequent, indirect contamination by faecal droppings may be an important way of transmission from birds to human, and especially in urban environment (Vlahović et al., 2010). Thus, accumulation of bird dropping in urban areas represents a potential risk of human infection with many pathogens (Cermeño et al., 2006a; Nyakundi and Mwangi, 2011). Moreover, water contaminated with bird's faeces is considered an important vector of many pathogens including antimicrobial resistant bacteria (Guenther et al., 2011). In this urban microbial community, horizontal gene transfers may induce the spreading in antibiotic resistance or the increase of pathogenicity between similar pathogens, such as Influenza A virus (Burgos and Burgos, 2007). In addition, the birds' migration and their great dispersal capabilities can expose populations to new/foreign pathogens (Sehgal, 2010). By systematically reviewing scientific publications on the topic, we aim in this manuscript to describe the current knowledge on how pathogens are transmitted from birds to human and especially understand the participation of birds in the spreading of antibiotics resistant pathogens.

Methods

We performed a NCBI Pub Med database query using the following search terms: "birds"; "wild birds"; "aquatic birds"; "pets birds"; "transmission", "human infection" that were associated with specific diseases or pathogens e.g. "Influenza virus", "*Aspergillus* spp.". The terms "waterborne" and "meat-borne" were also used find references dealing with transmission from migratory and non-migratory waterfowl or economic birds e.g. chickens, turkeys, respectively. We have searched whether birds could be infected with microorganisms that infect human, by using the keyword "birds infected" associated with the name of the pathogen. We also searched for articles using Google (Google scholar?) to expand our data sources.

Results

We will hereafter describe the role played by bird in viral, bacterial, fungal, and parasitic zoonoses.

Viral infections

The most important viral diseases that can be transmitted from birds to human are caused by influenza A viruses, West Nile virus and the avian paramyxovirus.

1-Influenza A viruses

Influenza virus is orthomyxoviruses and their main encoded proteins are hemagglutinin (HA) and neuramidase (N). The virus diversity comes from the combination between 16 subtypes of HA and 9 for N. All these subtypes have been detected in aquatic bird (Fouchier et al., 2005). Influenza virus can infect animals and human, the natural host of this virus is a wide variety of aquatic birds. Other species of animals such as chickens, turkeys, swine and horses including human acquired the infection through direct or indirect contact with aquatic birds (Fouchier, 2003). Ducks such as mallards (Anas platyrhynchos), are considered the main reservoir of low pathogenic avian influenza viruses (LPAIVs) in nature. Mallard was experimentally infected with LPAIVs, they excreted large amount of infectious virus via faeces and respiratory rout (Brown et al., 2012; Jourdain et al., 2010). These viruses replicate poorly in human, if the transmission directly occurs from birds to human but the viruses will be more appropriate to develop infection in human, if they replicate in an intermediate host (Webby and Webster, 2001), such as marine mammals and pigs, which are susceptible to be

infected by avian and human influenza virus, and have been documented as intermediate hosts (Parrish et al., 2015). Poultry is another host to highly pathogenic AIV that cause systemic infection in poultry. Subsequently, AIV can be present in meat products from infected birds (Harder et al., 2016). Several changes must occur in the composition of the virus (and especially in protein HA) before human infection. These mutations may occur during transmission from the intermediate host to human (Zambon, 1999; Ito and Kawaoka, 2000). However, Charadriiformes (gulls, terns, and shorebirds) are considered a major contributor to the transmission of the virus and the occurrence of infections (Wille et al., 2011), mainly because of their regular movements between aquatic and terrestrial environments where increasing chance of transmission of the virus to health birds through contact with infected birds or acquire the virus from contaminated water environment (Brown et al., 2008). Gulls could have played an important role in an outbreak of the influenza H5N1 virus among birds in British farm turkeys in 2007. Being scavengers they might have been in contact with infected bird corpse, and they have been recorded in large numbers around the infected farm, feeding on the poultry, processing waste and carrying the waste to other parts of the farm (National emergency epidemiology group, 2007; Department for Environment Food and Rural Affairs, 2007). The behavior of the most birds make them potentially able to spread influenza virus to and from wild and domestic birds, acting as an epidemiological bridge (Lee et al., 2008). The persistent presence of influenza virus in migratory or non-migratory waterfowl indicate that these birds act both as reservoir and vehicle for influenza virus and my play an important role in new pandemic outbreaks (Ferro et al., 2008).

2-West Nile virus

The first isolation of West Nile Virus (WNV) was in 1937 and in recent years, the virus was reported in Europe, Africa, parts of Asia, Australia, North America, and parts of Central America and the Caribbean (Hayes et al., 2005). Transmission to human occurs when a human is bitten by an infected mosquito (mainly *Culex* spp.). Wild reservoir of WNV are birds (which are also affected by the virus), the mosquito acts as a vector from birds to human. In 1999, an outbreak of human encephalitis occurred in North-East United State and was concomitant of an intensive mortality of crows (*Corvus* species), and other exotic avian species at a local zoological park. Genome analysis of WNV showed that the virus circulate from birds to mosquitoes and were responsible from human disease (Lanciotti et al., 1999). That was the first appearance to WNV in North America in 1999 causing human encephalitis.

Although the WNV infection may be asymptomatic, it produces mild fever, headache and bodily pain in some cases Meningitis/encephalitis can result from the serious WNV infections and the disease may be fatal (Abulreesh et al., 2007). The natural transmission of the virus is between several mosquito species and birds. Birds are considered the main host (Pradier, 2012). Usually mosquitoes infected with the virus when feeding on an infected bird. Infected mosquitoes spread the virus by biting to human and other animals (CDC, 2003). WNV infection in birds is commonly found in Europe, Africa and west Asia, but the mortality in birds associated with this infection is rare. In contrast, the virus is highly pathogenic for birds in the Americas. The virus has been detected in dead and dying birds of more than 250 species particularly members of the crow family (Corvidae) (WHO, 2011). Migratory birds have a role in spreading the virus distribution area, which was evaluated experimentally on two bird's species (Swainson's thrushes and gray catbirds). It was concluded that the birds spread the virus during the period of infection (Owen et al., 2006). Transmission of the virus via birds depends on the ability of the virus to adapt and causing infection in birds and the survival of the virus in the body of the bird (Brault et al., 2004) Otherwise virus in birds may be no active to infect the human after being transferred by mosquitoes (Rappole et al., 2000). While birds are considered competent reservoirs when they develop a viremic sufficient titer to transmit the infection to mosquitoes, which are serve as vector of virus to other animals as well as human (Komar et al., 2003). A few birds can be infected with the virus and becoming super spreaders for this multi-host pathogen, such as *Turdus migratorius*, the American robin (Kilpatrick et al., 2007; Savage et al., 2007). Other study identified the associations between human and dead bird cases by WNV in northern Virginia for 2002 and 2003, where was found that the infection started in birds, and then the infection appeared in human. This indicate that birds were the main caused in human infection (Liu et al., 2011).

3-Avian paramyxovirus (Newcastle virus)

Newcastle disease virus (NDV) is a member of the family paramyxoviridae (Samuel et al., 2009). Avian paramyxoviruses were designated in ten serotypes APMV-I to APMV-10 of which only the serotype APMV-I was included in NDV, which primarily infects poultry (Aldous et al., 2003; Hietala et al., 2005). The pathogenicity of NDV differs depending on clinical signs. The infection may be severe and lethal (Velogenic) or occasionally lethal (Mesogenic) or mild infection (lentogenic) (Alexander and Allan, 1974). Many species of birds, such as chickens, quails, ostrich, domestic pigeons, doves and ducks may be infected or

not but can serve as reservoirs of NDV to infect other animals (Sa'idu et al., 2004). According to genetic study of strains isolated from poultry and wild birds, the primary reservoir of NDV were wild birds and the secondary reservoir were domestic poultry (Czeglédi et al., 2006). Most of NDV strains circulating in wild birds are lentogenic, which cause mild infections (Shengqing et al., 2002). But they become more virulent in chicken (Czeglédi et al., 2006). The first documented human Newcastle disease case was conjunctivitis in a laboratory worker (Nelson et al., 1952). Subsequently, many cases of the same infection were reported (Trott and Pilsworth, 1965; Mustaffa-Babjee et al., 1976). Laboratory workers and poultry vaccination crews are more likely to be infected with NDV, because of their exposure to large quantities of velogenic NDV strains, which can cause conjunctivitis in human (Alexander, 2000).

Bacterial infections

Many species of bacteria can be transmitted by birds to human and in different ways depending on the species of the pathogen of which: chlamydiosis, Mycoplasmosis, avian cholera, salmonellosis, campylobacteriosis, colibacillosis, mycobacterial diseases, and *Clostridium difficile* infection.

1-Avian Chlamydiosis

Avian chlamydiosis (Psittacosis) is an infection caused by *Chlamydophila psittaci*, a gram negative, coccoid, obligate intracellular bacterium in the family Chlamydiaceae (Everett et al., 1999) *C. psittaci* was divided into 6 serotypes named A to F, which have been recognized on genetic differences in the outer membrane protein (Andersen, 1997; Geens et al., 2005). All serotypes have been documented in birds and can be transmitted to human and cause chlamydiosis, such as serotype A is endemic in psittacine birds and can infect human (Vanrompay et al., 1997; Andersen and Vanrompay, 2000), serotypes B and C are endemic in European non psittacine birds, which may represent an important reservoir for human psittacosis cases (Heddema et al., 2006).

Reservoir of bacteria: Birds are considered the primary reservoir for *C. psittaci*. However, Captive birds especially, psittacine as well as free living wild birds are important reservoirs of *C. psittaci* such as chicken, turkey, duck, goose, gulls and geese (Kaleta and Taday, 2003; Ghorbanpoor et al., 2015). Clinical signs of *C. psittaci* infection in birds, which is referred to as avian chlamydiosis, include "pneumo-enteritis" with respiratory signs, mucopurulent nasal

discharge, diarrhea, polyuria, dullness and yellow droppings, as well as central nervous system disturbances may be occasionally observed in Amazon parrots and macaws (Vanrompay et al., 1995).

Transmission: *C. psittaci* primarily transmits vertical and horizontal from bird to bird in close proximity by the Inhalation of airborne particles or infectious dust, and from bird to human share the same ecosystem (Erbeck and Nunn, 1999; Gelfand and Cleveland, 2013). Infected wild birds shed *C. psittaci* in their faeces for several months (Rehn et al., 2013). Wild aquatic birds are infected and excrete high concentrations of *C. psittaci* in water (Scientific Committee on Animal Health and Animal Welfare, 2002). Human infected with psittacosis shows clinical signs like fever, chills, headache, malaise, and myalgia, enlarged spleen and nonspecific rash are sometimes observed. Cough is usually present and can be accompanied by breathing difficulty and chest tightness (Smith et al., 2011). Transmission from person-to-person may occur (McGuigan et al., 2012). One familial case of psittacosis has been documented between two patients, the first patient had been in contact with parakeets but the second patient had not been in contact with any birds (Ito et al., 2002).

2- Mycoplasmosis

Mycoplasma species, which belong to the class Mollicutes, have a small genome and lack a cell wall. Certain species of this bacterium may be parasitic or commensal to human, animals and plants (Razin et al., 1998). Avian Mycoplasmosis, which is caused by *Mycoplasma gallisepticum* (MG) or *Mycoplasma* meleagridis or *Mycoplasma* synoviae or *Mycoplasma* iowae, is a respiratory infection basically found in poultry, especially chickens and turkeys (Vogl et al., 2008; Heleili et al., 2011; Wyrzykowski et al., 2013). MG infection is the most prevalent species of *Mycoplasma* in birds, poultry and domestic birds, but it has never been reported in human or other mammalian species (Williams et al., 2002; Delaney et al., 2012; Ammar et al., 2016). However, other *Mycoplasma* species, such as *M. pneumoniae*, *M. salivarium, M. arthritidis*, *M. canis*, *M. bovis*, *M. arginini*, *M. felis* and *M. edwardii*, can cause zoonotic infection that is transmitted between human and animals other than birds (Garg, 2009). As *Mycoplasma* ovis infection has recently been described in animals such as sheep, goats, deer, Japanese serows and dogs (Grazziotin et al., 2011; Ohtake et al., 2011; Varanat et al., 2011; Tagawa et al., 2012) and human (Sykes et al., 2010), these species may also cause zoonotic infections (Maggi et al., 2013). Currently, birds have not been shown to function as a reservoir of human *Mycoplasma* infection.

3- Avian cholera by *Pasteurella multocida*

Pasteurella multocida causes avian cholera (fowl cholera), which is considered one of the major infectious diseases affecting wild waterfowl (Descamps et al., 2012). *P. multocida* infects a variety of hosts such as cattle, cats, dogs and human as well as birds (Hendrie, 1974; Medani et al., 2004). Birds are considered as source to disseminate of *P. multocida* for other host and environment (Wang et al., 2009). The mortality rate among infected birds with *P. multocida* increases depending on the density of the bird population (i.e., bird to bird transmission) and the degree of exposure to contaminated water sources (Blanchong et al., 2006a). The *P. multocida* has four serotypes A, B, D and F, which are able to cause infection in poultry with low levels of antibiotic-resistance (Mohamed et al., 2012; Rigobelo et al., 2013). Birds can transmit *P. multocida* to other animals, but the transmission has not been reported from birds to human (Christensen and Bisgaard, 2000). *Pasteurella* infection can occur in human following via bite or scratch domestic animal (dog or cat), which serve as potential reservoirs of this bacterium (Boerlin et al., 2000; Liu et al., 2003). Once the infection has progressed in skin or soft tissue, it may provoke a systemic infection, such as meningitis or urinary tract infection (Ashley, 2004).

4-Salmonellosis

Salmonellosis is caused by a group of species of *Salmonella*, which infect both human and animals. Human acquires salmonellosis mostly with food-borne but infections can also acquire from direct or indirect contact with infected animals (Hoelzer et al., 2011; Gould et al., 2013). Numerous serotypes of *Salmonella* have been described in several hosts, but a few species are restricted to infect one animal species such as S. pullorum in chickens and *S. typhi* in human (Doyle et al., 2009).

Reservoir: Farm animals are considered the primary reservoir for important *Salmonella* serotypes as *S. typhimurium* and *S. enteritidis* , which are the most common serotypes causing human infection (Doyle et al., 2009; Eguale et al., 2016). In Europe, wild birds are a reservoir to the serotype typhimurium of *Salmonella* (Kapperud et al., 1998), which may develop salmonellosis causing a significant mortality in some species of birds (Hall and Saito, 2008). Human have acquired infection from infected birds, which serve as spreaders of *Salmonella* via contaminated faeces in the environment, including food, water surface (Lévesque et al., 2000). Health birds share the same environment with infected birds, can be

infected and establishing a reservoir of bacterium, which can transmit to human (Kapperud et al., 1998).

Transmission: *Salmonella* infections can transmit in different ways depending on strains varieties of bacteria, behavior and food patterns in birds as well as breeding practices by human (Friend and United States Geological Survey, 1999). *Salmonella* lives naturally in the intestines of human and other animals, especially free-living birds like; ducks, geese, pigeons and gulls that play an important role in the spread of various intestinal *Salmonella* serotypes, including multidrug-resistant strains (Kapperud and Rosef, 1983; Palmgren et al., 2006; Kobayashi et al., 2007) via faecal materials in aquatic environments (Cabral, 2010; Afema et al., 2016). However, identical *Salmonella* strains were isolated from both Black headed-gulls and water, which was resistant to antibiotics (Dolejská et al., 2009). Wild birds were also hosts of livestock *Salmonella* that can be transmitted to human (Epstein et al., 2007). *Salmonella* cause bacteremia in human (Percival, 2004; Gordon, 2008) and can be either asymptomatic or cause severe infection in birds (Mikaelian et al., 1997; Friend and United States Geological Survey, 1999).

5-*Campylobacter*iosis

*Campylobacter*iosis is an infectious disease caused by *Campylobacter* spp that was detected in 1970 (Shane, 2000). Some of *Campylobacter* species are thermophilic and bird-associated zoonotic bacteria such as *C. jejuni* subsp. *jejuni*, *C. coli* and *C. lari*, which are responsible of common infections in human (Fernandez et al., 1996).

Reservoir: The reservoirs of *Campylobacter* in industrialized and developing countries are domestic and wild animal species, including birds such as poultry that could be a source of contamination for human, other animals, food and environment (Stanley and Jones, 2003; Skarp et al., 2016). *Campylobacter* colonizes the gut of birds. Subsequently, birds shed the bacteria everywhere via their feces (Jorn et al., 2009). The fecal transmission route was the likely sources of human *Campylobacter* infections from the reservoirs of poultry and wild birds (Workman et al., 2005). Genetic studies have been suggested the transmission of *Campylobacter* from birds to human (Broman et al., 2002, 2004).

Transmission of resistant *Campylobacter*: several routes of *Campylobacter* transmission to human but most important of which handling pets' birds or consuming chickens contaminated with this pathogen or other products contaminated with feces of carrier animals (Kapperud, 2003). Recently, transmission of *Campylobacter* from slaughtered chicken to

abattoir workers was described (Ellström et al., 2014). Many studies have been confirmed that *Campylobacter* isolated from poultry and poultry meat of that was resistant to different antibiotics (Gallay et al., 2007; Stone et al., 2013; Tambur et al., 2013). *C. jejuni* were circulated in Canada geese and their environmental water, were susceptible to tetracycline, streptomycin, erythromycin, kanamycin, nalidexic acid and ciprofloxacin (Rutledge et al., 2013).

6- Colibacillosis

Escherichia coli are a normal flora of the gastrointestinal tract of animals and human. Some strains of *E. coli* adapt in intestine and may develop diarrhea and often migrate out of intestine to cause a range of diseases (Fairbrother & Nadeau, 2006). There are five main categories of harmful *E. coli* include enterotoxinogenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC) and Shiga toxin-producing *E. coli* (STEC). STEC is the only *E. coli* pathogenicity group (also named enterohaemorrhagic *E. coli* (EHEC)) can transmit to human via contaminated food-origin animals and cause severe zoonotic disease (Wasteson, 2001).

Reservoir and Transmission: Birds are potential vectors for STEC that are transmitted by faecal-oral route to other animals. In one study (Nielsen et al., 2004) same STEC serotype was isolated from the feces of wild birds (starling) and cattle, suggesting possible transmission of STEC strains between wild birds and animals farM. Strains of STEC O157 were isolated from three human cases in same family and rook birds. The authors suggested that rooks birds served as intermediate host between the farm of cattle and infected humans followed by person-to-person transmission, which occurred between family members (Ejidokun et al., 2005).

***E. coli* resistant to antibiotics:** birds play important role to spread antimicrobial resistant *E. coli* via fecal contamination in different water locations (Abdallah, 2005). (Bonnedahl et al., 2009) found that the level of general antibiotics resistance of *E. coli* isolates from fecal samples of Yellow-legged gulls in southern coastal region of France was 47.1 %. This indicates that the wild birds act as reservoir and transporter of resistant bacteria. Birds also serve as vector to transmission antibiotics resistant *E. coli* between contaminated sources (feces' animals or animals) to other environments (Cole et al., 2005), such as wild birds that have access to infected poultry brooder with multiple antibiotics resistant *E. coli*. Wild birds

had *E. coli* similar to poultry because they feed from poultry feeds or consume poultry feces (Krumperman, 1983).

7- Mycobacteriosis

The most of bird species can be infected with *Mycobacterium avium* subsp. *avium* causes a chronic infection in various organs of bird. Domestic hens are considered the principal source of *M. avium* subsp. avium for other susceptible hosts (Shitaye et al., 2008). Other *Mycobacterium* species, such as *M. scrofulaceum*, *M. intracellulare*, *M. fortuitum*, *M. bovis* and *M. tuberculosis* rarely infect birds (Dhama et al., 2011). Only Psittacine birds (principally the parrot's family) can be infected with *M. tuberculosis* that they acquire this species from infected human in close contact. However, the transmission is bilateral between human and parrots bird (Washko et al., 1998). African grey parrots and its owner infected with *M. tuberculosis*. The isolates that infected both human and parrots birds were genetically similar. The infection route likely was from human to parrots bird (Schmidt et al., 2008). *M. tuberculosis* in human may be asymptomatic for unlimited-time periods, and then develop a pulmonary disease (Hu et al., 2016). In parrots, symptoms of *M. tuberculosis* infections were nodular or diffuse keratinous skin lesions at the muco-cutaneous junction of the eyes and beak (Montali et al., 2001).

8- Clostridiosis

Clostridiosis is a disease caused by several species, belongs to the genus of *Clostridium* spp. *Clostridium difficile* are widely distributed in human, variety of animal species including birds. The most of hosts carry normally *C. difficile* in the intestinal tract (Borriello et al., 1983; Ferreira et al., 2003; Kiss and Bilkei, 2005). High percentage of poultry in Europe and North America were colonized by *C. difficile* indicating the negative impact on commercial operations of poultry (Rodriguez-Palacios, Borgmann, Terence R. Kline, et al., 2013). The transmission of *C. difficile* from animals to human has not been reported, but it has been suggested that animal meat, including poultry products, may play a role in *C. difficile* transmission to human via the food supply (Songer et al., 2009; Weese et al., 2010). Thus, for other species of *Clostridium* such as, *C. perfringens* and *C. botulinum* are also implicated in poultry diseases posing a threat for public health (Fossum et al., 2009; Hibberd et al., 2011; Sato et al., 2016). Many cases of Clostridiosis were reported in domestic and wild birds, such

as the infection of Moluccan cockatoo (*Cacatua moluccensis*) by *C. tertium* (Hess et al., 1998; Jang et al., 2014).

Fungal infections

1-Histoplasmosis

Histoplasmosis is systemic mycoses prevalent primarily in America as well as some parts of Asia and Africa, and most cases in Europe have been reported in Italy (Joseph Wheat, 2006). The majority of histoplasmosis cases display infections in respiratory system (acute pulmonary disease), which subsequently disseminate to other organs. Histoplasmosis may also be asymptotic (Ahuja et al., 2012; Ledtke et al., 2012). Histoplasmosis is caused by *Histoplasma capsulatum*, which is an intracellular pathogen and a dimorphic fungus (Kügler et al., 2000; Kauffman, 2007). Three varieties of *Histoplasma* have been reported in America, *H. capsulatum* var *capsulatum*, duboisii, and farciminosum (Kasuga et al., 1999). In Africa and Asia both capsulatum and duboisii varieties have been reported (Gugnani and Muotoe-Okafor, 1997; Pan et al., 2013). Birds or bats, which infected with Histoplasmosis in their gastrointestinal tract, contaminated the soil with *Histoplasma* via their droppings (Jorn et al., 2009). Therefore, dropping-contaminated soil is considered a reservoir for *H. capsulatum,* with a threat public health (Ashford et al., 1999; Gugnani, 2000). Recently, *H. capsulatum* was detected with high proportion (9.8%) from soil contaminated with droppings of pigeon and chicken compare with the soil contaminated (7.9%) with bat guano (Norkaew et al., 2013). *H. capsulatum* infection is acquired following inhalation of microconidia or hyphal fragments, which may result in severe disease development, particularly in immunocompromised or chemotherapy patients (Nosanchuk and Gacser, 2008). Disseminated-histoplasmosis has been documented in bats and Eclectus parrots (*Eclectus raratus*). The fungus was isolated from all internal organs of the animals (Taylor et al., 1999; Quist et al., 2011). *H. capsulatum* has also been isolated from dove excrement; therefore, the authors suggested, the exposure to dove excrement should be avoided (Cermeño et al., 2006b).

2- Aspergillosis

Aspergillosis is an non-contagious infection caused by opportunistic saprophytic genus *Aspergillus*, in particular, *Aspergillus fumigatus* (Agarwal, 2009) as well as other species of

Aspergillus that include *A. flavus*, *A nidulans*, *A. niger*, *A. clavatus*, *A. glaucus*, *A. oryzae*, *A. terreus*, *A. ustus* and *A. versicolor* (Geiser et al., 2007). *A. fumigatus* among all species of *Aspergillus* is considered the most commonly found in human and birds because it has smaller spores than the spores of other species (Tell, 2005; Beernaert et al., 2010) and the fungus capable to growth normally at 50 C° (Córdova et al., 2003; Hong et al., 2005; Dagenais and Keller, 2009). Aspergillosis is considered a major infection, which can occur in farm birds, wild birds and immunocompromised human by inhalation a large amount of the *Aspergillus* spores can be colonize the respiratory tract by intracellular germination and lysis of the phagocytic cells causing invasive aspergillosis (Tell, 2005; Arné et al., 2011; Olias et al., 2011; Van Waeyenberghe et al., 2012). *Aspergillus* can infect birds in captive and free-ranging environments, in all ages and whether immunocompetent or immunocompromised (Tell, 2005). *A. fumigatus* is considered the first fungal pathogen, which causes avian aspergillosis followed by *A. flavus* (Hadrich et al., 2013). Other *Aspergillus* species can have a role in occurring of infection, such as *A. niger* caused aspergillosis in ostriches (Perelman and Kuttin, 1992). Respiratory aspergillosis have been documented in trumpeter and tundra swans with *A. fumigatus* and *A. flavus* (Meyerholz et al., 2005; Degernes et al., 2006) and in commercial turkey with *A. flavus* (Stoute et al., 2009). Respiratory infection produces primarily clinical signs like lesions in both lungs and air sac. Necropsy of blue jay (Cyanocitta cristata) showed lesions in lung and air sac caused by *A. fumigatus* (Young et al., 1998). In poultry, the infection produces clinical signs such as, mild respiratory distress, ruffled feathers, weakness, diarrhea and a gross lesions on the surface of the lung and liver (Suleiman et al., 2012). Disseminated aspergillosis was reported in stitch bird (Notiomystis cincta) (Cork et al., 1999) and in ostriches (Khosravi et al., 2008). Aspergillosis can be transmitted horizontally among birds where *A. fumigatus* has spread in two flocks of broiler chickens (Zafra et al., 2008). Different genotypes of *A. fumigatus* were isolated from turkeys of which (C173D112 and C169D102), which are usually responsible for invasive aspergillosis in human (Lair-Fulleringer et al., 2003). Therefore, *Aspergillus* could be transmitted to human by handling infected birds and animals or eating meat of infected animals (Pattron, 2006) causing invasive aspergillosis among peoples especially with acquired immune deficiency syndrome (AIDS). Invasive aspergillosis is considered one of the more infections morbidity and mortality (Shen et al., 2007). A great genetic diversity was found among *A. fumigatus*, which recovered from both birds and their environment (Burco et al., 2012) and different genotypes of *A. fumigatus* can infect birds causing Aspergillosis

(Alvarez-Perez et al., 2010) and some of these birds may be reservoirs to genetically different strains of *Aspergillus*, some of which were resistant to antifungal drugs (Beernaert et al., 2009; Tokarzewski et al., 2012).

3-Candidiasis

Candida species is opportunistic yeasts, which can cause candidiasis in certain cases particularly, immunodeficiency patients (Pfaller et al., 2012). *Candida albicans*, which is considered as important pathogen among several *Candida* species, can cause different infections in mucous membranes of the mouth, nose, and vagina and may cause nosocomial diseases. *C. albicans* was also isolated from intestinal tract and moist areas of the skin without infection (Hazen, 1995; Kojic and Darouiche, 2004). However, the infections with non-albicans species of *Candida* (i.e. *C. tropicalis, C. glabrata, C. parapsilosis* and *C. krusei*) were increased in a few decades ago and were reduced susceptibility to antifungal drugs (Krcmery and Barnes, 2002; Arendrup, 2010; Pfaller et al., 2012). *Candida* is one of the natural flora in digestive tract of bird, similar to *Candida* in human (Kocan and Hasenclever, 1972) and can be isolated from different parts of birds without any infection (Cafarchia, Camarda, et al., 2006; Brilhante et al., 2010; Sidrim et al., 2010; Al-Barwari and Saeed, 2013). Many studies have been demonstrated, that *Candida* can infect the birds and cause superficial and systemic candidiasis (Kano et al., 2001; Sato et al., 2001; Lanteri et al., 2012). Sometimes a combination infection can occur with other pathogens (Quist et al., 2011). Furthermore, some *Candida* isolated from birds exhibited antifungal resistance (Lord et al., 2010; Sidrim et al., 2010). In general, birds act as carriers for pathogenic *Candida* spp (*C. albicans, C. tropicalis, C. glabrata* and others), and excrete these yeast in their droppings (Cafarchia, Romito, et al., 2006). Although, the genotypes were rather different between *Candida* isolated from human or animals including birds (Edelmann et al., 2005), but the close genetic relatedness was existing between C. dubliniensis isolates from Larus argentatus (gull) or human according to results of mating type, the transmission occurred between human and bird but the direction of transfer likely was from human to bird (McManus et al., 2009). All these publications precedent were evidences that the role of birds as reservoirs and spreading to antifungal pathogenic *Candida*.

4- Cryptococcosis

Cryptococcus spp is a pathogenic yeast, cause a systemic mycosis in human (Taramasso et al., 2016). The important species of this genus are C. neoformans var. grubii (serotype A), *C. neoformans* var. *neoformans* (serotype D) and a hybrid serotype AD (Nakamura, 2000). *Cryptococcus* is predominantly opportunistic yeast and can be isolated from soil, *Eucalyptus* spp and bird droppings (Escandon et al., 2006). Another important *Cryptococcus* species is *C. gattii* (serotype B, C) can be also isolated from soil and *Eucalyptus* trees (Ellis and Pfeiffer, 1990; Chen et al., 2014). The faeces of pigeon is a good medium to carry and spread *C. neoformans* that can infect healthy human and immunocompromised patients (Haag-Wackernagel, 2004). After the investigation of non-spreading *C. neoformans* in crops of pigeons, thus, the pigeon was not considered as a reservoir but can be act as a carrier for this species (Khan et al., 1978; Ellabib et al., 2016) and to other species of *Cryptococcus*, which have clinical interest (Rosario et al., 2005). Not only pigeons carry *C. neoformans*, but pet birds could carry the yeast in their gut and excreting in their droppings. Eight out of 25 pet bird breeding had a positive *C. neoformans* culture (Kielstein et al., 2000), Swallows (Hirundo rustica) (Hedayati et al., 2011), Munia birds (Pal, 1989), White face duck, eagle owl, peacock and spotted eagle owl (Irokanulo et al., 1997). In addition, the antifungal resistance of *Cryptococcus* species isolated from different birds (captive and wild birds) was confirmed. Therefore, birds serve as carriers for transmission resistant *Cryptococcus* species for human (Mattsson et al., 1999; Lord et al., 2010).

5- Microsporidiosis

Microsporidiosis is an infection caused by many fungal species such *as Enterocytozoon bieneusi, Encephalitozoon hellem, Encephalitozoon intestinalis* and *Encephalitozoon cuniculi*. These organisms are small single-celled, obligate, intracellular parasites, which were initially classified as eukaryotic protozoa, although they have been recently reclassified among fungi based on certain genetic characteristics (Didier, 2005). Pigeons and exotic birds are considered potential sources of human-*Microsporidia* infection due to *E. hellem, E. bieneusi, E. intestinalis* and *E. cuniculi* for human residing in urban areas (Bart et al., 2008; Lallo et al., 2012). The *Microsporidia* isolates from both human and pigeons were genetically similar indicating that pigeons are a reservoir of potential human-*Microsporidia* infections (Haro et al., 2005). Birds such as lovebirds, parrots, budgerigars and falcons can be infected with microsporidiosis. Then, infected birds in close to human are a threat public health

(Randall et al., 1986; Black et al., 1997; Barton et al., 2003; Lee et al., 2011). Waterfowl can also be infected with *Microsporidia*, which present on the water surface. Infected waterfowl shed the spores into uncontaminated waters, such as drinking water sources (Slodkowicz-Kowalska et al., 2006), which thereby provokes waterborne diarrheal disease in human (Graczyk et al., 2008). Overall, both aquatic and non-aquatic birds can contract microsporidiosis and may serve as a major source of human microsporidiosis (Lobo et al., 2006).

Parasitic infections

1- Cryptosporidiosis

Cryptosporidiosis is caused by the protozoan parasite *Cryptosporidium*, which belongs to the phylum Apicomplexa (Fayer, 2010). *Cryptosporidium* cause diarrheal diseases in human. Parasitic transmission to human occurs via water and food contaminated with infectious *Cryptosporidium* oocytes (Smith et al., 2007). Birds are considered a natural host of *Cryptosporidium parvum* particularly aquatic birds, which represent a major environmental disseminator of these pathogenic human parasites (Majewska et al., 2009). However, waterfowl can serve as a non-infected parasitic vector by transporting *Cryptosporidium* parvum oocytes in their intestine and shedding them in many aquatic environments (Graczyk et al., 1996; Kuhn et al., 2002). Such as, migratory Canada geese, which carried *Cryptosporidium parvum* along migratory routes (Graczyk et al., 1998). In other cases, *Cryptosporidium* infected a wild range from domesticated, wild and captive birds causing respiratory, enteric or renal infections (Plutzer and Tomor, 2009; Nguyen et al., 2013). Human-Cryptosporidial infection occurred via consummation of contaminated water as drinking water and for agricultural purposes (Ramirez et al., 2004; Carmena, 2010).

2-Giardiasis

Giardia spp. is classified in the phylum Sarcomastigophora (Carmena, 2010). *Giardia* infections in human are mainly diarrheal diseases. The transmission of parasite to human can be via water or food-*Giardia* contaminated (Smith et al., 2007). *Giardia* spp has been detected in faecal samples from ducks, geese, coots, crows and cormorants (Plutzer and Tomor, 2009), which can transport the parasite between different aquatic environments (Graczyk et al., 1996; Kuhn et al., 2002). Birds are not only serving as carrier to parasite, but

also infected as recorded in Budgerigars (Melopsittacus undulatus) and white stork (Filippich et al., 1998; Franssen et al., 2000).

3- Toxoplasmosis

Toxoplasma gondii is a protozoan parasite belonging to the phylum Apicomplexa, subclass coccidian (Elmore et al., 2010). Felids are considered the definitive host of *T. gondii* oocytes, although other mammals and birds may function as intermediate hosts (Elmore et al., 2010). *T. gondii* is a major foodborne pathogen. Poultry meat is considered the principal source of *T. gondii* infection in human (Rinaldi and Scala, 2008). *T. gondii* has been found in the meat of chickens, duck, goose and pigeon, sold in poultry markets (Goodwin et al., 1994; Cong et al., 2012; Yang et al., 2012). Chickens (free-range or farmed animals) living in areas close to human may be a risk factor of *T. gondii* transmission to human and other animals (Aboelhadid et al., 2013). *T. gondii* has also been detected in other birds such as parrots, penguins and ʻAlala (*Corvus hawaiiensis*) (Ratcliffe and Worth, 1951; Hartley and Dubey, 1991; Work et al., 2000). Birds may either develop a fatal toxoplasmosis (Dubey et al., 1994; Guillermo and DaMatta, 2004; Cooper et al., 2015) or function as asymptomatic carriers of the parasite as observed with pigeons (Mushi et al., 2000).

Discussion

Human encounters many threats via exposure to many pathogens circulating in the environment in which we live. This is due to the increase in population density on the globe, leading to increased interaction between the individuals from different populations. For example, increasing demand for animal-food products prompts human to animals husbandry, which are used their meat in daily meals. Therefore, man became meet these animal species daily. In addition, the ability of many pathogens to adapt living in different hosts (table 1), we see a great mutually movement of pathogens between human and animals because they live in the same ecosystem. Pets live in close contact with their owners experimentally shared the same bacterial community (Hille et al., 2014). Despite these pets subject to medical checkups and periodically vaccines in veterinary clinics, but we found many pathological accidents due to the presence of pets close to human (table 1). Noteworthy, Birds were in the most species-rich reported in the globe-pets trade (Bush et al., 2014). Human also share the same environment (water and air) with wild animals including wild birds. Therefore, the pollution which occurs in the water or air from either human or animals is an important factor contributes in spreading the pathogens that may be ineffective in the environment (Table 2).

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Table 1. Description of the pathogenicity in birds of bird-associated zoonoses agents.

Pathogens	Affected bird categories			References
	Pet	Farm	Wild	
Influenza virus	+	+	+	(Van Borm et al., 2005; Lierz et al., 2007; Amonsin et al., 2008; Jones et al., 2014)
West Nile virus	NA	+	+	(Allison et al., 2004)
New castle virus	NA	+	+	(Sa idu et al., 2004; Biswas et al., 2005)
Chlamydia psittaci	+	+	+	(W. Cong et al., 2014)
***Mycoplasma* spp**	+	+	+	(Fischer et al., 1997; Wyrzykowski et al., 2013)
Pasteurella multocida	NA	+	+	(Biswas et al., 2005)
***Salmonella* spp**	+	+	+	(Biswas et al., 2005; Jorn et al., 2009)
***Campylobacter* spp**	NA	+	+	(Hughes et al., 2009)
E. coli	NA	-	+	(Nielsen et al., 2004)
***Mycobacterium* spp**	+	+	+	(Portaels et al., 1996)
***Clostridium* difficile**	-	-	?	(Borriello et al., 1983; Weese et al., 2010)
***Aspergillus* spp**	NA	+	+	(Alvarez-Perez et al., 2010; Arné et al., 2011)
***Candida* spp**	+	+	+	(Lanteri et al., 2012)
***Cryptococcus* spp**	-	?	-	(Kielstein et al., 2000)
Histoplasma capsulatum	+	-	-	(Cermeño et al., 2006a; Quist et al., 2011)
***Microsporidia* spp**	+	-	+	(Randall et al., 1986)
***Cryptosporidium* sp**	+	+	+	(Pagès-Manté et al., 2007; Bougiouklis et al., 2013)
***Giardia* sp**	+	NA	+	(Filippich et al., 1998; Franssen et al., 2000)
***Toxoplasma* spp**	+	+	+	(Hartley and Dubey, 1991; Work et al., 2000; Yang et al., 2012; Wei Cong et al., 2014)

+ Infected bird, - Carriage without infection, NA not available

Table 2. Bird associated zoonoses agents, their route of transmission to human.

Pathogens	Air-borne	Food-borne	Water-borne	Direct	Vector-borne	References
Influenza virus	+	+	+	+	-	(Van Borm et al., 2005; Lierz et al., 2007; Amonsin et al., 2008; Corzo et al., 2013; Jones et al., 2014)
West Nile virus	-	-	-	-	+	(Fall et al., 2013)
New castle virus	-	+	-	-	-	(Sa idu et al., 2004)
Chlamydia psittaci	-	-	-	+	-	(W. Cong et al., 2014)
***Mycoplasma* spp**	+	-	-	+	-	(Fischer et al., 1997)
Pasteurella multocida	-	-	+	-	-	(Blanchong et al., 2006b)
***Salmonella* spp**	-	+	+	+	-	(Walters et al., 2014)
Campylobacter spp	-	+	+	+	-	(Mughini Gras et al., 2012; Laine et al., 2014)
Escherchia coli	-	+	+	-	-	(Horton et al., 2011)
***Mycobacterium* spp**	-	+	+	+	-	(Portaels et al., 1996)
***Clostridium* difficile**	+	+	+	-	-	(Rupnik, 2010; Hensgens et al., 2012; Rodriguez-Palacios, Borgmann, Terence R Kline, et al., 2013)
***Aspergillus* spp**	+	-	-	-	-	(Arné et al., 2011)
***Candida* spp**	-	-	-	+	-	(Quist et al., 2011)
***Cryptococcus* spp**	+	-	-	+	-	(Kielstein et al., 2000)
Histoplasma capsulatum	+	-	-	+	-	(Nosanchuk and Gacser, 2008; Quist et al., 2011)
***Microsporidia* spp**	-	-	+	-	-	(Thaddeus K. Graczyk et al., 2008)
Cryptosporidium* and *Giardia	-	+	+	-	-	(Thaddeus K Graczyk et al., 2008)
***Toxoplasma* spp**	-	+	-	-	-	(Opsteegh, 2011)

Chapter 2: Yellow-legged gulls

2.1 Yellow-legged gulls

The seabird, yellow-legged gull (*Larus michahellis*) is considered as competitor and predator for several bird species nesting in close proximately from its colonies. Gulls are predators of eggs, chicks and sometimes adults terns (Guillemette and Brousseau, 2001). Similarly, the Audouin's gull are expose to yellow-legged gulls predation (Paracuellos and Nevado, 2010). The yellow-legged gull has been divided into two subspecies; *L. michahellis michahellis* on the Mediterranean coast and *L. michahellis lusitanius* on the Atlantic coast. The Mediterranean gulls genetically related to the Atlantic gulls, as confirmed by the mitochondrial cytochrome *b* gene and microsatellite loci analysis. Herring gulls exhibit some phenotypic similarities with the yellow-legged gull (Pons *et al.*, 2004). Yet, the Mediterranean gull has less genetically similar to herring gull (*L. argentatus*) from Western Europe than the Atlantic gull.

On Mediterranean coast, yellow-legged gulls (*Larus michahellis*) populations have increased in number of individuals and colonies and they have particular feeding behavior, which is depending on access to dump areas from human activities (Duhem *et al.*, 2003; Ramos *et al.*, 2010). The abundance of food (dump) or nest site (roofs) leads to a demographic boost of yellow-legged gull populations. Their numbers, their density and their anthropophily represent a high opportunity of emergence, intraspecific transmission or host shift for pathogens (Slippers *et al.*, 2005) between yellow-legged gulls and human being at close distance. A recent study conducted in a neighboring geographic area (Bonnedahl *et al.*, 2009) found that yellow-legged gulls contributed to the zoonotic dissemination of antibiotic resistant *Escherichia coli* isolates with Extended Spectrum β-Lactamase in southern of France. Yellow-legged gulls represent an interesting model for studying the circulation of fungi in natural populations as well as in their colonies close to human settlements, because these birds can construct their breeding colonies on building roof tops inside the city. The colonies of gulls relatively increase during the breeding season. Gulls lay their eggs from mid-March to early May and the individuals remain close to their birth area throughout the year, the farthest distance recorded was less than 50 km from their birth colonies (Arizaga *et al.*, 2010). The movement of gulls depends on available food sources in the environment (Telailia *et al.*, 2015), and their anthropophilic characteristics allows them to exploit the food sources of human activities (Pons and Migot, 1995). However, the role of yellow-legged gulls

as reservoir and, partly, vector of potentially pathogenic human fungi is unknown. Because data on the characterization of yellow-legged gull's gastrointestinal fungal biota are scarce, the aim of this study was to identify the species diversity and abundance of the gut mycobiota in the yellow-legged gulls, and to detect the carriage of antifungal resistant isolates.

2.2 Study areas and sample collection

Yellow-legged gull's fecal samples were collected from April to September 2013 in two departments (Hérault and Bouches-du-Rhone) on the Mediterranean coast from southern of France. The first collection was in April 2013, with a team from "Centre d'information de la réserve nationale de Camargue" in Villeneuve-lès-Maguelone, we could access a natural reserve at the lagoon of Pierre-Blanche located on the coast of the Mediterranean Sea. There were many migratory birds as well as two colonies of yellow-legged gulls. We collected from inside the yellow-legged gulls colonies 60 fecal samples (30 samples from each colony) (Figure 2.1). With the same team from the center above, we also collected 55 faecal samples from the colonies of nesting gulls on the roof tops of buildings in the two cities, Palavas-les-Flots and La Grande-Motte (26 samples from gulls in Palavas-les-flots and 29 samples from gulls in La Grande-Motte). The faecal samples in Marseille area were collected from gulls' colonies on two archipelagos, Frioul and Riou. Firstly, after obtaining permission to enter the natural reserve of the Riou Archipelago, we collected 31 faecal samples in July 2013. In September 2013, we collected 31 faecal samples from gulls' colonies on the Frioul Archipelago. All faecal samples were collected with sterile cotton-tipped swabs dipped 3 ml of sterile saline solution (0.9 % NaCl) supplemented with 0.5 g/L chloramphenicol (to prevent bacterial growth) and labeled according to site and date. We transferred and analyzed the samples in the same day of collection in the laboratory of Parasitology and Mycology, Hospital La Timone, Marseille city.

Figure 2.1. Pictures show sampling areas.
A, B, C Lagoon of Pierre Blanche / Villeneuve les Maguelone
D, E, F Riou Island/ Marseille
G, H, I Frioul Island/ Marseille

2.3 Samples processing

The swabs were vortexed and allowed to sediment for at least 10 min. Then the sticks were removed and the supernatant was transferred to sterile conical tubes. Then 100 µl supernatant was plated in each of the Five culture media plates (Figure 2.2): Sabouraud agar, L-Dopamine agar (Liu *et al.*, 2014), Scedo-select III agar (Table 2.1) and Dichloran Rose Bengal Benomyl agar (Sigma-Aldrich, France) with or without 4 mg/L itraconazole. The cultures were incubated at 30°C. The colony-forming units (CFU) were subsequently counted in each Petri dish after 48 h for the yeast colonies and after 5 days for the filamentous fungi colonies. The remain of supernatant was conserved at – 20 °C, which was then sent to virology laboratory / faculty of medicine / Aix-Marseille University for further study in virology.

2.4 Culture media

Our investigation was looking for fungal biota at faecal samples of yellow-legged gulls. Therefore, we used four culture media to isolation diverse fungal species from the samples and one more culture medium to isolate the ant-itraconazole species resistant.

2.4.1. Sabouraud agar with chloramphenicol was used for general fungal isolation including yeast and molds. Yeast grows on sabouraud agar in creamy or white colonies and mold grow with filamentous colonies and different colors.

2.4.2. Dichloran Rose Bengal Chloramphenicol agar (DRBC) is used to grow pathogenic yeast and molds. The accumulation effects of the ingredients initially inhibit bacterial growth and reduce the diffusion of *Mucor* and *Rhizopus*. In current study, we used DRBC for two purposes i) to isolate fungal flora in general ii) we assessed the antifungal resistance via added 4 mg/L of itraconazole.

2.4.3. Scedo-select III agar (Table 2.1) supports the growth of *Scedosporium* species complex (Semi-selective medium) through several carbon and nitrogen sources and prevents the growth of other fungal species via benomyl and dichloran.

2.4.4. L-Dopamine agar (Table 2.1) is a selective medium to isolation *Cryptococcus* spp. This medium supports with L-3,4-dihdroxyphenylalanine (L-Dopa), which stimulate to melanin formation by *Cryptococcus* resulting to display the black color in yeast colony and in culture.

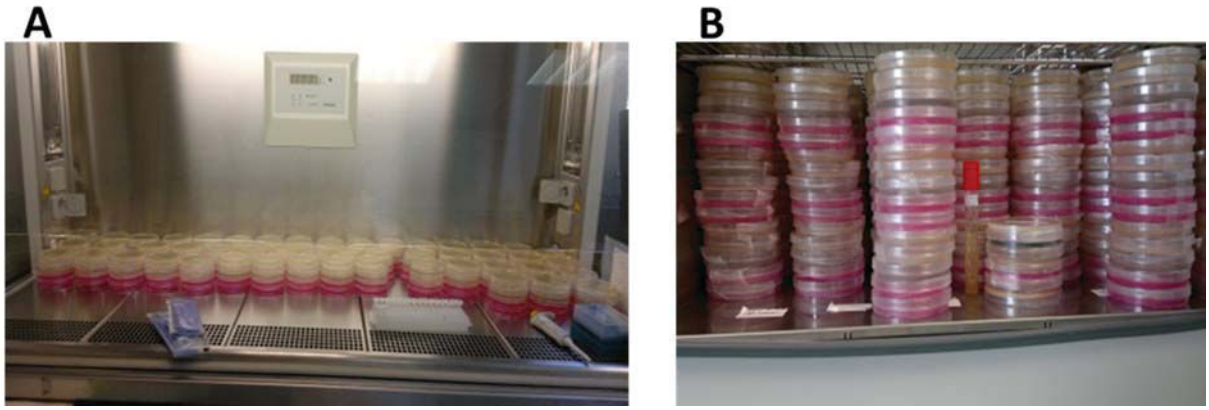

Figure 2.2. Pictures show the plates of culture media.

A. **Inside the laminar**
B. **Inside the incubator**

Table 2.1. Composition of semi-selective culture medium Scedo-Select III and L-Dopamine agar

Scedo-select III agar	Amount
4-hydroxybenzoate (4-HOC_6H4CO_2Na)	0.9 g
Ammonium sulfate (NH4)2SO_4)	5 g
potassium dihydrogen phosphate (KH_2PO_4)	1.25 g
Magnesium sulfate heptahydrate ($MgSO_4$-7H_2O)	0.625 g
chloramphenicol	0.5 g
Dichloran[a]	2 mg
Benomyl[b]	8 mg
Agar	20 g
Distilled water	1 L
L-Dopamine agar	
3,4-dihydroxy-phenilalanine (L-DOPA)	0.2 g
Yeast nitrogen base without amino acids and ammonium sulphate	6.7 g
Asparagine	1 g
Glucose	1 g
Gentamycin (40U/ml) and Penicillin (20U/ml) (Panapharma / France)	0.5 ml
Agar	20 g
Distilled water	1 L

Chloramphenicol = Dilute in 5 ml ethanol 96%
Dichloran = 1 ml of a 0.2% solution in ethanol
Benomyl = dilute in 5 ml methanol, add to the 55◦C agar

2.5 Fungal identification

We performed macroscopic examination in Petri dishes to describe the characteristics of diverse colonies growing on agar with necked eye. Then, we used microscope as supplementary step with MALDI-TOF MS (Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer) technique to determine final identification of fungi through describe the morphology of yeast cells and filamentous fungi.

The identification by MALDI-TOF MS was carried out using yeast and filamentous fungi colonies obtained after incubation at 30°C on original petri dishes. Protein extraction was performed according to the manufacturer's instructions with one yeast colony or a small part of the filamentous fungi colony placed in sterile conical tubes containing 300 µl distilled water and 900 µl absolute ethanol (70%; Sigma-Aldrich, France). The tube was centrifuged at 13,000 rpm for 5 minutes for yeasts and 10 minutes for filamentous fungi. The supernatant was aspirated and the cell pellet was retained, and the tubes were centrifuged again for 2 minutes. The remaining supernatant was removed by micropipette, and the cell pellet was dried and resuspended in 10 µl formic acid (70%; Sigma-Aldrich, France), mixed well and incubated at room temperature (RT) for 5 min. Subsequently, it was followed by addition of 10 µl acetonitrile (Sigma-Aldrich) mixed well and incubated at room temperature (RT) for 5 min. This suspension was centrifuged at 13,000 rpm for 2 min, one spot (1µl) from the supernatant of yeast sample and 4 spots filamentous fungus samples were removed and placed on a target spot on a metallic plate (Anchorchip 96-spot; Bruker Daltonics, Inc.) and dried. Matrix solution (alpha-cyano-4-hydroxycinnamic acid; Bruker Daltonics, Inc; one spot (1.2µl) of a concentration of 10 mg/ml was then added to the fungal protein extract and redried. The Microflex LT™ (Bruker Daltonics, Germany) mass spectrometer with the MALDI Biotyper software v2.1 (Bruker Daltonics) is used routinely for yeast and Fungal isolates identification (Cassagne *et al.*, 2013). As recommended by the company, *Escherichia coli* isolate is generally employed as a positive control. The identification was carried out using the Bruker database ver. 3.2.1.1 combined with a homemade rarer yeast species reference spectra database.

2.6 Nucleic acid extraction

NucliSENS easyMAG technology was used to nucleic acid extraction. This technique based on Boom technique using Magnetic silica particles to capture of nucleic acid from lysis suspension. Firstly, fungi sample was placed in 800 ml of lysis buffer to direct sample lysis. The first step in the machine began manually by adding 50 µl of Magnetic silica suspensions on each sample and homogenized to adsorption of nucleic acid on silica matrix. Then, sixty minutes programmed in the machine to wash the nucleic acid adsorbed on the silica matrix using a buffer containing guanidinium thiocyanate to maintain high stringency. To maintain the nucleic acids adsorbed on the silica matrix, all the steps were performed at a neutral pH level with high salt concentrations. The final step was the elution of nucleic acid in a volume 50 ml at 70 C^{o} and pH > 8.0 (Jeddi *et al.*, 2013).

2.7 Identification with Polymerase Chain Reaction (PCR)

We sequenced D1/D2 domain, Internal Transcript Spacer (ITS1- ITS4) and β-tubulins regions for some species that we could not identify them with MALDI-TOF MS due to there were no reference database for them. 1 µl of genomic DNA were added to 12 µl of light cycler® 480 Probes Master, 11 distilled water and 0.5µl from each forward and reverse primers. PCR amplification was performed in T3-thermocycler (Biometra, Germany) with specific conditions for both D1-D2 and ITS 1-4 primers. The reactions products were purified by using purification kit (Neo-Biotech-France). For sequencing reactions, we added 1 µl from purified DNA to 19 µl from the mix (Distilled water 14 µl, Mix (BigDye terminator V1.1) 2 µl, sequence buffer 5x 3 µl, primer Fwd. or Rev. 0.3 µl), then Amplification cycle was 96 C˚ for 2 min, 25 cycles of (96 C˚ for 10 sec, 50 C˚ for 5 sec, 60 C˚ for 4min). The reactions products were diluted in 19 µl distilled water and were added to 55µl of (10µl Big Dye® X-terminator (Applied Biosystems, USA) and 45 µl SAM) in plate 96 wells. The plate was vortexed for 30 min and centrifuged for 1 min. DNA sequence (Genetic Analyzer 3130, Applied Biosystems) was used to DNA sequencing. Then the sequences were edited by using SEQUENCHER version 5.1.

First Article: Yeast communities structure in various *Larus michahellis* breeding colonies

The infections with pathogenic yeast species are higher than filamentous fungi infections in human. In general, fungal infections mostly occur before fungal biota species, particular, in immunocompromised patients. Fungal infections may be superficial or cutaneous or systemic depending on the ability of fungi species to adapt and infect the specific part of human body. The spreading of pathogenic fungi in human environment can be by intermediate host, such as birds. Therefore, we evaluated the yeast gut communities in five yellow-legged gulls breeding colonies on Mediterranean area, southern France. These gull colonies exposed to a variable anthropogenic impact ranging from building roofs in two cities, La Grande-Motte (GM) and Palavas-les-Flots (PF), to a natural reserve, the lagoon of Pierre-Blanche (PB), and including two suburban ecoclines, the Frioul (FR) and Riou (RI) archipelagos. The latter is being exposed to sewers pollution. High richness and diversity of yeast species were found according to geographical locations and probably, bird's diet. Gulls breeding on Frioul and Pierre-Blanche displayed the highest richness and diversity of yeast species. The lowest diversity of yeast species was observed in gulls breeding on Riou archipelago, which may share a similar type of diet with gulls on both La Grande-Motte and Palavas-les-flots. *C. krusei* was the major species isolated from Frioul and Riou archipelagos of Marseille city, and the second only after *Galactomyces geotrichum*, which was mainly isolated in gulls from Pierre Blanche. High prevalence of *C. glabrata* and *C. albicans* was detected in gulls breeding colonies on building roofs in two cities; La Grande-Motte and Palavas-les-flots. The two frequent *Candida* species isolated from human infections, particularly in bloodstream infections. The yeast communities of gulls gut on Frioul and Pierre Blanche involved *Saccharomyces cerevisiae*, which is most frequent yeast species in human food. Rarer yeast species, such as *C. lusitaniae* and *C. tropicalis,* were isolated here as well as other species *C. lambica, Pichia kluyveri* and *Candida guilliermondii*. In contrast, *Cryptococcus neoformans* was not isolated in our study despite the use the selective L-dopamine agar culture medium. This indicates that the yellow-legged gull sample here is not a reservoir host for *Cryptococcus* spp. The anti-itraconazole resistance was observed in *C. glabrata*, *C. albicans, C. krusei,* C. *kefyr*, *C. lusitaniae*, *G. geotrichum*, *S. cerevisiae*, and *Kloeckera apiculata*, indicating that gulls act as reservoir and spreader for antifungal resistant yeast species that may be potential human infection.

Article status: submitted

Gut yeast communities in Larus michahellis from various breeding colonies

Journal:	*Medical Mycology*
Manuscript ID	MM-2016-0109
Manuscript Type:	Original Article
Date Submitted by the Author:	10-Apr-2016
Complete List of Authors:	AL-YASIRI, Mohammed Hashim Yasir; Aix-Marseille Université, IP-TPT UMR MD3, 13885 Marseille, France, Faculty of medecine Normand, Anne-Cecile; Assistance Publique Hopitaux de Marseille, Parasitology - Mycology Piarroux, Renaud; Aix-Marseille Universite Faculte de Medecine, IP-TPT UMR MD3 Ranque, Stéphane; Aix Marseille Université, Parasitologie Mycologie Mauffrey, Jean-Francois; Aix Marseille Université, LPED
Keyword:	Yellow-legged gulls, yeast communities, antifungal resistance, Candida krusei
Abstract:	Yellow-legged gulls have been reported to carry antibiotic-resistant Enterobacteriaceae; however, the gut mycobiota of these birds has not yet been described. In this study, we analyzed the gut yeast communities in five yellow-legged gull breeding colonies along the Mediterranean littoral in southern France. Gull fecal samples were inoculated onto four types of culture media, including one supplemented with itraconazole. Yeast species richness, abundance and diversity were estimated, and factorial analysis was used to highlight correspondences between breeding colonies. Yeast grew in 113 of 177 cultures, and 17 distinct yeast species were identified. The most frequent species were Candida krusei (53.5%), Galactomyces geotrichum (44.1%), C. glabrata (40.9%), C. albicans (20.5%) and Saccharomyces cerevisiae (18.1%). Gut yeast community structure in the gulls at both Pierre-Blanche Lagoon and Frioul Archipelago were characterized by greater species richness and diversity than in those at the two cities of La Grande-Motte and Palavas-les-Flots as well as Riou Archipelago. Gulls in these latter three sites probably share a similar type of anthropogenic diet. Notably, the proportion of anthropic yeast species, including C. albicans and C. glabrata, in the gull mycobiota increased with gull colony synanthropy. Antifungal resistance was found in each of the five

	most frequent yeast species. We found that the gut yeast communities of these yellow-legged gulls include antifungal-resistant human pathogens. Further studies should assess the public health impact of these common synanthropic seabirds, which represent a reservoir and disseminator of drug-resistant human pathogenic yeast into the environment.

Gut yeast communities in *Larus michahellis* from various breeding colonies

Mohammed Hashim Al-Yasiri[a], Anne-Cécile Normand[b], Renaud Piarroux[a,b], Stéphane Ranque[a,b,*], Jean-François Mauffrey[c]

a. Aix-Marseille Université, IP-TPT UMR MD3, 13885 Marseille, France
b. Laboratory of Parasitology - Mycology, APHM CHU Timone, 13005 Marseille, France
c. Laboratoire Population Environnement Développement (LPED)- UMR-151, Aix-Marseille Université – IRD, 13331 Marseille, France

*** Corresponding author**

Dr Stéphane Ranque
Laboratoire de Parasitologie Mycologie
APHM CHU Timone
264 rue Saint Pierre
13385 Marseille CEDEX 5
Phone: +33491386090
Fax: +33491384957
E-mail: stephane.ranque@ap-hm.fr

Abstract

Yellow-legged gulls have been reported to carry antibiotic-resistant Enterobacteriaceae; however, the gut mycobiota of these birds has not yet been described. In this study, we analyzed the gut yeast communities in five yellow-legged gull breeding colonies along the Mediterranean littoral in southern France.

Gull fecal samples were inoculated onto four types of culture media, including one supplemented with itraconazole. Yeast species richness, abundance and diversity were estimated, and factorial analysis was used to highlight correspondences between breeding colonies.

Yeast grew in 113 of 177 cultures, and 17 distinct yeast species were identified. The most frequent species were *Candida krusei* (53.5%), *Galactomyces geotrichum* (44.1%), *C. glabrata* (40.9%), *C. albicans* (20.5%) and *Saccharomyces cerevisiae* (18.1%). Gut yeast community structure in the gulls at both Pierre-Blanche Lagoon and Frioul Archipelago were characterized by greater species richness and diversity than in those at the two cities of La Grande-Motte and Palavas-les-Flots as well as Riou Archipelago. Gulls in these latter three sites probably share a similar type of anthropogenic diet. Notably, the proportion of anthropic yeast species, including *C. albicans* and *C. glabrata*, in the gull mycobiota increased with gull colony synanthropy. Antifungal resistance was found in each of the five most frequent yeast species.

We found that the gut yeast communities of these yellow-legged gulls include antifungal-resistant human pathogens. Further studies should assess the public health impact of these common synanthropic seabirds, which represent a reservoir and disseminator of drug-resistant human pathogenic yeast into the environment.

Keywords: Yellow-legged gulls, yeast communities, *Candida krusei*, *Galactomyces geotrichum,* antifungal resistance

Introduction

Several bird species are involved in dissemination and transmission of many zoonosis agents. For example, pet birds have an important impact on human health (especially owners and animal health care workers), as these animals carry and diffuse pathogens such as the yeast *Cryptococcus neoformans*.[1] Additionally, several wild bird species are the reservoir of various human pathogenic agents such as viruses, *Salmonella*, *Candida* and *Cryptococcus*.[2–4] Migratory birds are particularly important vectors of various zoonoses.[5] The yellow-legged gull (*Larus michahellis*) is the most prevalent seabird in the French Mediterranean area. Gull population numbers have greatly expanded over the course of the last few decades, primarily due to the increased availability of food derived from human activities.[6–8] This species has a profound impact on the local ecosystem due to a great degree of ecological adaptability and particularly aggressive behavior.[9] Yellow-legged gulls from two colonies also in the French Mediterranean area (in which one colony fed from a city dump and the other fed offshore) were found to carry antibiotic-resistant Enterobacteriaceae.[10] These seabirds are known to carry human-pathogenic yeast species within their gut.[11,12] However, the gut mycobiota of these birds has never been studied. Whether breeding biotope influences these gut yeast communities remains unknown. Therefore, the primary objective of this study was to characterize the yeast community in the gut yellow-legged gulls sampled in five distinct breeding colonies. We also aimed to assess whether the birds carry azole-resistant yeasts.

Materials and Methods

Study area (Fig. 1). The yellow-legged gull colonies included in the study were located in three sites in the department of Hérault: a natural reserve at Pierre Blanche Lagoon

(43.494028N, 3.860266E) and on the building rooftops of two cities, Palavas-les-Flots (43.527767N, 3.92581E) and La Grande-Motte (433341.72N, 40501.81E). The other two sites were in the department of Bouches-du-Rhône: two archipelagos off the coast of Marseille, namely Frioul (431613.03N, 51753.43E) and Riou (431041.20N, 52249.45E). The principal dietary resources of birds in Pierre Blanche, Frioul and Riou were likely offshore sites, while the gulls in Palavas-les-Flots and La Grande-Motte primarily obtained nutrients from garbage from domestic refuse or outdoor city dumps. Overall, these study sites were characterized by a gradient of anthropogenic influence. The sites in the cities of Palavas-les-Flots and La Grande-Motte, which represent entirely man-made environments, were exposed to high anthropogenic influence. The suburban zone of the Riou and Frioul Archipelagos were exposed to an intermediate anthropogenic impact. Finally, Pierre Blanche Lagoon, a natural reserve distant from urban settlement, displayed very little anthropogenic influence. Notably, the Riou Archipelago, which is located just off coast from the Marseille sewage outlet at the Calanque of Cortiou, is exposed to anthropogenic pollution distinct from that at the Frioul Archipelago.[13]

Sample collection and processing. Fecal samples from yellow-legged gulls were collected from breeding colonies during the reproduction period at each site: in April 2013 at Pierre Blanche, Palavas-les-Flots and La Grande-Motte; in July 2013 at the Riou Archipelago; and in September 2013 at the Frioul Archipelago. When possible, fresh droppings were selected to avoid a possible selection bias towards yeast species that resist desiccation. Each sample was collected with a sterile cotton-tipped swab dipped in 3 ml of sterile saline (0.9% NaCl) supplemented with 0.5 g/L chloramphenicol transport medium. The samples were labelled according to site and date. Each swab was vortexed in the transport medium and then allowed to sediment for at least 10 min. After removing the swab, the supernatant was transferred to a sterile conical tube. Next, 100 µl supernatant was inoculated onto each of the four culture

media plates: Sabouraud agar, L-dopamine agar for isolation of *Cryptococcus* species (1 g asparagine, 1 g glucose, 0.2 g L-DOPA (3,4-dihydroxy-phenylalanine), 6.7 g yeast nitrogen base without amino acids and ammonium sulfate, 20 g agar, and 1000 ml distilled water; all reagents available from Sigma Aldrich, France), and Dichloran Rose Bengal Chloramphenicol (DRBC, Sigma Aldrich) agar + 10 mg/L Benomyl (Sigma Aldrich) with or without 4 mg/L itraconazole (Sigma Aldrich). The cultures were incubated for 48 h at 30°C, and the yeast colony-forming units (CFUs) were subsequently quantified.

Yeast identification and nomenclature. Yeasts were identified via MALDI-TOF MS (matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry), as previously described,[14] using a Microflex LT™ (Bruker Daltonics, Germany) mass spectrometer with the MALDI Biotyper software v. 2.1. (Bruker Daltonics). The species were identified using the Bruker database v. 3.2.1.1 supplemented with an in-house-generated reference spectra database including rarer yeast species, as previously described.[15] As fungal nomenclature is constantly evolving, we used the most common name of each yeast species. The names used were the current names according to MycoBank, the website of the International Mycological Association (www.mycobank.org, accessed on 26 March 2016), with the exception of the following species: *Candida krusei*: *Candida acidothermophilum*; *Candida albicans*: *Candida albicans* var. *albicans*; *Candida tropicalis*: *Candida tropicalis* var. *tropicalis*; *Candida pelliculosa*: *Candida beverwijkiae*; *Candida lambica*: *Candida fimetaria* var. *fimetaria*; *Candida zeylanoides*: *Candida zeylanoides* var. *zeylanoides; Candida kefyr*: *Atelosaccharomyces pseudotropicalis; Rhodotorula mucilaginosa*: *Rhodotorula mucilaginosa* var. *mucilaginosa*; *Kloeckera apiculata*: *Cryptococcus vini;* and *Cryptococcus uniguttulatus*: *Cryptococcus neoformans* var. *uniguttulatus*.

Statistical Analyses

Species richness for each breeding colony was estimated using the Chao 1 statistic either by cumulating or averaging positive sample values. Abundance was estimated by using either the number of positive cultures (incidence) for a given species or the CFU counts for each species. Yeast species diversity for each breeding colony was estimated using the Shannon–Wiener index, which calculates species diversity taking into account the relative abundance of each species at each site, and evenness using the Buzas and Gibson's index (evenness= e^H/S; in which H is the Shannon index and S represents species richness) (available in PAST 3.05).[16] Yeast community structure similarity between breeding colonies was estimated by assessing the presence/absence of taxa using the Sorensen index[17] and the relative abundance of each species using the Bray-Curtis index.[16] We first tested the effect of dropping sample state (dry or fresh), to assess whether sample state had an effect on culture results, using the Chi-square test. The Akaike information criterion statistics was used to select the best-fitting statistical model for yeast species diversity, richness and abundance.[18] Next, yeast species diversity, richness and abundance at each breeding colony were compared using proc Genmod in SAS, version 9.2 (SAS Institute, Cary, NC, USA). Factorial correspondence analysis was computed using PAST 3.05.[16] In this analysis, the information for all sites was condensed into a two-dimensional graphical representation of similarity between study sites, with a minimum loss of information with respect to gut yeast community structure (species composition and relative abundance). Graphical proximity between sites indicated gut yeast mycobiota similarity.

Results

The geographical location of the five yellow-legged gull breeding colonies is presented on Fig. 1. Yeast grew in 113 of 177 collected samples from the five study sites: Pierre Blanche, n=60; Palavas-les-Flots, n=26; La Grande-Motte, n=29; Riou, n=31; and Frioul, n=31. The best-fitting statistical distributions were: normal for the yeast species diversity, poisson for

species richness (Chao 1), and negative binomial for abundance. From all positive samples, 90 samples were fresh and 23 samples were relatively dry droppings. Whether dropping samples were dry or fresh did not significantly (p=0.60) influence the growth of colonies upon culture. Moreover, when taking into account the gull colony effect, there was no significant difference in yeast species diversity (p=0.55), abundance (p=0.56) or richness (p=0.91) between dry and fresh samples. Therefore, the status of samples (dry or fresh) was not included in further analyses.

Overall, 17 distinct yeast species were isolated. The number of distinct yeast species from each site was 14 in Pierre Blanche, 8 in Palavas-les-Flots, 6 in La Grande-Motte, 5 on the Riou Archipelago, and 13 on the Frioul Archipelago (Table 1). The highest degree of yeast species richness was observed in gulls on the Frioul Archipelago, where up to 7 distinct species were isolated per sample (Fig. 2). Details concerning the yeast communities of each study site are presented in Table 1. *C. krusei* was the most frequent species found in Frioul (95.7%) and Riou (81.3%); *C. glabrata* (53.3%) was the most frequent species observed in La Grande-Motte, *G. geotrichum* was the most frequent species complex in Pierre Blanche (79.1%), and *C. albicans* (43.8%) was the most frequent species found in Palavas-les-Flots.

Notably, the yeast community structure in the guts of these gulls was similar at Pierre-Blanche and the Frioul Archipelago. Both harbored rich communities of 14 and 13 species, respectively. Likewise, populations from both sites displayed similar proportions of very abundant, moderately abundant, and particularly rare species (Table 1). In contrast, the structure of the gut yeast communities in gulls at the other three breeding colonies was characterized by a relative scarcity in rarer species and a relatively poor species correlation between the three breeding colonies. The characteristics of the gut yeast communities at each study site are illustrated in Fig. 3. The richness of yeast communities varied widely between the breeding colonies (p=0.0002). This was partly explained by the observation that the yeast

communities in gulls from the breeding colonies of Pierre Blanche and Frioul displayed a similar (p=0.39) mean (SD) richness, with 2.91 (0.35) and 3.30 (0.67) distinct species, respectively. Furthermore, this varied richness was also due to similar (p=0.52) species diversity in the two sites, which was significantly higher than in all other sites, with pairwise comparison test p values ranging from 0.0013 to $<10^{-4}$. In contrast, evenness at both Pierre-Blanche and Frioul was lower than at the other sites due to the presence of the distinct predominant species *G. geotrichum* and *C. krusei*, respectively. The high level of evenness observed in Riou, Palavas-les-Flots and La Grande-Motte was due to lack of rare species in these breeding colonies.

The similarities in yeast community structure between gulls from distinct breeding colonies are presented in Table 2. Regarding the presence/absence of taxa, the community structure of the gulls at Pierre-Blanche shared 81.5% similarity with those at Frioul; while those at La Grande-Motte shared 66.7% similarity with gulls at Riou and Palavas-les-Flots. Concerning the relative abundance of each species, the community structure at Frioul clustered with Riou (79.2% similarity) and Pierre-Blanche (59.6% similarity). Overall, yeast species structure was similar at Frioul and Pierre-Blanche, whereas the relative abundance of species was similar at the Riou and Frioul Archipelagos.

Factorial correspondence analysis (Fig. 4) illustrated the correspondence between yellow-legged gull breeding colony locations and the associated yeast community structure, taking into account the relative abundance of each species. Pierre-Blanche, Frioul and Palavas-les-Flots were the most divergent sites; each was characterized by either specific species (which were not isolated in any other location, i.e., *C. lambica* and *C. pelliculosa* at Pierre-Blanche or *Cryptococcus uniguttulatus* and *Hanseniaspora guilliermondii* at Frioul) or particular abundant species combinations (i.e., an abundance of *Candida zeylanoides* and *Aureobasidium pullulans* as well as the absence of *Galactomyces geotrichum* at Palavas-les-

Flots). The large majority of the *C. krusei* isolates originated from yellow-legged gull breeding colonies located on the two archipelagos off the coast of Marseille: Riou and Frioul. Assessment of the gut yeast community structure in these two archipelagos, the yeast community in the gulls of Riou corresponded to a subset of those in Frioul. The most abundant yeast species at La Grande-Motte were also abundant at all other sites; and *C. glabrata* was the predominant species at this site as illustrated in Fig. 4.

Analysis of the yeast community structures grown on DRBC and DRBC media supplemented with itraconazole (Fig. 5) revealed azole-resistant isolates among the following species: *C. glabrata*, *C. albicans, C. krusei, C. kefyr*, *C. lusitaniae*, *G. geotrichum*, *S. cerevisiae* and *Kloeckera apiculata*. No resistant isolates were detected among the *C. lambica*, *C. pelliculosa*, *C. tropicalis*, *C. zeylanoides* and *Cryptococcus uniguttulatus* isolates. In contrast, a few species, including *C. kefyr*, *Hanseniaspora. guilliermondii* and *Pichia kluyveri*, were isolated exclusively in the presence of itraconazole. Notably, only a single *Cryptococcus* sp. isolate (*C. uniguttulatus*) was detected in our study, despite of the use of specific dopamine agar medium.

Discussion

This is the first study of gut yeast communities in yellow-legged gulls (*Larus michahellis*), which highlights the richness of the yeast species communities and the striking differences with respect to geographic location of the gull breeding colonies. These observations are in line with the findings of a similar study conducted on South American kelp gulls (*Larus dominicanus*).[19] We observed the highest degree of yeast species richness and diversity in gulls breeding on the Frioul Archipelago, followed by those in the natural reserve of Pierre Blanche. In contrast, the lowest species diversity was observed in gulls breeding on the Riou Archipelago. The reasons behind the clear dissimilarity between these two archipelagos off

the coast of Marseille are unknown. Recently, Duhem *et al.* have analyzed yellow-legged gull pellets on these two archipelagos and concluded that they shared a similar type of diet.[20] In contrast, our findings of varied species diversity between the gut yeast communities of yellow-legged gulls on these two archipelagos suggest that the gulls breeding on the Frioul Archipelago have easier access to a more varied diet (hence the high yeast species diversity) than those breeding on the Riou Archipelago. The pollution of the marine environment of Riou might influence the gull mycobiota, as the archipelago is approximately 4 km off coast from the inlet of Cortiou, where the Marseille sewage outlet is located.[13] In summary, the gut yeast communities of these yellow-legged gulls displayed significantly higher diversity in Pierre-Blanche and Frioul than in the other breeding colonies. We therefore hypothesize that the gulls in La Grande-Motte, Palavas-les-Flots and Riou shared a similar type of diet, likely primarily derived from garbage from domestic refuse or outdoor dumps. Whether the predominance of either *Galactomyces* or *Candida* spp. in the gull mycobiota depends on the bird's principal dietary resource remains to be elucidated; nevertheless, we discuss this hypothesis with a focus on species particularities.

It has been demonstrated that these yeast communities can survive in the high-temperature (41 ± 2 C°) and low-pH environment of the gull's gut.[2] The prevalence of *C. krusei*, *C. glabrata*, *C. albicans* and *S. cerevisiae* was higher in the current study than previously observed in other gull species, including lesser black-backed gulls (*Larus fuscus*), slender-billed gulls (*L. genei*), herring gulls (*L. argentatus*) and black-headed gulls (*L. ridibundus*).[21] In contrast to the kelp gull (*Larus dominicanus*), in which the predominant yeast species was *C. lambica*,[19] the predominant species in the yellow-legged gull were *G. geotrichum*, *C. krusei*, *C. glabrata* or *C. albicans* depending on breeding colony location. Interestingly, *C. krusei* was the predominant species in the colonies on the two archipelagos off the coast of Marseille; and it was also second only to *G. geotrichum* in the natural reserve of Pierre Blanche. In the kelp

gull, *C. krusei* was second only to *C. lambica*.[19] In seawater, *C. krusei* is considered an indicator of pollution from terrestrial sources.[22] Moreover, Duhem *et al.* have shown that the diet of the yellow-legged gulls breeding in the Frioul and Riou Archipelagos consists mainly of nutrients from refuse dumps and terrestrial sources.[20] We thus hypothesize that *Candida krusei* colonizes the yellow-legged gull's gut due to consumption of polluted seawater, refuse dumps and/or other terrestrial sources in the vicinity of the metropolis of Marseille. The second most frequent species observed in this study was *G. geotrichum*, which was predominant in the gulls from Pierre Blanche, relatively frequent in those from the Riou and Frioul Archipelagos, and very rare in those from La Grande-Motte. *G. geotrichum* is present in many foods and considered a normal component of the human gut microbiota.[23] We found no data concerning the diet of yellow-legged gulls in Pierre Blanche. However, the presence of *G. geotrichum* likely stems from a food-borne origin as these gulls commonly feed from both refuse dumps and terrestrial sources, and less frequently from marine sources.[20]

Interestingly, the highest prevalence of *C. glabrata* and *C. albicans* was observed in the gull colonies located on building rooftops at Palavas-les-Flots and La Grande-Motte. Notably, these two species also represent the most frequent yeast species involved in human infections, particularly in bloodstream infections.[24] Whether the presence of these anthropic yeasts is associated with the close proximity of these gulls to humans and/or anthropogenic food resources remains to be confirmed. In correlation this hypothesis, we found that the prevalence of *C. albicans*, the major commensal and pathogenic yeast species in humans, increased with proximity of gull colonies to human settlements and activities. This finding is in agreement with a study conducted in a remote human community in French Guiana, which highlighted a very low carriage rate of *C. albicans* and a high carriage rate of *C. krusei*, in both Amerindians and various animals in their environment.[25]

Saccharomyces cerevisiae was primarily isolated in gulls from Pierre Blanche and the Frioul Archipelago, the two locations that displayed the highest degree of species diversity and richness. In kelp gull, the presence of this species has been associated with gull diets that were closely associated with human activities.[19] Indeed, *S. cerevisiae* is probably the most frequent yeast species found in human food, and it sometimes colonizes the human gut and can cause infections in immunodeficient patients.[26,27] Rarer yeast species, such as *C. lusitaniae* and *C. tropicalis,* have also been isolated from synanthropic wild bird droppings in the suburb of Kuala Lumpur.[12] Remarkably, we did not isolate the human pathogen *Cryptococcus neoformans*, which is commonly associated with birds, especially pigeons,[28–31] despite the use the specific dopamine agar culture medium. *C. uniguttulatus*, which is rarely involved in human diseases, was only isolated from one sample collected on the Frioul Archipelago. Thus, we conclude that the yellow-legged gull is unlikely to be reservoir for *Cryptococcus* spp.

Itraconazole-resistant isolates were observed in *C. glabrata C. albicans* and *C. krusei* as previously reported.[12,32,33] The most abundant yeast species isolated on DRBC media supplemented with itraconazole were *C. glabrata*, *G. geotrichum*, *C. krusei*, *S. cerevisiae* and *C. albicans*. These species and most of the others identified in this study have been shown to be involved in human mycoses and thus share zoonotic potential.[34] A recent study has also reported many antifungal-resistant yeast species isolated from synanthropic wild bird droppings.[12] Indeed, our findings and other reports indicate that the yellow-legged gull may act as a reservoir and vector of drug-resistant human pathogenic yeasts, including some drug-resistant species.

Conclusions

The yeast mycobiota composition of yellow-legged gulls varies with respect to the geographical location and, probably, the bird's diet. Among the diverse yeast species

communities identified in this study, human pathogens such as *C. krusei*, *C. glabrata* and *C. albicans*, some of which are highly resistant to antifungal drugs, were frequently isolated. The frequency of anthropic yeast species in the gut of these gulls increased with synanthropy of the gull colonies. The yellow-legged gull may thus act as reservoir and contribute to the dissemination of many yeast species, including antifungal drug-resistant isolates of species involved in human infections. Further genetic studies are warranted to investigate the gene flow between yeast populations carried by sympatric humans and gulls.

Acknowledgements

We gratefully acknowledge the help of Stanislas Rebaudet (APHM), Olivier Scher (Conservatoire des Espaces Naturels du Languedoc-Roussillon), Alain Mante (Parc National des Calanques) and Lucile Yrles (Ligue de Protection des Oiseaux, Hérault). We thank Sandra Moore (Aix-Marseille University) for English editing of the manuscript.

Mohammed Hashim Al-Yasiri was funded by the Higher Education Ministry of Iraq.

Disclosure of Conflict of Interest

No conflict of interest.

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Table legends

Table1. Yeast species distribution and relative abundance estimated via either the proportion of positive samples (incidence) or the total number of colony forming units (CFUs) in the five study areas (red = the highest percentage, dark brown ≥ 20 %, light brown ≤ 20 %, yellow ≤ 5 %).

Table 2. Gut yeast species similarity between the five yellow-legged gull breeding colonies, estimated via either presence/absence data, using the Sorensen index (upper triangle), or abundance data, using the Bray-Curtis index (lower triangle).

Figure legends

Fig. 1 Map illustrating the geographical location of the five yellow-legged gull (*Larus michahellis*) breeding colonies along the French Mediterranean shore. The pie charts illustrate the repartition of the five predominant yeast species (*Candida krusei*, *C. glabrata*, *C. albicans*, *Galactomyces geotrichum* and *Saccharomyces cerevisiae*) found in the gut communities of the yellow-legged gulls in the two archipelagos off the coast of Marseille (Frioul (FR) and Riou (RI)), the two cities of La Grande-Motte (GM) and Palavas-les-Flots (PA), and Pierre-Blanche Lagoon (PB).

Fig. 2 Yeast species richness per sample in the two archipelagos in Marseille (Frioul (FR) and Riou (RI)), the two cities of La Grande-Motte (GM) and Palavas-les-Flots (PA), and Pierre-Blanche Lagoon (PB). Y axis: the percentage of samples in each site depending on the

frequency of yeast species in each sample. X axis: the frequency of yeast species in each sample.

Fig. 3 A. Average species richness (mean number per sample), **B.** Evenness (Buzas and Gibson's index) and **C.** Diversity (Shannon index) of the gut yeast communities in yellow-legged gulls from the five breeding colonies, located in the two Archipelagos off the coast of Marseille (Frioul (FR) and Riou (RI)), in the two cities of La Grande-Motte (GM) and Palavas-les-Flots (PA), and Pierre-Blanche Lagoon (PB).

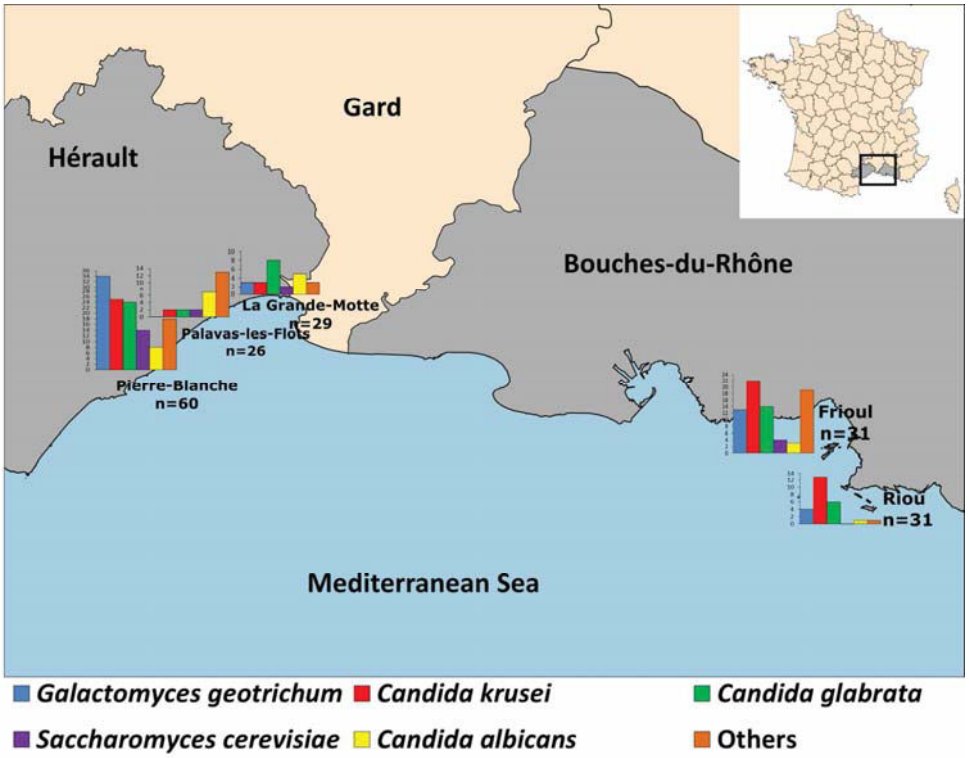
Fig. 4 Correspondence analysis of the presence/absence data for each location. First and second axis plot representing 62% and 16% of total variance, respectively. Locations (FR=Frioul; GM=Grande-Motte; PA=Palavas-les-Flots; PB=Pierre-Blanche; RI=Riou) are dispersed according to species composition similarities and specificities. Each yeast species is indicated with an acronym: the first two letters of the genus in uppercase and the species in lowercase (*e.g.*, *Candida zeylanoides* = CAze).

Fig. 5 Number of positive samples for each yeast species cultured on Dichloran Rose Bengal Chloramphenicol (DRBC) medium either without (gray bars) or with itraconazole (DRBC+I, black bars).

Table1. Yeast species distribution and relative abundance estimated either via the proportion of positive samples or the total number of colony forming units (CFU) in the five study areas. (Red = the highest percentage, Dark brown = > 20 %, Light brown = < 20 %, Yellow = < 5 %)

Positive samples	Pierre-Blanche	Palavas-les-Flots	Grande-Motte	Riou	Frioul	N
Candida krusei	58.1	12.5	20	81.3	95.7	65
Candida glabrata	55.8	12.5	53.3	37.5	60.9	54
Candida albicans	18.6	43.8	33.3	6.3	13.0	24
Candida tropicalis	2.3		6.7		13.0	5
Candida pelliculosa	2.3					2
Candida lambica	9.3					4
Candida zeylanoides	2.3	12.5				3
Candida lusitaniae	4.7	12.5			26.1	10
Saccharomyces cerevisiae	32.6	12.5	13.3		17.4	22
Candida kefyr	2.3				13.0	4
Galactomyces geotrichum	79.1		20.0	25.0	56.5	54
Pichia kluyveri	2.3			6.3	13.0	5
Rhodotorula mucilaginosa	7.0	25.0			4.3	8
Kloeckera apiculata	9.3				4.3	5
Cryptococcus uniguttulatus					4.3	1
Aureobasidium pullulans		31.3	13.3			7
Hanseniaspora guilliermondii					4.3	1
Total number of CFU, N (%)						
Candida krusei	2467(31.5)	149(13.0)	15(1.4)	2414(71.6)	4774(52.3)	9819
Candida glabrata	1201(15.4)	129(11.2)	652(61.6)	803(23.8)	2039(22.3)	4824
Candida albicans	325(4.2)	248(21.6)	179(16.9)	10(0.3)	21(0.3)	783
Candida tropicalis	100(1.3)		1(0.1)		165(1.8)	266
Candida pelliculosa	40(0.5)					40
Candida lambica	221(2.8)					221
Candida zeylanoides	50(0.6)	4(0.3)				54
Candida lusitaniae	106(1.4)	400(34.8)			552(6.0)	1058
Saccharomyces cerevisiae	558(7.1)	90(7.8)	203(19.2)		231(2.5)	1082
Candida kefyr	12(0.2)				240(2.6)	252
Galactomyces geotrichum	2652(33.9)		5(0.5)	45(1.3)	550(6.0)	3252
Pichia kluyveri	10(0.1)			100(3.0)	340(3.7)	450
Rhodotorula mucilaginosa	5(0.1)	10(0.9)			4(0.04)	19
Kloeckera apiculata	75(1)				200(2.2)	275
Cryptococcus uniguttulatus					7(0.1)	7
Aureobasidium pullulans		119(10.4)	3(0.3)			122
Hanseniaspora guilliermondii					10(0.1)	10
Total number of CFU	7822	1149	1058	3372	9133	22534
Total richness	14	8	6	5	13	
No positive samples	43	16	15	16	23	113

Sorensen / Bray-Curtis	Pierre-Blanche	Palavas-les-Flots	Grande-Motte	Riou	Frioul
Pierre-Blanche		63.6	57.1	52.6	**81.5**
Palavas-les-Flots	37.3		**66.7**	46.2	57.1
Grande-Motte	28.7	37.6		**66.7**	60
Riou	48.6	24.5	26		55.6
Frioul	**59.6**	32.9	27.0	**79.2**	



Map illustrating the geographical location of the five yellow-legged gull (Larus michahellis) breeding colonies along the French Mediterranean shore. The pie charts illustrate the repartition of the five predominant yeast species (Candida krusei, C. glabrata, C. albicans, Galactomyces geotrichum and Saccharomyces cerevisiae) found in the gut communities of the yellow-legged gulls in the two archipelagos off the coast of Marseille (Frioul (FR) and Riou (RI)), the two cities of La Grande-Motte (GM) and Palavas-les-Flots (PA), and Pierre-Blanche Lagoon (PB).
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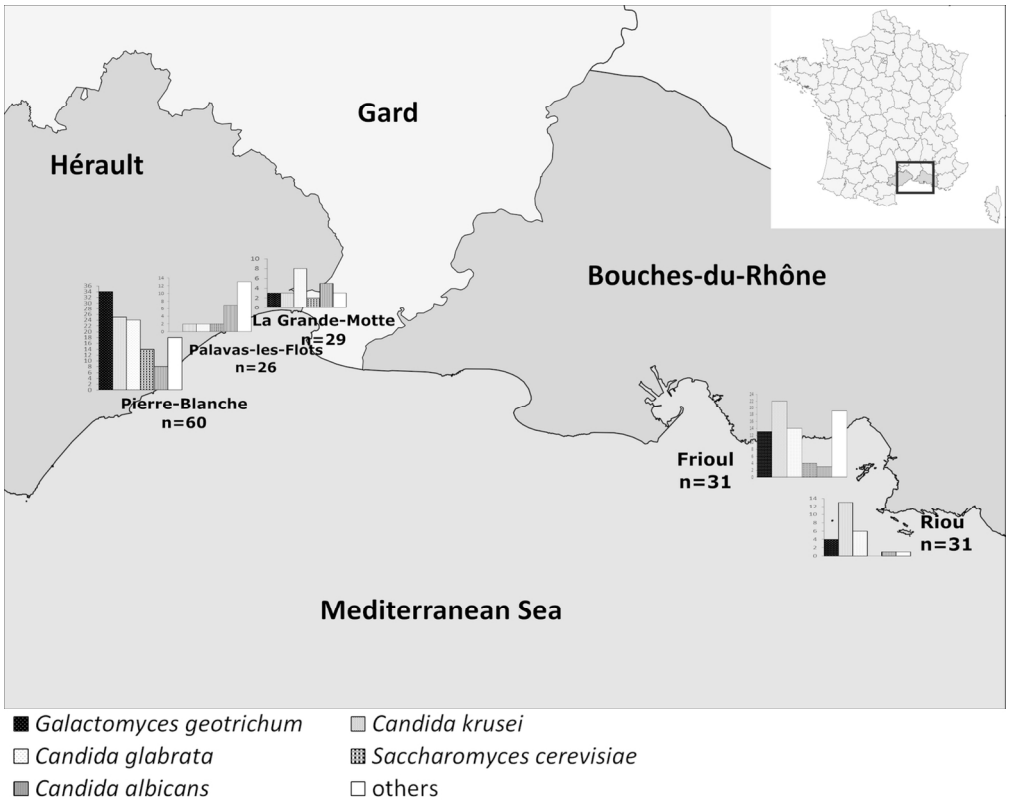

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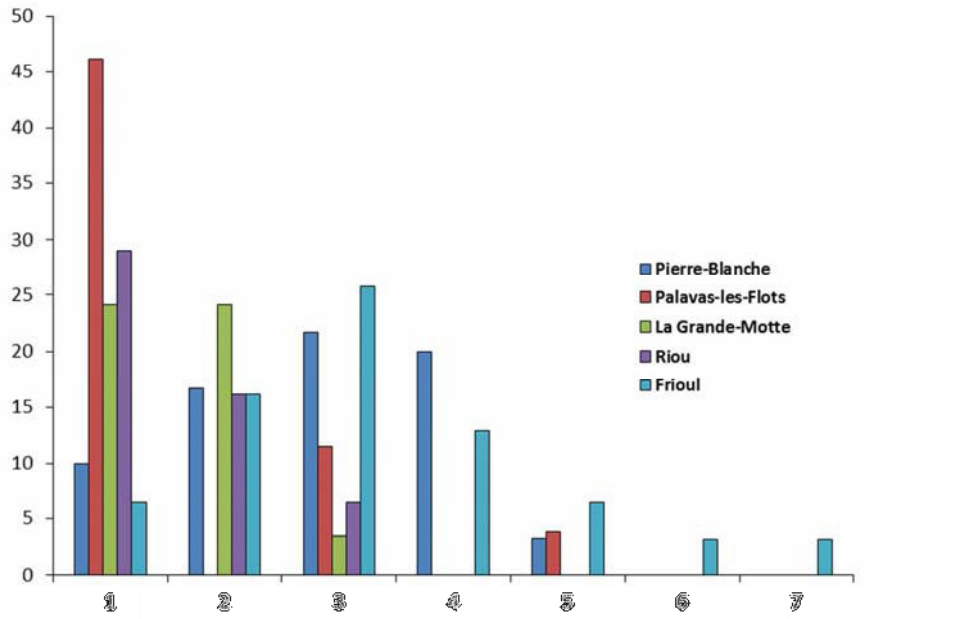

Yeast species richness per sample in the two archipelagos in Marseille (Frioul (FR) and Riou (RI)), the two cities of La Grande-Motte (GM) and Palavas-les-Flots (PA), and Pierre-Blanche Lagoon (PB). Y axis: the percentage of samples in each site depending on the frequency of yeast species in each sample. X axis: the frequency of yeast species in each sample.
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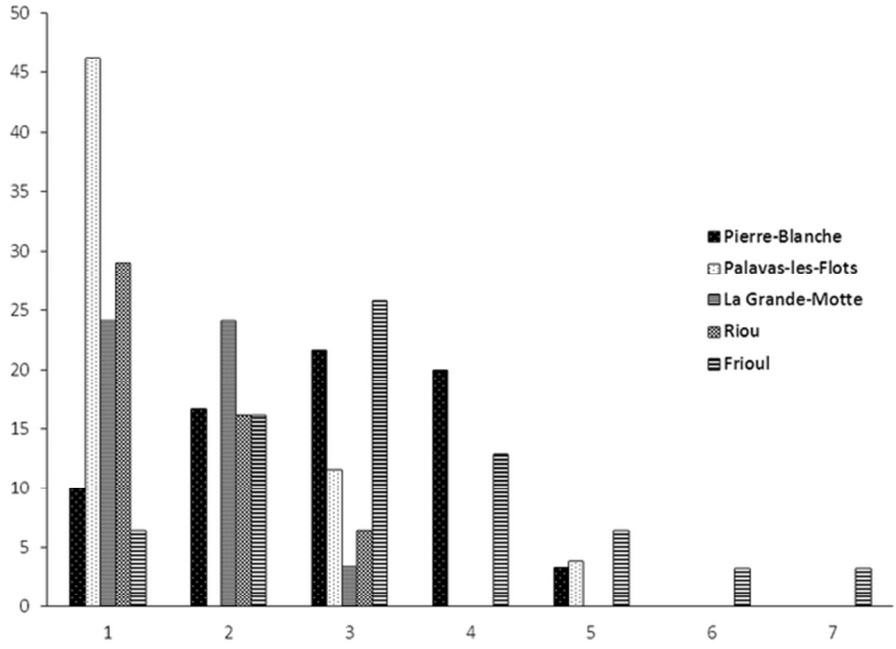

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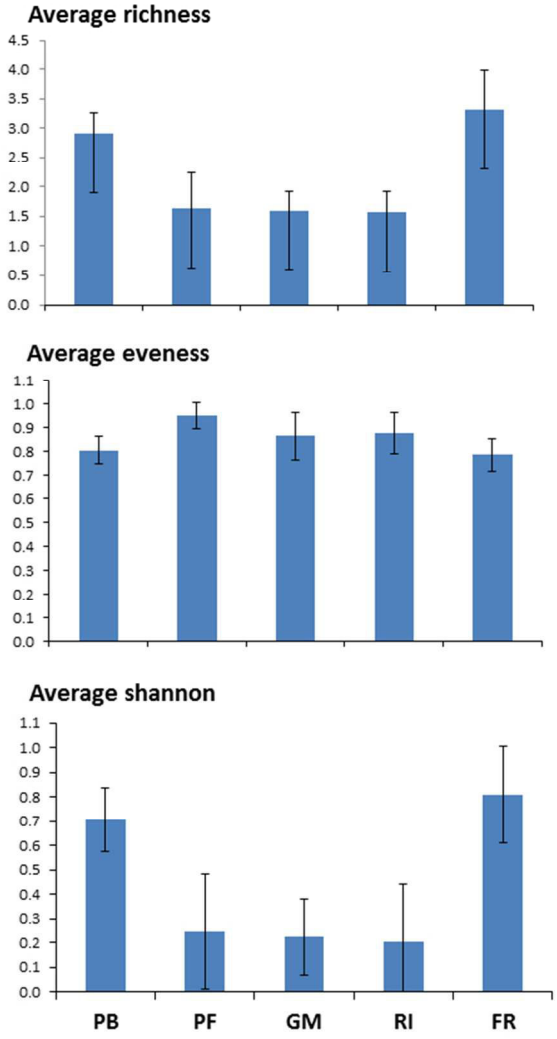

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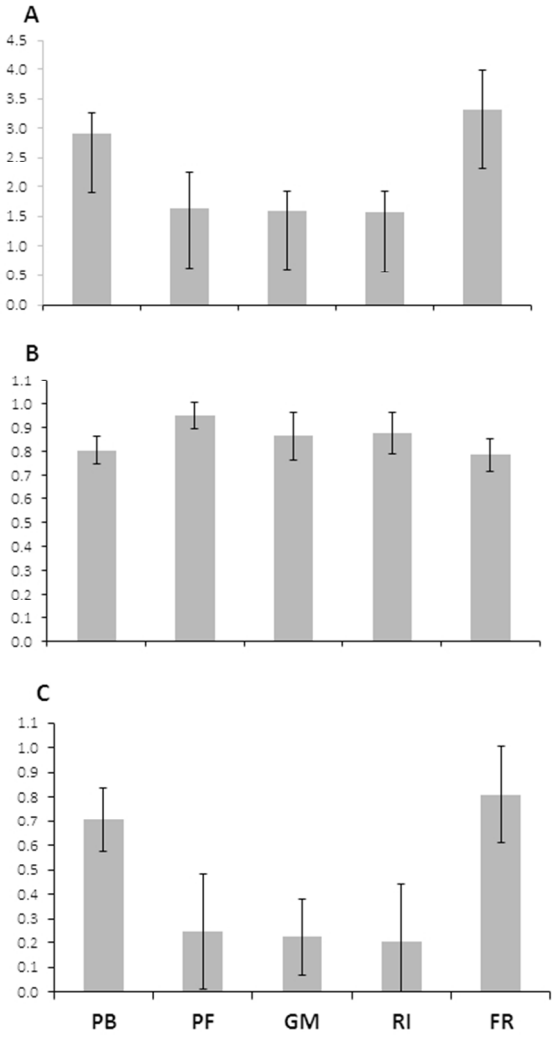

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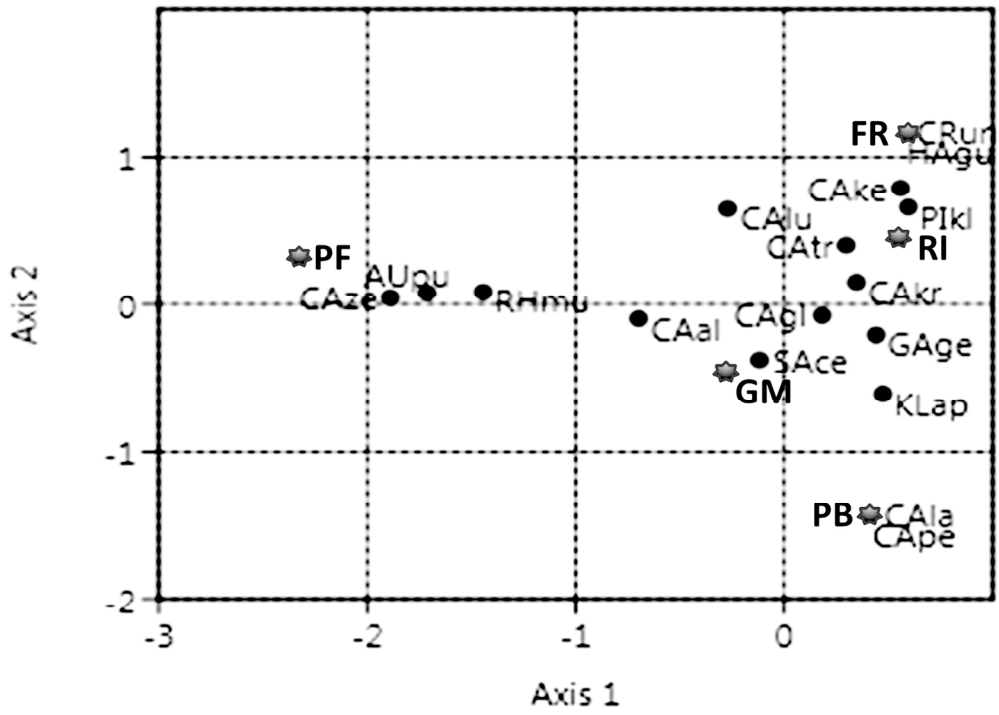

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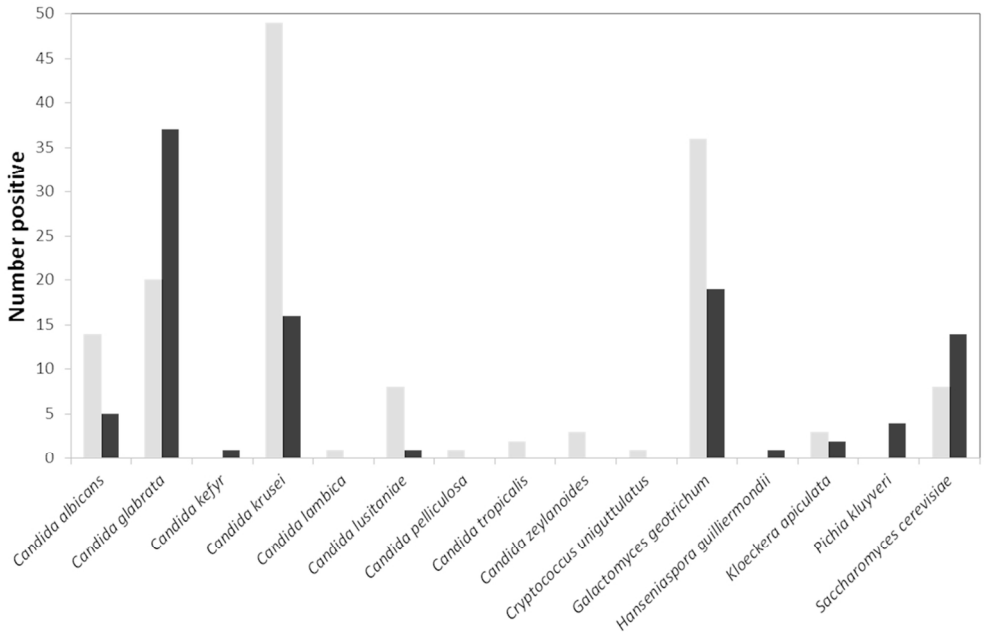

Fig. 5 Number of positive samples for each yeast species cultured on Dichloran Rose Bengal Chloramphenicol (DRBC) medium either without (gray bars) or with itraconazole (DRBC+I, black bars).
118x77mm (300 x 300 DPI)

Second Article: Anthropogenic Impact on Environmental Filamentous Fungi Communities along the Mediterranean Littoral

Article status: submitted

OXFORD UNIVERSITY PRESS | FEMS Microbiology Ecology

http://mc.manuscriptcentral.com/fems

Anthropogenic Impact on Environmental Filamentous Fungi Communities along the Mediterranean Littoral

Journal:	*FEMS Microbiology Ecology*
Manuscript ID	Draft
Manuscript Type:	Research article
Date Submitted by the Author:	n/a
Complete List of Authors:	AL-YASIRI, Mohammed Hashim; Aix-Marseille Universite Faculte de Medecine, Parasitology Mycology Normand, Anne-Cécile; Assistance Publique Hopitaux de Marseille, Parasitology Mycology Mauffrey, Jean-Francois; Aix-Marseille Universite, LPED Ranque, Stéphane; Aix-Marseille Universite Faculte de Medecine, Parasitology Mycology; Assistance Publique Hopitaux de Marseille, Parasitology Mycology
Keywords:	filamentous fungi, moulds, Synathropy

Anthropogenic Impact on Environmental Filamentous Fungi Communities along the Mediterranean Littoral

Running title: Synanthropy of filamentous fungi communities

Mohammed Hashim Al-Yasiri[a], Anne-Cécile Normand[b], Jean-François Mauffrey[c], Stéphane Ranque[a,b,*]

a. Aix-Marseille Université, IP-TPT UMR MD3, 13885 Marseille, France
b. Laboratory of Parasitology - Mycology, APHM CHU Timone, 13005 Marseille, France
c. Laboratoire Population Environnement Développement (LPED)- UMR-151, Aix-Marseille Université – IRD, France

*** Corresponding author**

Dr Stéphane Ranque
Laboratoire de Parasitologie-Mycologie
APHM CHU Timone
264 rue Saint Pierre
13385 Marseille CEDEX 5, France
Phone: +33491386090
Fax: +33491384957
E-mail: stephane.ranque@ap-hm.fr

Abstract

We hypothesize that anthropogenic influences have an effect of the community structure of filamentous fungi and that particular species or species patterns might serve as markers to characterize ecosystems. We sampled a filamentous fungi from yellow-legged gull fecal samples at five study sites along the Mediterranean littoral in southern France. The sites were characterized by variable anthropogenic influence, ranging from building rooftops in two cities to a natural reserve. The sites also included two suburban ecoclines, one of which was exposed to sewer pollution. Filamentous fungal colonies were quantified and identified via MALDI-TOF mass spectrometry.

Our results provide further insight into the structure of filamentous fungi communities of various biotopes along the Mediterranean shore. We also describe the manner in which anthropogenic influence might impact these communities. Interestingly, we found that both fungal diversity and abundance are low in urban areas compared with suburban ecocline or environments little affected by anthropogenic influence. We also reveal that some fungal species are clearly associated with particular environments. In particular, *Mucor circinelloides* was associated with a natural environment with little anthropogenic impact and distant from human settlements. Whereas *Scedosporium apiosperma* was associated with an ecocline polluted by sewage.

One-sentence Summary

The community structure of filamentous micromycete is dependent on anthropogenic influence and may be useful to characterize ecosystems.

Introduction

Mankind has become one of the major if not the most important driving factor of environmental changes, thereby engendering significant ecological shifts (Ellis *et al.* 2010; Ellis 2011). Many species are thus associated with environmental conditions influenced by man, which are referred to as synanthropes (Nuorteva 1963). Consequently, species or populations might be categorized into synanthropic groups. Eusynanthropic species are preferentially associated with human settlements, asynanthropic species avoid human populations, and hemisynanthropic species live in ecotones or transition zones (Nuorteva 1963; Linhares 1981; Schnack *et al.* 1995). This classification has been primarily applied in entomology (Centeno, Almorza and Arnillas 2004). However, data concerning anthropogenic influence on environmental filamentous fungi communities are scarce. In a study describing the mycobiota of yellow-legged gulls (*Larus michahellis*) (Al-Yasiri *et al.*), we analyzed fungal cultures yielded from bird droppings collected in breeding colonies located in each of the five study sites. We found that yeast community structure in these common seabirds was influenced by synanthropy, as the frequency of anthropogenic yeast species in the mycobiota increased with gull synanthropy (Al-Yasiri *et al.*). In addition to yeast species, which are a normal mycobiota component of the gut of these birds, we also cultured various filamentous fungi species derived from bird droppings, which likely reflects environmental airborne fungal contamination rather than the gut mycobiota of these birds. In the current study, we aimed to describe the community structure of filamentous fungi in geographically distinct sites along the Mediterranean littoral, characterized by a gradient of anthropogenic influence.

Materials and Methods

Study sites

The samples were collected from five yellow-legged gull colonies located along the French Mediterranean coast (Fig. 2). Three sites were located in the department of Hérault: a natural reserve at Pierre Blanche Lagoon (43.494028N, 3.860266E) and the building rooftops of two cities, Palavas-les-Flots (43.527767N, 3.92581E) and La Grande-Motte (433341.72N, 40501.81E). The two remaining sites, which were located in the department of Bouches-du-Rhône, included two archipelagos off the coast of Marseille: Frioul (431613.03N, 51753.43E) and Riou (431041.20N, 52249.45E). Overall, these study sites were characterized by a gradient of anthropogenic influence. The sites in the cities of Palavas-les-Flots and La Grande-Motte, which represent entirely man-made environments, were exposed to high anthropogenic influence. The suburban zone of the Riou and Frioul Archipelagos were exposed to an intermediate anthropogenic impact, and the Pierre Blanche Lagoon, a natural reserve distant from urban settlement, displayed very little anthropogenic influence. This lagoon supports the growth of the halophyte *Salicornia europaea*, which is characteristic of high salinity, silty soil. Notably, the Riou Archipelago is off the coast of Cortiou Calanque, where the sewage of Marseille is released into the sea (Fig. 1). Due to frequent sewage overflow, this Archipelago is exposed to anthropogenic pollution distinct from that at the Frioul (Sarrazin *et al.* 2004).

Sample collection and processing

Yellow-legged gull fecal samples were collected from breeding colonies in April 2013 at Pierre Blanche, Palavas-les-Flots and La Grande-Motte, in July 2013 at the Riou

Archipelago, and in September 2013 at the Frioul Archipelago. Each fecal sample was considered independent and constituted the sampling unit to assess the community structure of filamentous fungi in each ecological environment. Each sample was collected with a sterile cotton-tipped swab dipped in 3 ml of sterile saline (0.9% NaCl) and supplemented with 0.5 g/L chloramphenicol transport medium. Samples were labelled according to site and date. Each swab was vortexed in the transport medium and then allowed to sediment for at least 10 min. After removing the swab, the supernatant was transferred to a sterile conical tube. Next, 100 µl of supernatant (per plate) was inoculated onto five different culture media plates: Sabouraud agar, Dopamine agar (Liu *et al.* 2014), Scedo-select III (Pham *et al.* 2015), and Dichloran Rose Bengal Benomyl Chloramphenicol (DRBC) agar (Madrid *et al.* 2009) with or without 4 mg/L itraconazole. The cultures were incubated at 30°C. The filamentous fungi colony-forming units (CFUs) were quantified after 5 days of incubation.

Fungal species identification

Fungi were identified via MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry), as previously described (Cassagne *et al.* 2013), using a Microflex LT™ mass spectrometer (Bruker Daltonics GmbH, Germany) with MALDI Biotyper software v2.1. (Bruker Daltonics). Species identification was performed using an in-house developed filamentous fungi reference spectra database as previously described (Normand *et al.* 2013; Becker *et al.* 2014).

Statistical analyses

The species richness of filamentous fungi in each breeding colony was estimated using the Chao 1 statistic by either cumulating or averaging all sample values. Abundance

was estimated by using either the number of positive cultures or the CFU counts for a given species. Fungal species diversity in each breeding colony was estimated using the Shannon–Wiener index (*H*), as available in PAST 3.05 (Hammer, Harper and Tayan 2001), which calculated the species diversity depending on the total number of species and the relative abundance of species at each site. Species similarity (presence/absence) between breeding colonies was estimated using the Sorensen index (Santiago, Santos and Maia 2013). Abundance-based species similarity was estimated via the Bray-Curtis Index (Hammer, Harper and Tayan 2001).

Anthropogenic pressure and synanthropy

We considered Palavas-les-Flots and La Grande-Motte as synanthropic urban zones, Riou and Frioul as suburban ecotones, and Pierre Blanche as a biotope with little anthropogenic influence. The species primarily isolated from urban zones were classified eusynanthropic, while those mainly isolated from the suburban ecotones were classified hemisynanthropic. Species predominantly absent from urban areas were classified asynanthropic.

Measure of synanthropy

The level of synanthropy for each species was estimated via the Synanthropy Index (*SI*) proposed by Nuorteva (1963) and recently adapted by Mollov:

$$SI = \frac{2\mathrm{a} + \mathrm{b} - 2\mathrm{c}}{2} * 100$$

In which a is the percentage of the individuals in urbanized areas (human settlements), b represents the percentage of individuals in suburban ecotones, and c is the

percentage of individuals in biotopes with little anthropogenic influence (Nuorteva 1963; Mollov 2014).

Results

Fungal diversity and community structure

At least one filamentous fungi colony grew from 78 of 177 samples collected in the five study sites (Fig. 3; Table 1). The most frequent genus observed was *Mucor* (30.6%), followed by *Aspergillus* (29.1%) and *Penicillium* (14.2%) (Fig. 3). Altogether, 35 filamentous fungi species in 16 genera were isolated, including 13 species in six genera from the Pierre Blanche lagoon, four species in three genera from Palavas-les-Flots, eight species in five genera from La Grande-Motte, 18 species in 11 genera from the Riou Archipelago, and 18 species in nine genera from the Frioul Archipelago (Table 1). The highest mean in filamentous fungi species richness per sample was observed in samples from the Riou (2.31) and Frioul (2.19) Archipelagos. We observed a low mean in filamentous fungi species richness in samples from the Pierre Blanche lagoon, Palavas-les-Flots and La Grande-Motte (Hérault) (Fig. 4). The similarities in filamentous fungi species structure observed between breeding colonies are detailed in Table 2. The highest Sorensen index was observed between the Frioul and Riou Archipelagos (54.1%). Meanwhile, Pierre Blanche shared 53.3% similarity with Frioul but only 32.3% similarity with Riou (Table 2). The Bray-Curtis index was also highest (90.5%) between the Frioul and Riou Archipelagos. The similarities, based on the Bray-Curtis index, when comparing Pierre Blanche with Frioul and Riou were 54.6% and 62.4%, respectively (Table 2). Overall, these findings highlight the relatively high degree of similarity between fungi populations on the Frioul and Riou Archipelagos.

The most frequent species isolated irrespective of geographical site was *Mucor circinelloides*, which totaled 66.1%, 62.5% and 35.2% of all filamentous fungi isolates collected at Pierre Blanche, Palavas-les-Flots and the Frioul Archipelago, respectively. *Penicillium crustosum* was the predominant (45.2%) filamentous fungi species at La Grande-Motte, while *Aspergillus fumigatus* was the predominant (23.1%) species at the Riou Archipelago. Compared with the sites in Hérault, the archipelagos off Marseille were characterized by a higher diversity (H=2.9 and 27 taxa *vs*. H=2.1 and 18 taxa; $P<10^{-4}$). *Scedosporium apiosperma* was much more abundant on Riou (16%) than on Frioul (1%).

Anthropic biotopes characteristics

Fungal communities in urban environments (La Grande Motte and Palavas-les-Flots) were characterized by very low species abundance and diversity. The suburban ecotones, including the Archipelagos of Riou, which is highly exposed to anthropogenic pollution, and Frioul were both characterized by the highest levels of fungal species abundance and diversity. The Pierre Blanche lagoon, a biotope with little anthropogenic influence, was also characterized by high fungal species abundance and diversity.

The fungal species *Alternaria citri*, *Purpureocillium lilacinus*, *Aspergillus versicolor*, *Trichoderma harzianum* and *Fusarium incarnatum* were rarely isolated and only in densely populated urban areas and settlements. These species might therefore be classified eusynanthropic. In contrast, *Mucor circinelloides*, *Mucor plumbeus* and *Penicillium crustosum* were frequently isolated and predominantly found at Pierre Blanche lagoon. As this site lacks human settlements, these species were classified asynanthropic.

The major human pathogens, *A. fumigatus*, *A. niger* and *A. tubingensis* (Gautier *et al.* 2016), displayed a slight association with urban and human settlements and were classified hemisynanthropic. These three fungi were predominately isolated in the suburban ecotones. Interestingly however, in contrast to the two other *Aspergillus* species, *A. fumigatus* predominated in Riou. Similarly, *S. apiosperma*, *Scopulariopsis brevicaulis* and *Stachybotrys chartarum* were almost exclusively isolated from the polluted ecotone of Riou.

Discussion

Overall, our results provide further insight into the community structure of filamentous fungi species, according to various biotopes, along the Mediterranean shore of southern France. To our knowledge, these results provide the first appreciation of anthropogenic influences on these fungal communities. We found that fungal diversity and abundance are low in urban areas compared with suburban ecoclines or environments characterized by little anthropogenic impact. We also reveal that some fungal species are clearly associated with particular environments, such as heavily polluted ecoclines (*S. apiosperma*) or natural environments without human settlements (*M. circinelloides*). Further studies are warranted to investigate whether filamentous fungi communities may represent a robust marker of particular biotopes.

As we applied a novel eco-epidemiological approach to analyze the manner in which anthropogenic modifications impact airborne filamentous fungi communities, we were unable to compare our findings with those of previous studies. Our findings therefore warrant similar studies extended to other biotopes and eco-geographical areas. Indeed,

apart from anthropic influences, further consideration of ecological confounding factors is warranted, such as comparisons of the filamentous fungal communities in lagoon or rocky coasts as well as mainland or island areas. Other confounding factors should also be assessed such as exposure to sewage outlets, upwind exposure of urban areas, distance from the sea shore, altitude or seasonal variations in airborne fungal spores.

One limitation of our study lies in the relatively low abundance of some filamentous fungi species, in particular those cultured from samples collected in urban areas, which ultimately rendered the synanthropy categorization less robust. The type of samples collected may also be considered a limitation. Our interesting findings of varying fungi population structure stemmed from ancillary observations of a previous study assessing the impact of synanthropy on yeast communities of the yellow-legged gull mycobiota (Al-Yasiri *et al.*). Gull droppings may not be the most convenient or practical type of sample to assess airborne fungal spores in the environment, as they might contain foodborne filamentous fungi spores. Nevertheless, although humans often consume filamentous fungi along with food in-take, they are seldom isolated from stool samples in the clinical laboratory. We understand that human-based findings can only be extrapolated to birds with caution. Regardless, it is very likely that the filamentous fungi cultured in this study were only of airborne origin, as bird droppings represent a unique collection and preservation substance for difficult to gather air-borne spores. Indeed, a previous study has shown that *Scedosporium* species were readily isolated from soil samples but seldom collected from air sampling. Further studies should compare the present culture results with those from air-borne sampling.

In contrast, analyzing bird droppings might be a strength rather than a limitation of our study. This type of sample is relatively standardized, widely available in very diverse biotopes, and capable of detecting fungi species that are seldom isolated from air samples. Our study also highlights the recent progress in filamentous fungi identification via MALDI-TOF MS technology (Ranque *et al.* 2014), which dramatically strengthened the accuracy of high-throughput species identification of filamentous fungal colonies collected in large-scale studies.

We observed a relatively higher level of species richness and diversity in the two Archipelagos off the coast of Marseille compared with the three sites located in the Hérault department. The genus *Aspergillus* was the most frequently observed in our study, which is probably because *Aspergillus* spp. are ubiquitous and produce large numbers of small airborne conidia that can be easily cultured from various samples, including air, water and soil (Sabino *et al.* 2014). In this study, the prominent *Aspergillus* species were *A. fumigatus*, *A. niger* and *A. tubingensis*, which were mainly isolated on the Frioul and Riou Archipelagos. These species are opportunistic pathogens, which can cause invasive aspergillosis in both humans (Dagenais and Keller 2009) and gulls (Nardoni *et al.* 2006). Interestingly, *A. fumigatus*, the predominant human aspergillosis agent, was the most prevalent species on the polluted Riou Archipelago. Nine distinct species of the Mucorales family were isolated in the five gull colonies. The most abundant, *M. circinelloides* was more frequently recovered from Pierre Blanche, Palavas-les-Flots and Frioul Archipelago (66.1%, 62.5% and 35.2%, respectively), followed by *Rhizopus* spp, *Lichtheimia (Absidia) corymbifera* and *Actinomucor elegans*. The *Mucor*, *Rhizopus* and *Lichtheimia* genera, which were the most common within the

Mucorales family, can cause life-threatening infections in diabetic or immunocompromised patients (Pfaller and Diekema 2004). The species *M. circinelloides* and *M. plumbeus* can be classified as asynanthropic, while *L. corymbifera*, which was isolated in the suburban ecotones, can thus be classified as hemisynanthropic. Among the five *Penicillium* species identified in the current study, *P. crustosum* can be classified asynanthropic, whereas *P. aurantiogriseum*, which showed preference to urban areas and human settlements, can be classified hemisynanthropic. *S. apiosperma*, which was only recovered in the two archipelagos off the coast of Marseille, was frequently recovered in Riou and rarely in Frioul. *S. apiosperma* is also an opportunistic pathogen that can colonize the airways of patients with chronic lung disease and cause invasive fungal diseases in immunocompromised patients (Pihet *et al.* 2009; Hirschi *et al.* 2012). The association of *Scedosporium* sp. with the Riou Archipelago corresponds with previous results showing that this species was absent from natural habitats but was found in agricultural soils and, with even higher frequencies, urban parks, playgrounds and industrial areas (Kaltseis, Rainer and DeHoog 2009). Moreover, *Scedosporium* spp. abundance clearly correlated with anthropogenic impact, increased nitrogen concentration and decreased pH of the soil. We thus propose that *Scedosporium* spp. might be used as a long-term indicator of environmental health and pollution, e.g., in swimming facilities and surface waters (Kaltseis, Rainer and DeHoog 2009). The highly polluted Riou Archipelago was characterized by a distinct fungal community; seven species were isolated exclusively from this location.

Fungi play an important role in ecosystem resistance or resilience to anthropogenic pollution. Biotope alteration thus likely selects particularly resistant or adapted fungal communities. This might be the case in Riou, which is exposed to a relatively high level of anthropogenic pollution due to its proximity to the Marseille sewage outlet. Our findings strongly suggest that species such as *Scedosporium apiosperma*, *Scopulariopsis brevicaulis* and *Stachybotrys chartarum*, which were relatively abundant in the polluted ecotone of Riou, might serve as indicators of anthropogenic pollution.

Conclusion

Compared with suburban ecoclines or environments with little anthropogenic influence, urban areas were characterized by low diversity and abundance in filamentous fungi communities. Our interesting finding indicating that particular fungal species might serve as markers to characterize ecosystems, in particular those exposed to environmental anthropogenic pollution, warrant further investigation.

Acknowledgments

The authors gratefully acknowledge the assistance of Stanislas Rebaudet (APHM), Olivier Scher (Conservatoire des Espaces Naturels du Languedoc-Roussillon), Alain Mante (Parc national des Calanques) and Lucile Yrles (Ligue de Protection des Oiseaux, Hérault). We thank Sandra Moore (Aix-Marseille University) for English editing of the manuscript.

Conflict of interest

There is none.

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Figure legends

Figure 1. A photo of the Marseille sewage outlet at the Calanque of Cortiou showing the wastewater stream heading towards the Riou Archipelago (photo obtained from http://www.topo-calanques.com/proteger_nature.htm, with written agreement).

Figure 2. Map illustrating the number of analyzed samples and the geographical location of the five Yellow-legged gull (*Larus michahellis*) breeding colonies in the two archipelagos off the coast of Marseille, Frioul and Riou, the two cities of La Grande-Motte and Palavas-les-Flots, and Pierre-Blanche lagoon. The bars show filamentous fungi genera distribution at each study site; the vertical axis indicates the number of positive samples for each genus. The map was drawn using Inkscape ver. 0.91 (available at https://inkscape.org).

Figure 3. Relative frequencies of filamentous fungi genera isolated along the Mediterranean coast.

Table1. Filamentous fungi species distribution and relative abundance estimated either via the total number of colony forming units (CFU) or the proportion of positive samples in the five study areas: the tidal lagoon of Pierre-Blanche (PB), the two cities of Palavas-les-Flots (PF) and La Grande-Motte (GM), and the two Archipelagos off Marseille, Riou (RI) and Frioul (FR). In bold = the dominant species, in italics rare ≤ 5% species.

Total number of CFU, N (%)	PB	PF	GM	FR	RI	Total
Lichtheimia corymbifera				6 (3.7%)	10 (7.5%)	16
Actinomucor elegans	*3 (0.8%)*			1 (0.6%)		4
Alternaria alternata	*2 (0.6%)*					2
Alternaria citri		1 (12.5%)				1
Alternaria longipes	*3 (0.8%)*	1 (12.5%)				4
Aspergillus allahabidii	*1 (0.3%)*					1
Aspergillus flavus			1 (3.2%)		*2 (1.5%)*	3
Aspergillus fumigatus	*4 (1.1%)*			4 (2.5%)	**31 (23.1%)**	39
Aspergillus nidulans				1 (0.6%)		1
Aspergillus niger	*3 (0.8%)*		1 (13.2%)	41 (25.3%)	15 (11.2%)	60
Aspergillus niveus					*1 (0.7%)*	1
Aspergillus oryzae				4 (2.5%)		4
Aspergillus terreus				1 (0.6%)		1
Aspergillus versicolor			2 (6.5%)			2
Aspergillus tubingensis	*1 (0.3%)*			15 (9.3%)	*1 (0.7%)*	17
Chaetomium globosum					*2 (1.5%)*	2
Fusarium incarnatum			1 (3.2%)			1
Fusarium verticillioides				1 (0.6%)		1
Mucor circinelloides	**236 (66.1%)**	**5 (62.5%)**	4 (12.9%)	**57 (35.2%)**		302
Mucor fragilis	*10 (2.8%)*			19 (11.7%)	*4 (3.0%)*	33
Mucor plumbeus	*11 (3.1%)*					11
Purpureocillium lilacinus		1 (12.5%)				1
Penicillium aurantiogriseum			6 (19.4%)	4 (2.5%)	*4 (3.0%)*	14
Penicillium chrysogenum				1 (0.6%)		1
Penicillium citrinum					*1 (0.7%)*	1
Penicillium crustosum	80 (22.4%)		**14 (45.2%)**	1 (0.6%)		95
Penicillium glabrum				1 (0.6%)	*1 (0.7%)*	2
Rhizomucor pusillus				2 (1.2%)	*1 (0.7%)*	3
Rhizopus microsporus					*3 (2.2%)*	3
Rhizopus oryzae	*3 (0.8%)*			2 (1.2%)	*1 (0.7%)*	6
Scedosporium apiosperma				1 (0.6%)	21 (15.7%)	22
Scopulariopsis brevicaulis					15 (11.2%)	15
Stachybotrys chartarum					16 (11.9%)	16
Trichoderma harzianum			2 (6.5%)			2
Trichosporiella cerebriformis					*5 (3.7%)*	5

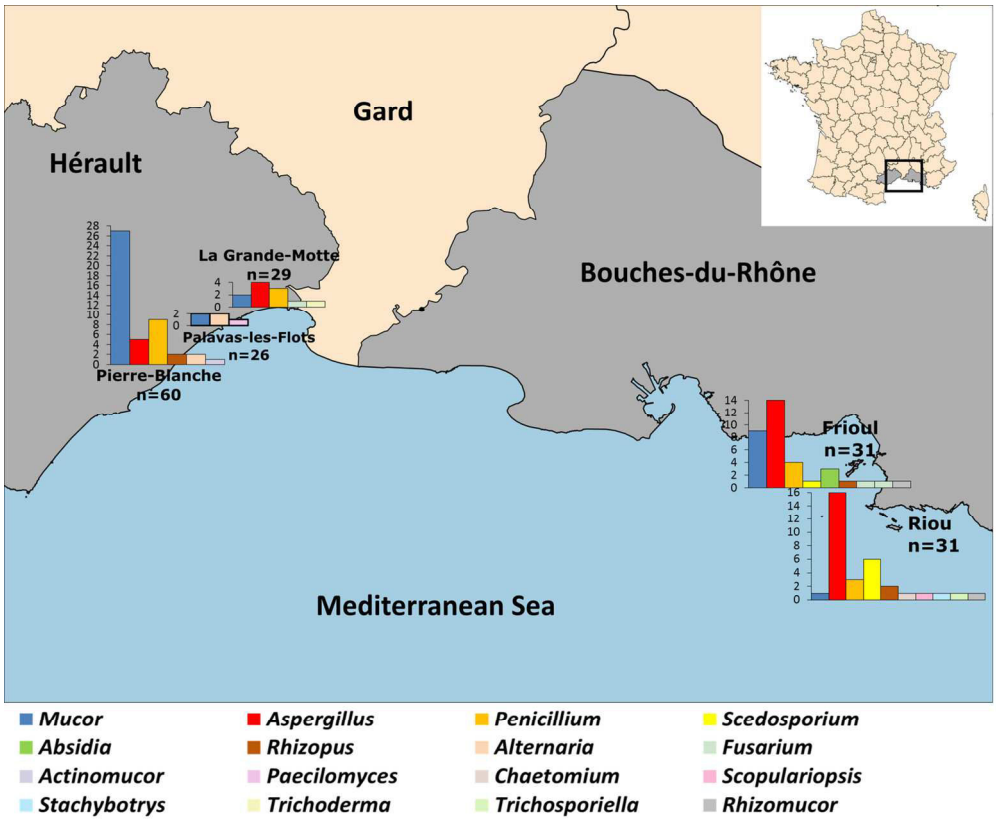
Table 2. Filamentous fungi species similarity matrix, estimated via either the Sorensen index (presence/absence, upper triangle) or the Bray-Curtis index (abundance, lower triangle), between the five study sites. Index values > 50 are bolded.

Sorenson / Bray-Curtis	Pierre Blanche	Palavas-les-Flots	La Grande Motte	Riou	Frioul
Pierre Blanche	**-**	47.1	31.6	32.3	**53.3**
Palavas-les-Flots	4.4	-	33.3	0	17.4
Grande Motte	15.9	41	-	30.8	32
Riou	**54.6**	11.3	37.6	-	**54.1**
Frioul	**62.4**	9.4	32.1	**90.5**	-

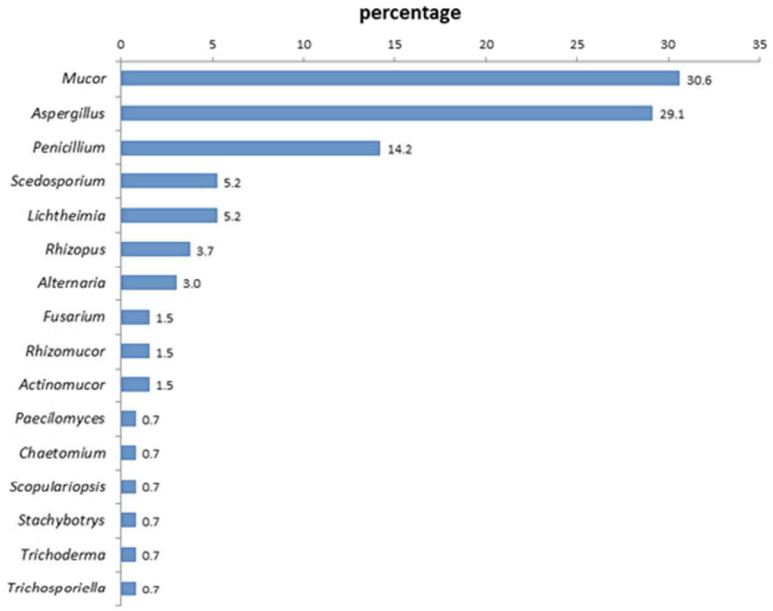
Table 3. Classification of the filamentous fungi species according to their Synanthropy Index (SI) (Mollov 2014), a measures their synanthropy level. Eusynanthropic species show a clear preference to densely populated urban areas and settlements; Hemisynanthropic species show a slight preference or indifference to urban areas and human settlements; Asynanthropic species show a clear avoidance of urban areas and human settlements.

Species	SI	Ecological preferences
Alternaria citri	**100.00**	Full preference to densely populated urban areas and human settlements, (SI [75 to 100])
Purpureocillium lilacinus	**100.00**	
Aspergillus versicolor	**100.00**	
Trichoderma harzianum	**100.00**	
Fusarium incarnatum	**100.00**	
-	-	Clear preference to urban areas and human settlements, (SI [50 to 75[)
Penicillium aurantiogriseum	**71.43**	Slight preference to urban areas, (SI]0 to 50[)
Aspergillus flavus	**66.67**	
Lichtheimia corymbifera	**50.00**	
Rhizomucor pusillus	**50.00**	
Aspergillus niveus	**50.00**	
Trichosporiella cerebriformis	**50.00**	
Stachybotrys chartarum	**50.00**	
Penicillium glabrum	**50.00**	
Penicillium citrinum	**50.00**	
Scedosporium apiosperma	**50.00**	
Chaetomium globosum	**50.00**	
Rhizopus microsporus	**50.00**	
Scopulariopsis brevicaulis	**50.00**	
Aspergillus terreus	**50.00**	
Aspergillus nidulans	**50.00**	
Aspergillus oryzae	**50.00**	
Penicillium chrysogenum	**50.00**	
Fusarium verticillioides	**50.00**	
Aspergillus niger	**43.33**	
Aspergillus tubingensis	**41.18**	
Aspergillus fumigatus	**34.62**	
Mucor fragilis	**4.55**	
-	**0**	**Indifference to urban areas and human settlements**
Rhizopus oryzae	**-25.00**	Preference to non-populated areas, (SI [-25 to 0[)
Alternaria longipes	**-50.00**	Avoidance of urban areas and human settlements, (SI [-50 to -25[)
Actinomucor elegans	**-62.50**	Clear avoidance of urban areas and human settlements, (SI [-75 to -50[)
Mucor circinelloides	**-65.73**	
Penicillium crustosum	**-68.95**	
Mucor plumbeus	**-100.00**	Complete absence in urban areas, (SI [-100 to -75[)
Aspergillus allahabadii	**-100.00**	
Alternaria alternata	**-100.00**	

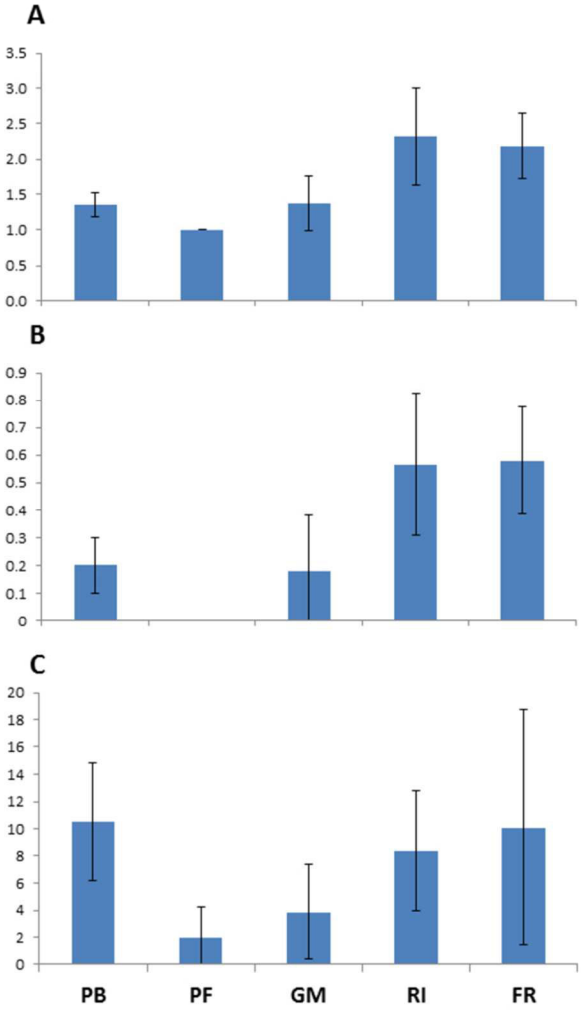
A photoview of the Marseille's city sewage outlet at the cove of Cortiou Creek showing the wastewater stream heading towards the Riou Archipelago. (photo obtainedCourtesy from http://www.topo-calanques.com/proteger_nature.htm, with written agreement).
82x61mm (300 x 300 DPI)



Map illustrating the number of analyzed samples and the geographical location of the five Yellow-legged gulls (Larus michahellis) breeding colonies in the two archipelagos off the coastcity of Marseille, Frioul and Riou, the two cities of La Grande-Motte and Palavas-les-Flots, and the lagoon of Pierre-Blanche lagoon. The bars show the filamentous fungi genera distribution at each study site; the vertical axis indicates the number of positive samples for each genus.. T (The map was drawn with using the Inkscape ver. 0.91 (software available at https://inkscape.org)).
136x110mm (300 x 300 DPI)



Relative frequencies of filamentous fungi genera isolated alongon the Mediterranean coast.
59x44mm (300 x 300 DPI)



Average (confidence intervaI) filamentous fungi richness (Chao 1, pannel A), diversity (Shannon index, pannel B) and species abundance (panel C), atin each study site: the lagoon of Pierre-Blanche (PB), the two cities of La Grande-Motte (GM) and Palavas-les-Flots (PA), and the two archipelagos off the coastcity of Marseille, Riou (RI) and Frioul (FR).
80x107mm (300 x 300 DPI)

101x57mm (300 x 300 DPI)

Chapter 3: Population genetic of *Candida glabrata*

3.1 Introduction

In this chapter, we analysed the genetic structure of *C. glabrata* isolates from human and gulls. Population genetics is a study the changes in gene frequencies over time. These genetic changes lead to genetic evolution in the population (Nielsen and Slatkin, 2013). Many factors contribute in genetic changes in population, such as mutation, mating systems, gene flow or migration, population size, and selection (McDonald, 1997). These genetics changes can be related to selective environmental processes or random changes from one generation to the next or migration or genetic mutations. In addition, studying the pattern of genetic variations within population has a major impact on the diagnosis of fungal infections and lead to dramatic improvements to understand the population of pathogenic fungi (Xu, 2006). With population genetics, we can access phylogenetic relationships between strains, evolutionary history of genetics or circulation of strains between hosts. Recently, some studies presented genetic evidences that the same fungal genotypes were isolated from both bird and humans using various typing methods such as Multilocus sequence typing and Microsatellite typing (McManus *et al.*, 2009; Litvintseva *et al.*, 2011; Van Waeyenberghe *et al.*, 2012). Our objective in this section of the thesis was to describe the potential circulation of fungal strains between human and gull populations using Microsatellite typing. Eight markers were used to type the *C. glabrata* isolates from both human and gull populations.

3.2 *Candida glabrata* isolates

3.2.1 *C. glabrata* isolates from gull's faecal samples

After the identification and analyze of fungal species diversity in all study sites, we selected the colonies of *C. glabrata* from original plates. The yeast colonies were collected in sterile conical tubes that contain a solution of NaCl 0.9% and conserved at Room temperature to next step.

3.2.2 *C. glabrata* isolates from patients

Clinical *C. glabrata* isolates collected in the same time period (2013) and a geographic region (Marseille, Montpelier and Nimes) with gull's *C. glabrata* isolates (Table 2.2).

3.3 Culture media

All isolates were subcultured to confirm the identification and for nucleic acid extraction. Therefore, we used **Malt extract agar** (as available in our Lab.) to prepare the isolates for the identification by MALDI-TOF MS. Then, **Chromogenic agar** was used to confirm the purification of isolates before send them to nucleic acid extraction.

3.4 Nucleic acid extraction:

Described in Chapter 1.

3.5 Multiple-locus variable number tandem repeat analysis (MLVA)

MLVA is a molecular typing method used to determine the variable copy of numbers of tandem repeats (VNTR) within a genome. VNTR loci or is called microsatellite markers consist of repeating sequences of 2-5 nucleotides formed as tandem repeats of di-, tri-, tetra-, or penta-nucleotide distributed in multiple copies (5-50 times) within the genome (Turnpenny and Ellard, 2012). DNA target is amplified via PCR and followed by amplicon sizing using capillary electrophoresis to measure the size of number of repeat copies (allele size) at each locus. Allele size is the sum of the size of the tandem repeat plus conserved primers, which are designed outside the tandem repeat (the offsets at both ends) (Nadon *et al.*, 2013). MLVA based on the variation of numbers of copy repetitions is an accurate method to study the population genetics of pathogenic microorganisms (Hardy *et al.*, 2003).

All 190 *C. glabrata* isolates were typed for eight microsatellite markers (Brisse *et al.*, 2009). Amplification reactions were performed using lightcycler® 480 Probes Master (Roche Diagnostics, GmbH, Germany). The primer sequence, fluorophores and hybridization temperatures were described in our article. To visualize replicated DNA, gel electrophoresis and safe DNA gel stain (Invitrogen, USA) on 2% agarose gels (Euromedex, France) in 1X of Tris borate EDTA buffer (Euromedex, France) were used. The PCR products were diluted 1:100 in sterile water. Then 1µl of diluted PCR products was mixed with a solution containing 25 µl HiDi formamide (Applied Biosystems, France) and 0.5 µl Gene Scan™ (Applied Biosystems, UK) in plate 96 wells. Then the plate was subjected to ABI 3130 genetic analyzer (Applied Biosystem, France) to fragment size analysis and the results were analyzed with GeneMapper software v 4.0.

3.6 Anti-fluconazole susceptibility testing

C. glabrata has increased in invasive *Candida* infections particular, in immunocompromised patients (Pfaller *et al.*, 2012). This species is mostly less susceptible to azoles than other *Candida* species in particular after develop anti-fluconazole resistance (Shin *et al.*, 2007; Pfeiffer *et al.*, 2010). We should investigate about the clonal origin of the resistant *C. glabrata* genotypes and the horizontal spread of these genotypes among hosts.

In current study, *C. glabrata* anti-fluconazole susceptibility testing was performed using RPMI microbroth plates (PAN-Biotech, GmbH, Germany) dilution as described by Clinical Laboratory Standards Institute (CLSI) M27-S4 document guidelines (CLSI, 2012). *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were tested to quality control in our isolates. Then, reading of result has done visually after 48 h of incubation.

Table 2.2. The strains of *Candida glabrata,* isolated from patients with their susceptibility to fluconazole.

Sample	Hospital	Sampling site	Anti-fluconazole
1300577	Marseille	Urine	NA
1304148	Marseille	Urine	NA
1303938	Marseille	Stool	S
1321293	Marseille	Urine	S
1321552	Marseille	Urine	S
1323059	Marseille	Stool	NA
1324704	Marseille	Stool	S
1325590	Marseille	Urine	NR
1329774	Marseille	Urine	S
1331313	Marseille	Stool	R
1331564	Marseille	Stool	R
1332525	Marseille	Urine	S
1334346	Marseille	Urine	R
1334463	Marseille	Stool	NA
1336535	Marseille	Urine	NA
1337656	Marseille	Urine	R
1337959	Marseille	Urine	R
1338100	Marseille	Urine	S
1338109	Marseille	Urine	R
1341438	Marseille	Urine	NA
1341280	Marseille	Urine	R
1341878	Marseille	Urine	S
1343155	Marseille	Stool	S
1343887	Marseille	Urine	R
1344601	Marseille	Stool	S
1345019	Marseille	Urine	S
1346027	Marseille	Urine	R
1346028	Marseille	Urine	S
1347401	Marseille	Urine	R
1347924	Marseille	Urine	R
1349384	Marseille	Urine	R
1350011	Marseille	Urine	R
1350926	Marseille	Urine	S
1353355	Marseille	Stool	R
1353665	Marseille	Urine	S
1353666	Marseille	Urine	NA
1356805	Marseille	Urine	NA
1356777	Marseille	Stool	S

1357301	Marseille	Urine	NA
1910	Montpellier	Bile	NA
5468	Montpellier	Liquid graft preserving	NA
6610	Montpellier	Collection abdominal wall	NA
2910	Montpellier	Blood	NA
3020	Montpellier	Bronchoalveolar lavage	NA
4887	Montpellier	Bronchoalveolar lavage	NA
9465	Montpellier	Blood	NA
9482	Montpellier	hepatic collection	NA
3908	Montpellier	liver abscess	NA
5673	Montpellier	Content rear Omentum cavity	NA
6214	Montpellier	Blood	NA
7218	Montpellier	buccal swab	NA
1908	Montpellier	Blood	NA
4407	Montpellier	peritoneal fluid	NA
5616	Montpellier	Blood	NA
6278	Montpellier	Blood	NA
6283	Montpellier	peritoneal fluid	NA
8076	Montpellier	Blood	NA
8297	Montpellier	intra-abdominal abscess	NA
8469	Montpellier	peritoneal fluid	NA
1-GLA	Nimes	organic liquid	NA
4-GLA	Nimes	urine	NA
6-GLA	Nimes	CBB	NA
8-GLA	Nimes	urine	NA
19-GLA	Nimes	fistula flow	NA
38-GLA	Nimes	CBB	NA
39-GLA	Nimes	bile liquid	NA
47-GLA	Nimes	urine	NA
51-GLA	Nimes	urine	NA
65-GLA	Nimes	urine	NA

68-GLA	Nimes	cervical fistula	NA
70-GLA	Nimes	stools	NA
76-GLA	Nimes	urine	NA
78-GLA	Nimes	urine	NA
81-GLA	Nimes	urine	NA
101-GLA	Nimes	expectoration	NA
112-GLA	Nimes	urine	NA
115-GLA	Nimes	aspiration bronchium	NA
121-GLA	Nimes	hemoculture	NA
125-GLA	Nimes	urine	NA

S= Sensitive, R= Resistant, NA= Not Available

Article 3: Pathogenic Yeast *Candida glabrata* circulates between Humans and Yellow-legged gulls

In this article, we studied the population genetics of 190 *C. glabrata* isolates from gulls and human (111 isolates from gulls and 79 isolates from human) isolated in the same geographic area and within the same time period. All isolates were typed using Multiple-locus variable number tandem repeat analysis (MLVA), using eight microsatellite markers. High genetic diversity was found in MLVA *C. glabrata* genotypes in both human and gulls isolates. Moreover, we analysed all allelic profiles with the STRUCTURE v. 2.3.1 software to identify distinct genetic clusters. Then, two distinct genetic clusters were identified by estimating the individual's membership coefficient in each cluster. The pairwise fixation index (F_{ST}), P-value and analysis of molecular variance (AMOVA) were computed with ARLEQUIN v 3.5 software, in order to find the genetic differentiation between and among *C. glabrata* clusters and populations. A significant effect of genetic structure in population differentiation was found between clusters (F_{ST} = 0.27, $p<10^{-5}$). In contrast, a relatively low *C. glabrata* population differentiation was observed between gull and human isolates (F_{ST} = 0.03, $p<10^{-5}$). According to the AMOVA results, the highest genetic variance 90% ($p<10^{-5}$) was found within populations. We also tested the effect of geographic distances on genetic differentiation via the Mantel test, which showed that geographical distances explained 15.5% of *C. glabrata* genetic differentiation (p=0.023). Additionally, *in vitro* fluconazole susceptibility was tested on 54 independent *C. glabrata* isolates, collected within the same time period in the area of Marseille. Both, MST and UPGMA trees showed the absence of genetic clustering according to the host (gull or human) or *C. glabrata* isolates fluconazole susceptibility or resistant phenotype.

Article status: submitted

The Pathogenic Yeast *Candida glabrata* circulates between Humans and Yellow-legged gulls

Mohammed Hashim Al-Yasiri[a], Anne-Cécile Normand[b], Coralie L'Ollivier, Laurence Lachaud[c,d], Nathalie Bourgeois[c], Stanislas Rebaudet[a], Renaud Piarroux[a,b], Jean-François Mauffrey[e], Stéphane Ranque[a,b,*]

a. Aix-Marseille Université, IP-TPT UMR MD3, 13885 Marseille, France
b. Laboratory of Parasitology - Mycology, APHM CHU Timone, 13005 Marseille, France
c. Département de Parasitologie-Mycologie, CHU de Montpellier, Montpellier, France,
d. Faculté de Médecine de Montpellier-Nîmes, Nîmes, France
e. Laboratoire Population Environnement Développement (LPED) - UMR-151, Aix-Marseille Université – IRD, Marseille, France

Keywords: synanthropy; reservoir; transmission; genetic structure; MLVA; population genetics; antifungal resistance

Abstract

The pathogenic yeast *Candida glabrata* is a component of the mycobiota of both humans and Yellow-legged gulls that is prone to develop fluconazole resistance. Whether gulls are a reservoir of the yeast and facilitate the dissemination of human *C. glabrata* strains remains an open question.

In this study, MLVA genotyping highlighted the lack of genetic structure of 190 *C. glabrata* strains isolated from either patients in three hospitals or fecal samples collected from gull breeding colonies located in five distinct areas along the French Mediterranean littoral. Fluconazole-resistant isolates were evenly distributed between both gull and human populations.

These findings demonstrate that gulls are a reservoir of this species and facilitate the diffusion of *C. glabrata* and indirect transmission to human or animal hosts via environmental contamination. This eco-epidemiological view, which can be applied to other species, broadens our perspective regarding the reservoirs and dissemination patterns of antifungal-resistant human pathogenic yeast.

Candida glabrata is one of the most frequently identified yeast species of the human gut mycobiota[1]. This species has also emerged as a major agent of human mucosal, systemic and bloodstream yeast infections, second only to *C. albicans*[2–4]. *C. glabrata* infections are characterized by a high (40-70%) fatality rate, especially in immunocompromised patients[5]. However, the reservoir of *C. glabrata* has not been well characterized. Apart from humans, this yeast is a common commensal organism of many species of pet birds, such as Cockatiels, Parakeets, Lovebirds and Cockatoos or migratory birds such as the Common whitethroat (*Sylvia communis*) and the Spotted flycatcher (*Muscicapa striata*)[6–9]. A growing number of studies has demonstrated that birds act as a transporter and facilitate the spread of many pathogens, including viruses, bacteria, fungi and parasites[8,10–14]. Several pathogenic microorganisms have been shown to be transmitted over vast distances between humans and birds, such as the aquatic bird-borne influenza virus[15]Studies have highlighted the risk of spreading various antibiotic resistant pathogens via contaminated bird feces[16–18].

In recent decades, Yellow-legged gull population numbers have greatly increased throughout the Mediterranean littoral concurrent with an increase in anthropic refuse production. Consequently, they have displayed an increase in interaction with the environment in general and in particular with humans and other animals[19–22]. These birds feed on maritime or terrestrial matter, including human refuse, as demonstrated by an analysis of the composition of Yellow-legged gull pellets in the Marseille region[23].

Among pathogenic yeasts, *C. glabrata* is particularly prone to develop resistance to antifungal drugs, in particular fluconazole, which is the first-line antifungal treatment for

yeast infections[24]. The extended use of fluconazole treatment in humans has been associated with an increase in fluconazole-resistant *C. glabrata* clinical isolates[25–27]. Yellow-legged gulls are known to be a reservoir of antibacterial-resistant Enterobacteriaceae (recently reviewed in[28]). In a previous study, we have shown that *C. glabrata* constitutes part of the gut mycobiota of Yellow-legged gulls[29]. However, whether Yellow-legged gulls act as a reservoir and facilitate the dissemination of *C. glabrata* remains to be determined. Moreover, no data is available concerning the presence of antifungal-resistant yeast within the gut mycobiota of these birds. This study therefore aimed to assess whether *C. glabrata* isolates derived from sympatric Yellow-legged gull and human populations are genetically distinct. We also determined whether fluconazole-resistant *C. glabrata* strains are present in the gut mycobiota of these birds.

Results

MLVA revealed high genetic diversity with 129 distinct genotypes among the 190 *C. glabrata* isolates (Table 2). Allelic richness, diversity and evenness for each study site are detailed in Table 3. We found 100 singleton genotypes, of which 44 were isolated from humans and 56 were derived from gulls. Among the 29 non-singleton genotypes, 13 were present in both human and gull isolates, while 4 and 12 were only isolated in humans or gulls, respectively (Table 2).

Two distinct genetic clusters, including 101 and 89 isolates, were identified by estimating the individual membership coefficient for each cluster using STRUCTURE (Fig. 3). The between-cluster F_{ST} was 0.27 ($P<10^{-5}$), thereby indicating, as expected, a significant effect of genetic structure on population differentiation. In contrast, the

between-host F_{ST} was 0.03 ($P<10^{-5}$), which indicates a relatively low *C. glabrata* population differentiation due to genetic structure between gull and human isolates. The MST (Fig. 1) highlighted the high genetic diversity of *C. glabrata* isolates. Neither host (gull or human) nor study site was associated with any genetic cluster. In particular, gull and human isolates were distributed between both genetic clusters, with an exception for the 11 isolates collected from gulls at La Grande-Motte, which all belonged to the first cluster (Fig. 2).

AMOVA revealed that study sites, within-host and among-host populations explained 90% ($P<10^{-5}$), 9% ($P<10^{-5}$) and 1% ($P=0.11$) of the genetic variance, respectively. We also calculated F_{ST} for three patient populations and five gull populations; the between site pairwise F_{ST} values are specified in Fig. 2 and Table 4. The population differentiation due to genetic structure between sites (hospitals) of human isolates was relatively low; the highest of these F_{ST} values was 0.037 ($P=0.04$) between populations from Montpellier and Marseille (Fig. 2, Table 4). The population differentiation due to genetic structure between sites (breeding colonies) of Yellow-legged gull isolates was comparatively higher than among the human isolates. In particular, differentiation due to genetic structure was particularly high between the population from the Riou Archipelago and those from all other study sites. The overall highest pairwise F_{ST} value was 0.615 ($P<0.001$) between Riou and La Grande-Motte (Fig. 2, Table 4). The Mantel test showed that geographical distances explained 14.4% of *C. glabrata* genetic differentiation ($P=0.023$).

In vitro fluconazole susceptibility was assessed in 54 *C. glabrata* isolates, all of which were collected within the same time period in the Marseille area, including 25 samples

collected from Yellow-legged gull breeding colonies on the Frioul and Riou Archipelagos and 29 isolates collected from patients at the university hospital of Marseille. Overall, 23 isolates were classified fluconazole resistant (MIC ≥ 64 mg/L); 9 and 14 (36.5%, 95%CI [18.0-57.5] vs. 50%, 95%CI [29.5-67.5], *P*=0.53) were isolated from gull or human hosts, respectively. The absence of genetic clustering according to the host or fluconazole susceptibility is depicted in the MLVA-based MST tree (Fig. 4).

Discussion

Overall, this study highlights the absence of significant genetic differentiation between *C. glabrata* populations in humans or Yellow-legged gulls. We also demonstrated that antifungal-resistant pathogenic yeasts are present within the gut mycobiota of Yellow-legged gulls. The low differentiation between human and gull *C. glabrata* populations is in agreement with a previous study that has shown that *C. dubliniensis* populations isolated from Herring gulls (*Larus argentatus*) or humans were genetically similar[30]. In contrast, geographic location of the collection site was the major factor in genetic variance. Furthermore, Mantel test analysis showed a trend of increasing genetic differentiation with increasing geographical distance. Similarly, de Meeûs et al.[31] have shown using both multilocus enzyme electrophoresis and randomly amplified polymorphic DNA that *C. glabrata* populations isolated from patients in Paris and Montpellier, which are 800 km apart, displayed genetic differentiation (F_{ST}=0.11, *P*=0.054). Using the same MLVA scheme as in the present study, Dhieb et al.[32] have shown highly significant genetic differentiation (F_{ST}=0.359, $P<10^{-5}$) between *C. glabrata* populations isolated from patients in France or Tunisia. Although the geographical scale

of our study was much more limited than in the previous studies, we detected either relatively high or low genetic differentiation according to the study sites. As we found evidence for both dispersion and differentiation, our study was indeed adequately scaled to dissect transmission profiles and detect reservoirs.

C. glabrata is a component of the human gut mycobiota. We hypothesize that Yellow-legged gulls inadvertently ingest yeast such as *C. glabrata* with their food, which might be contaminated with human excreta in highly anthropic marine and terrestrial environments. Compared with other sea birds, Yellow-legged gulls are highly synanthropic. This is a major reason that gull populations have grown concomitantly with human-made environments, including human refuse sites, along the Mediterranean littoral [33]. The birds become a reservoir as the pathogen develops into a component of the gut microbiota. The Yellow-legged gull can fly relatively extended distances along the Mediterranean littoral to feed on landfills. Therefore, garbage dumps in urbanized areas may be a potential source of clinically important yeast transmitted by gulls. Due to the high mobility of gulls, the birds facilitate the dissemination of the pathogen by releasing their droppings over an expansive area of the marine and terrestrial environment.

Humans may be infected with *C. glabrata* originating directly (via bird droppings in their direct environment) or, more frequently, indirectly by ingesting food that has been contaminated with bird droppings. The genetic homogeny of human and bird isolates clearly suggests that Yellow-legged gulls play a role in the diffusion of pathogenic *C. glabrata* acquired from an anthropic environment. In line with this hypothesis, Yellow-legged gulls transmit and spread pathogens in various environments. Indeed,

Bonnedahl et al. [16] have demonstrated that Yellow-legged gulls disseminate antibiotic-resistant *Escherichia coli* isolates not far from Pierre Blanche. Similarly, we demonstrated the presence of genetically homogeneous fluconazole-resistant *C. glabrata* populations in both gulls and humans in Marseille. Therefore, our findings show that Yellow-legged gulls act as a carrier, reservoir and disseminator of *C. glabrata*. These birds may thus contribute to the transmission of pathogenic yeast to humans and likely other animal hosts. Moreover, the likelihood of infectious disease transmission via Yellow-legged gulls has therefore increased due to the marked demographic growth of gull populations along the Mediterranean littoral. Our findings also demonstrate that yellow-legged gulls represent a reservoir of antifungal-resistant *Candida* strains and disseminate antifungal-resistant isolates.

Methods

C. glabrata isolates

In this study, we analyzed 190 *C. glabrata* isolates. One hundred eleven samples were isolated from feces collected at five yellow-legged gull breeding colonies as previously described [29]. Briefly, the sampled breeding colonies were located in the departments of Hérault and Bouches-du-Rhône, in the South of France. In Hérault, the colonies were located in a natural reserve at the lagoon of Pierre Blanche and in two cities, Palavas-les-Flots and La Grande-Motte. In Bouches-du-Rhône, two breeding colonies were located on the Frioul and Riou Archipelagos off the coast of Marseille. Yellow-legged gulls may yet be exposed to varying levels of anthropogenic pressure. In this study for instance, birds breeding at the lagoon of Pierre Blanche were exposed to a relatively

low anthropogenic influence compared with those breeding on the building rooftops in the cities of Palavas-les-Flots, La Grande-Motte and Marseille. Riou and Frioul Archipelagos are suburban ecocline exposed to an intermediate anthropogenic influence. Seventy-nine *C. glabrata* samples were isolated from patients at three university hospitals in Marseille, Montpellier and Nimes, which were located in the same geographical area and sampled during the same time period as those isolated from gulls. All isolates were subcultured on Malt extract agar (Sigma Aldrich, USA). The samples were identified via the MALDI-TOF MS (matrix-assisted laser desorption /ionization-time-of-flight mass spectrometry) technique, as previously described [34], and subcultured onto chromogenic medium plates (CHROMagar™, Becton Dickinson, France) to verify isolate purity[35].

Multiple-locus variable number tandem repeat analysis (MLVA)

All *C. glabrata* isolates were typed with eight microsatellite markers (GLA2, GLA3, GLA4, GLA5, GLA6, GLA7, GLA8 and GLA9) as previously described[36]. Genomic DNA was extracted using the NucliSENS™ EasyMAG™ (bioMérieux) system[37], eluted in 50 µl and stored at -20 C°. Amplification reactions were performed using a Lightcycler™ 480 (Roche Diagnostics, GmbH, Germany) instrument with Lightcycler™ 480 Probes Master (Roche Diagnostics, GmbH, Germany). The loci, primer sequences, fluorophores and hybridization temperatures are described in Table 1. The PCR products were visualized using 2% agarose gel electrophoresis in 1X of Tris borate EDTA buffer (Euromedex, France) with safe DNA gel stain (Invitrogen, USA). Next, 1 µl of 1:100 diluted PCR products was mixed with a solution containing 25 µl HiDi formamide (Life Technologies, France) and 0.5 µl Gene Scan™ 500 LIZ™ size

standard (Applied Biosystems, UK). The fragment length was determined via capillary electrophoresis using an ABI 3130 Genetic Analyzer (Applied Biosystems, France) and analyzed using GeneMapper software v4.0 (Applied Biosystems, France).

Population genetic analysis

Several indices of clonal diversity were estimated using the *poppr* R package[38], including the genetic richness (or the number of multilocus genotypes observed per population (MLG) and the number of expected MLG at the smallest sample size based on rarefaction (eMLG)); the genetic evenness E_5, (ranging from 0, a population composed of a single genotype, to 1, all genotypes having equal frequency); Simpson's (lambda) diversity index[39] corrected by the number of isolates in a population; and the clonal fraction [40].

STRUCTURE v. 2.3.1 was used to identify genetically distinct clusters via estimation of the proportion of membership (Q) of each isolate in each cluster. This software applies a Bayesian model-based clustering approach[41] to model K populations that are characterized by a set of allele frequencies at each locus and probabilistically assigns isolates to clusters based on the multilocus genotypes. For each K value, ranging from 1 to 10, the posterior probability of the data was measured using an admixture model with correlated allele frequencies over 10 independent runs with a burn-in period of 5000 followed by 50000 Markov Chain Monte Carlo steps. The number of clusters was estimated by the value of K that maximizes the posterior probability of the data. The highest ΔK value corresponds to the most pronounced partition of the data and indicates the likely number of clusters.

The among- and between-population differentiation due to genetic structure was estimated via Slatkin's linearized pairwise fixation index (F_{ST}) and Analysis of MOlecular VAriance (AMOVA) using ARLEQUIN v3.5 software. The effect of geographical distances on genetic differentiation was tested via Mantel test. MLVA-based Minimum Spanning Tree (MST) was constructed using BIONUMERICS software v7.1.

Anti-fluconazole susceptibility testing

C. glabrata anti-fluconazole susceptibility was assessed as described by Clinical Laboratory Standards Institute (CLSI) M27-S4 document[42]. Fluconazole resistance was defined as a minimal inhibitory concentration of ≥64 mg/L. Each assay was validated using ATCC 22019 (*Candida parapsilosis*) and ATCC 6258 (*Candida krusei*) quality control strains.

Conclusion

The close proximity and interaction between very dense human and Yellow-legged gull populations in cities of the Mediterranean littoral facilitates the circulation of pathogens between the two hosts. Gulls likely ingest pathogenic *C. glabrata* by eating or drinking in environments contaminated with human excreta. The yeast eventually evolves to be incorporated into their gut mycobiota and is spread via gull feces to human environment. Moreover, Yellow-legged gulls represent a reservoir of fluconazole-resistant *C. glabrata* isolates, which may have a potential impact on public health, including food safety and potable water source protection.

Acknowledgments

The authors gratefully acknowledge the help of Olivier Scher (Conservatoire des Espaces Naturels du Languedoc-Roussillon), Alain Mante (Parc national des Calanques), Katleen Vranckx (BioNumerics, Applied Maths), Lucile Yrles (Ligue de Protection des Oiseaux, Hérault), Philippe Rispail for graciously providing clinical strains and Sandra Moore for English editing.

Author Contributions Statement

F.M., A.-C.N. and S.R. designed the study. A.-C.N., M.H.A.-Y., F.M and S. Ra. collected gulls' isolates; C.L., L.L., N.B. and S. Ra. provided patients' isolates; M.H.A.-Y. and A.-C.N. performed the culture, identification and typing of *C. glabrata* isolates;. M.H.A.-Y., F.M, S. Re. and S. Ra. analyzed the data; .M.H.A.-Y., F.M, R.P., S.Re. and S.Ra. wrote the main manuscript text and S.Re. prepared figures 1-2. All authors reviewed the manuscript.

Competing financial interests

There are none.

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Table 1. Characteristics of the eight microsatellite loci used for *C. glabrata* MLVA genotyping.

locus	Chromosome	Gene or intergene	repeat in CBS138	Forward primer	Reverse primer	Temperature of hybridization
GLA2	I	Intergene	$(GCT)_8$	6-FAM-GCACTCTGTCTACTTATAC	CGAATCCGTGATCCCTTC	54
GLA3	D	Intergene	$(AAC)_6$	6-FAM-ACACCTACGAGAAACCAACA	TAGCGGTCATCCAGCATCA	56
GLA4	A	Intergene	$(ATC)_8$	VIC-ATGCTGTATTTAACGATGCC	ACCAATGGTAACAGAGT	54
GLA5	H	CAGL0H02783g	$(ATC)_{10}$	VIC-TTATTACTCTTTCGGGTCAGG	CGAAACAACGTCAGAAACTC	54
GLA6	J	CAGL0J10560g	$(ATC)_9$	VIC-CCTTTAAGGATGAGCTACTTC	GCTGGTGGTTTAAAGGAAAC	54
GLA7	K	CAGL0K05423g	$(GTT)_{13}$	PET-GTCGTACCTTTGTATAATGTTG	AATGCGTTAGATGCCTT GAGA	50
GLA8	C	CAGL0C01265g	$(TAA)_{11}$	PET-ATTTACTAATAATACAGCTCAC	CTCAGCAGAACTTTCTT TAGT	50
GLA9	I	Intergene	$(ACTC)_5$	PET-CCTTCCCTCTGCGGATACT	TCACTGGACCTCTGTAG TGGT	56

Table 2. Frequency distribution of the 111 MLVA genotypes identified for the 190 *C. glabrata* samples isolated from Yellow-legged gulls or humans.

Genotypes	Gull	Human	Total
65	4	1	5
66	4	5	9
68	5	2	7
98	1	4	5
121	2	4	6
17	2	1	3
42	1	2	3
43	2	1	3
70	2	1	3
77	1	2	3
24	1	1	2
86	1	1	2
124	1	1	2
102	3	0	3
41	5	0	5
27	2	0	2
39	2	0	2
40	2	0	2
57	2	0	2
67	2	0	2
75	2	0	2
100	2	0	2
101	2	0	2
105	2	0	2
122	2	0	2
3	0	3	3
88	0	2	2
112	0	2	2
113	0	2	2
Singletons*	56	44	100
Total	**111**	**79**	**190**

*A singleton is a genotype that has been found only once in the study population.

Table 3. Allelic richness, diversity and evenness of the 111 MLVA genotypes identified for the 190 *C. glabrata* samples isolated from Yellow-legged gulls or humans were estimated for each study site. Allelic richness was specified using the number of multilocus genotypes observed per population (MLG) and the number of expected MLG at the smallest sample size based on rarefaction (eMLG). Genetic evenness was estimated using the E5 index. Diversity was calculated using the Simpson's (lambda) index corrected by the number of isolates in a population.

Pop	N	MLG	eMLG	E_5	Corrected lambda	Clonal fraction
PB	33	30	10.61	0.93	0.99	0.09
GM	11	11	11.00	1.00	1.00	0.00
PF	19	17	10.36	0.95	0.99	0.11
RI	14	12	9.79	0.94	0.98	0.14
FR	34	25	10.05	0.92	0.98	0.26
MR	39	31	10.29	0.90	0.99	0.21
MO	20	20	11.00	1.00	1.00	0.00
NI	20	18	10.28	0.90	0.98	0.10
Total	**190**	**131**	**10.62**	**0.74**	**0.99**	**0.31**

Table 4. **Pairwise *C. glabrata* population differentiation matrix according to the study sites**. Slatkin's linearized F_{ST} fixation index values are tabulated in the lower triangle, and the corresponding *P* values are indicated in the upper triangle. Statistically significant F_{ST} values in indicated in bold text.

	PB	**PF**	**GM**	**RI**	**FR**	**MA**	**MO**	**NI**
PB		0.35	0.08	< 0.001	< 0.001	< 0.001	0.01	< 0.001
PF	0.003		0.16	< 0.001	< 0.001	< 0.001	0.001	< 0.001
GM	0.043	0.036		< 0.001	< 0.001	< 0.001	0.009	< 0.001
RI	0.262	0.43	0.615		< 0.002	< 0.001	< 0.001	< 0.001
FR	0.074	0.146	0.198	0.112		0.009	0.012	0.06
MA	0.071	0.132	0.215	0.157	0.044		0.04	0.06
MO	0.050	0.104	0.11	0.229	0.052	0.037		0.17
NI	0.085	0.139	0.248	0.205	0.035	0.032	0.022	

Study site abbreviations. Yellow-legged gulls' breeding colonies: PB= lagoon of Pierre Blanche; PF= Palavas-les-Flots; GM= La Grande-Motte; RI= Riou Archipelago and FR= Frioul Archipelago. University hospitals of MA= Marseille, MO= Montpellier and NI= Nîmes.

Figure legends

Figure 1. Minimum spanning tree of the 190 *C. glabrata* isolates collected from gulls or patients. Each node represents a unique MLVA genotype, and the various colors of the nodes indicate the study site. The single-locus variants are linked with thick solid lines, double-locus with thin solid line, while the triple-locus variants are linked with dashed lines. The two genetic clusters identified using STRUCTURE software are outlined in red (cluster 1; n= 101) and green (cluster 2; n= 89).

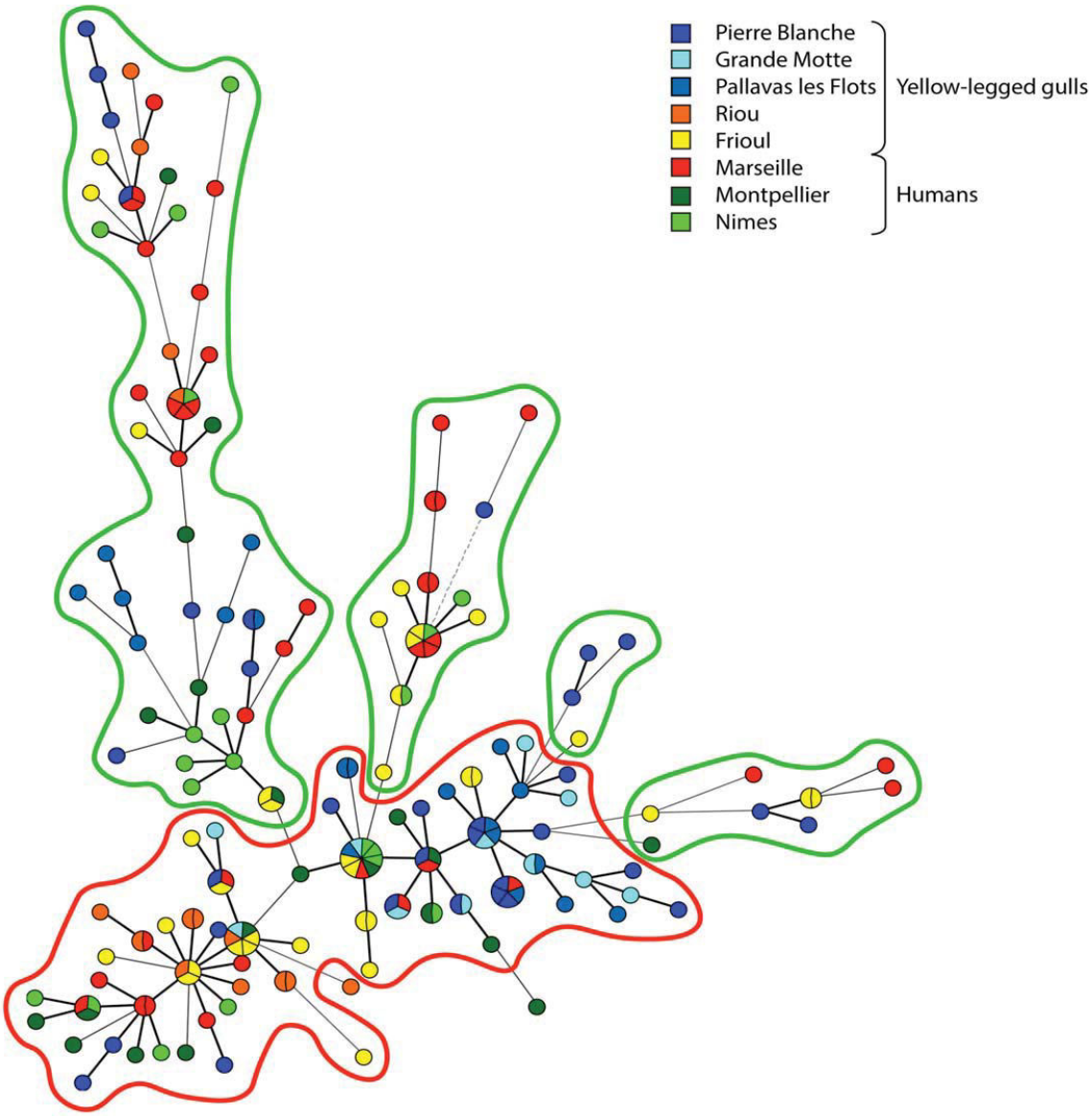


Figure 2. Map of the three cities, Montpellier, Nîmes and Marseille, and the Yellow-legged gulls' breeding colonies in the Mediterranean areas where isolates were sampled. The nodes indicate the relative number of isolates sampled in the respective region. Pie chart colors correspond to the proportion of two genetic clusters of the 190 *Candida glabrata* isolates at each site. *C. glabrata* population differentiation between study sites was measured via calculation of pairwise F_{ST}. Statistically significant F_{ST} values are indicated in bold. The locations of the Yellow-legged gulls' breeding colonies are abbreviated as: PB= lagoon of Pierre Blanche; PF= Palavas-les-Flots; GM= La Grande-Motte; FR= Frioul Archipelago; and RI= Riou Archipelago). *[This map was created on the open source QGIS Geographic Information System software version 2.12.1-Lyon (http://qgis.osgeo.org), using an open license shapefile of French departments obtained from IGN (http://professionnels.ign.fr/geofla).]*

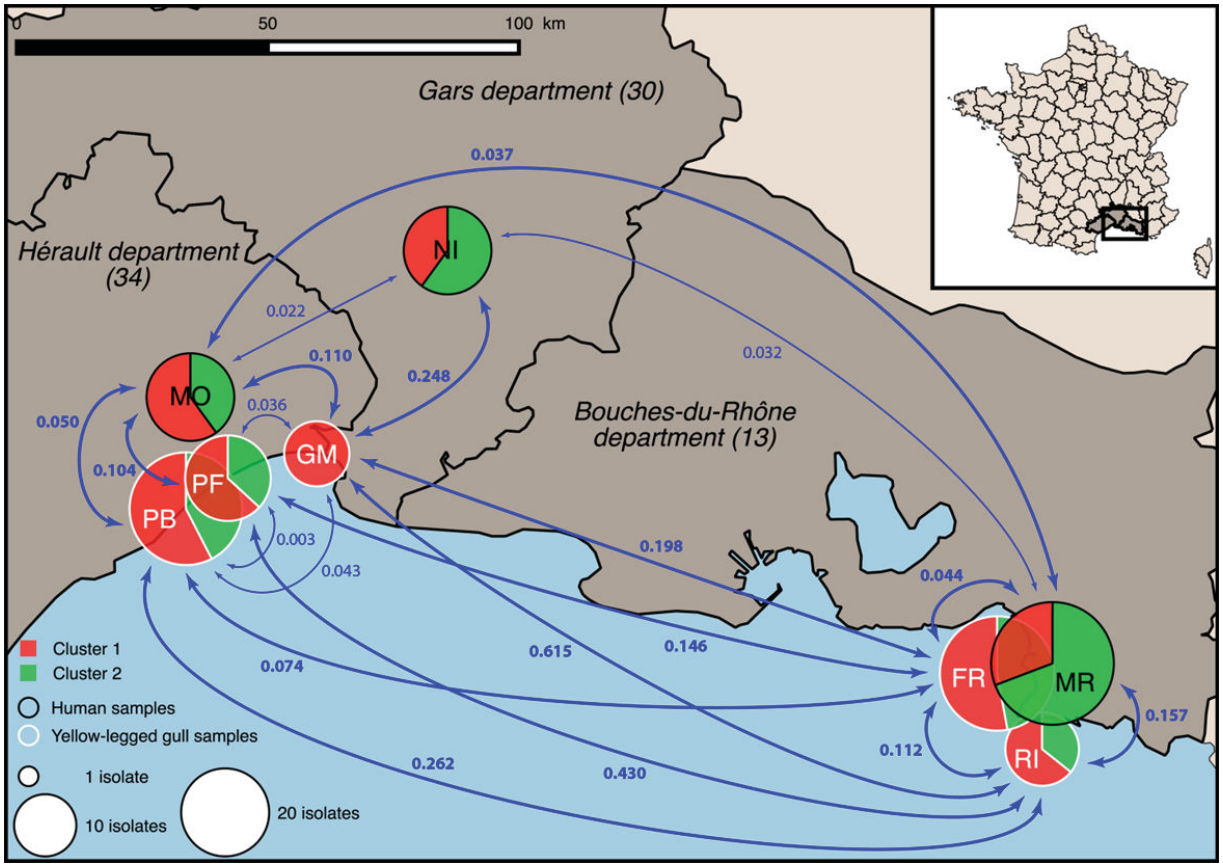


Figure 3. STRUCTURE clustering (admixture) in which each isolate is represented by a single vertical line that is partitioned into K=2 colored segments. The segment length represents the individual's estimated membership fractions in cluster 1 (red) and cluster 2 (green). Isolates with multiple colors have admixed genotypes from each cluster.

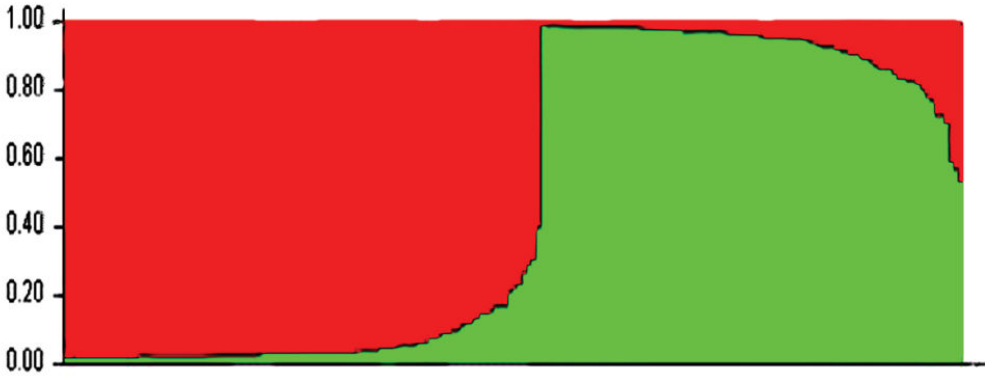
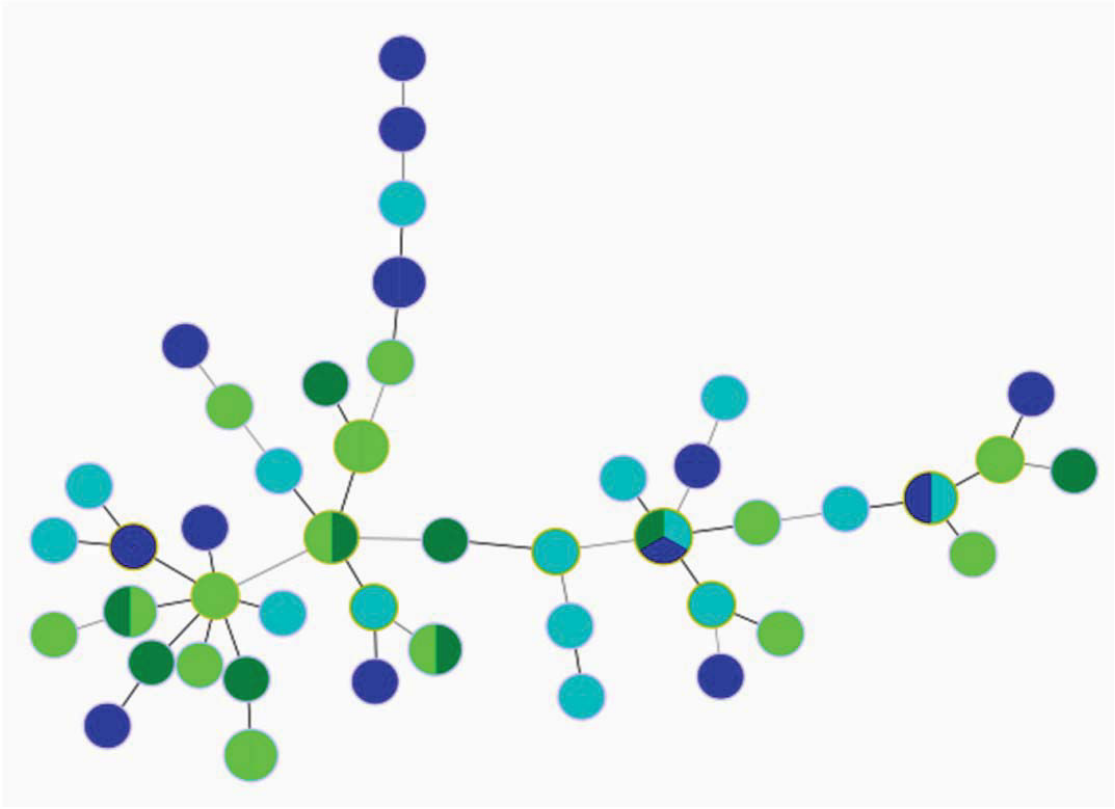


Figure 4. MLVA-based minimum spanning tree of 54 *C. glabrata* isolates from Marseille hospital patients and gulls from the Riou and Frioul archipelagos off the coast of Marseille. In vitro resistance to the antifungal fluconazole was found in 9 and 14 isolates from gull and human hosts, respectively**.** Blue nodes indicate human isolates; green nodes indicate gull isolates; dark colors indicate resistance to fluconazole and light colors indicate susceptibility to fluconazole.



Conclusion

For this dissertation, I successfully collected 177 feces samples of yellow-legged gull colonies, which is the common sea bird on the Mediterranean coast that is well adapted to anthropogenic environments. After two days of incubation at 30 C^{o}, I found high proportion of samples were positive and a huge growth of yeast. I started to count the colony forming unit for different colonies on plates depending on their morphological characteristics followed by the identification with MALDI TOF, which was very easy and cheap. In particular, the database of our MALDI TOF is supported with a great fungi species mass spectrum data, which was created in our laboratory. In addition, I sequenced ITS and D1D2 fragment for all fungi species that I could not identify with MALDI TOF due to missing database. When I finished the identification of yeast I re-incubated the petri dishes for further three days to allow the growth of mold colonies. Then, I did the same protocol of identification with MALDI TOF. The database of MALDI TOF for mold identification was completely created in our laboratory.

I had a huge data including yeast and mold species colony forming unit on five culture media and itraconazole resistance. Therefore, I simply focus on several biodiversity indices to analyze my result. I calculated the abundance using either the number of positive culture or CFU counts for each species. I computed Shannon-Wiener index for species diversity in each breeding colony. Chao 1 index was also calculated for species richness in each feces sample, and evenness using the Buzas and Gibson's index was computed depending on Shannon-Wiener index and Chao 1 index. I also estimated fungi community structure similarity between breeding colonies by assessing the presence/absence of taxa using the Sorenson index and the relative abundance of each species using the Bray-Curtis index.

Firstly, I identified 17 yeast species in the 177 samples. The highest diversity and richness of yeast species were observed at both Pierre Blanche and Frioul archipelago, which displayed similar proportions of very abundant species. The yeast community structure at both Pierre Blanche and Frioul was characterized by high species similarity. In contrast, the structure of gut yeast communities varied between the other three gull colonies at La Grande-Motte, Palavas-les-Flots and Riou archipelago due to low diversity and richness. Interestingly, I found that the yeast species similarity was 66.7% between the yeast community of gulls at La Grande-Motte and Riou archipelago. Despite Riou archipelago is considered as natural

reserve with a little anthropogenic influence it is exposed to sewage pollution. I concluded that human pathogenic yeast such as; *Candida glabrata*, *C. krusei* and *C. albicans* can colonize the gut of yellow-legged gull Larus michahellis. The frequency of yeast species increased with the synanthropy of the gull colonies. Gulls probably acquire pathogenic yeast, including antifungal-drugs resistant isolates, from human environment by feeding on human garbage refuse contaminated with this yeast.

Secondly, I identified 35 filamentous fungi species cultured in the same culture media plates. The filamentous fungi species community likely reflected environmental airborne fungal contamination of the study site. I also found that the diversity and the abundance were low at the synanthropic urban sites, La Grande-Motte and Palavas-les-Flots, compared with hemisynanthropic ecotones sites, Frioul and Riou archipelagos and asynanthropic site, Pierre Blanche. The community of filamentous fungi species at Frioul, Riou and Pierre Blanche were characterized by a high abundance and diversity. I found that the presence/absence of some species in particular was associated with specific biotopes and with the anthropogenic influence on these biotopes.

Finally, I studied the population genetics of 111 *C. glabrata* isolates from gulls and *79 C. glabrata* isolates from patients at three hospitals (Marseille, Montpellier and Nimes) using eight microsatellite markers for Multi-Locus Variable number tandem repeat Analysis (MLVA). I implemented the 190 *C. glabrata* genotype profiles into STRUCTURE v2.3.4 software to identify the two probable population genetics via estimation of the proportion of membership of each isolate in each population. Several indices in poppr R package were used to estimate the clonal diversity and genetic richness. I calculated Slatkin's linearized pairwise fixation index (F_{ST}) and analysis molecular variance (AMOVA) using ARLEQUIN v3.5 software. AMOVA within-host and among-host populations explained 90%, 9% and 1% of the genetic variance, respectively. According to the F_{ST} values, I found that the genetic differentiation between yellow-legged gull isolates was comparatively higher than between human isolates. I used a Mantel test to test the link between the geographical distances and *C. glabrata* genetic differentiations. I assessed in vitro fluconazole susceptibility in 54 *C. glabrata* isolates, and showed that fluconazole resistance was present in both yellow-legged gull and human isolates. Due to high genetic diversity in both human and *gull C. glabrata* populations, there was no distinct population or genetic cluster according to the host or geographical profile as well as to fluconazole-resistance profile.

Gulls likely ingest *C. glabrata*, including fluconazole-resistant isolates, by eating or drinking in environments contaminated with human excreta. These *C. glabrata*, including antifungal-drug resistant isolates, adapt in the gulls gut for order that make them able to amplify. Therefore, we found high *C. glabrata* abundance, which clearly increased in feces samples of gull's colonies at La Grande-Motte (synanthropy). In addition, gulls can access and disseminate *C. glabrata* in very diverse and relatively distant environments. These birds are commonly seen anywhere, and particularly on building roofs, in coastal urban areas, and in water environments. They contaminate these environments via their feces. Hence, human as well as wild and domestic animals may acquire pathogenic yeast via foodborne and waterborne transmission in gull's feces-contaminated environments.

Perspectives

The current results of this work give us a future vision for searching the role of other animal's species in spreading of pathogenic fungi to other hosts, which share the same environment. Our results confirm the carriage of pathogenic yeast by the gut of yellow-legged gulls, which colonize in different distances environments from human settlement on Mediterranean coast. Particularly, the circulation of *Candida glabrata* isolates between gulls and human was genetically demonstrated. We did not stopped at *C. glabrata*, but we started to search other yeast species isolated from gulls gut such as; Candida krusei. We started to type almost 150 *C. krusei* isolates from gulls gut using one microsatellite marker but we were not able to distinguish among the isolates due to absent the genetic diversity with one locus. Then we changed to use another method of typing, Multilocus sequence type (MLST) with 6 loci. We used MLST for studying the population genetics of *C. krusei* isolates from gulls and human, but we have not analyzed the results yet. Therefore, the question remains, will we find the same results that are found in *C. glabrata* isolates? Or will there be different results due to using another method or pathogenic yeast?

Although, *C. albicans* was few isolated from gulls, we can study their population genetics by comparing the genotypes profiles with those, which isolated from human.

The idea of using the filamentous fungi as environmental pollution marker with various hosts and ecosystems remains under testing. In this regards, we will need to perform some experiments in different environments to prove or refute this idea.

We can emphasize the idea of yeast proliferation into the bird gut. We can investigate from this idea by seasonal sampling of bird cloacae for five years. In each season, we observe the yeast community in gulls gut, after five years, we will have a great yeast isolates, which can be genetically compared together, to observe the continuation of yeast genotypes in birds gut. Birds can be marked during the study period. In the same context, we could follow the vertical and horizontal transmission of yeast species among bird individuals (from parents to chicks) and other hosts.

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Annex

Annex 1: MALDI-TOF typing highlights geographical and fluconazole resistance clusters in *Candida glabrata*

This study aimed to apply MALDI-TOF mass spectra for the strain typing of *C. glabrata* clinical isolates derived from both Marseille (France) and Tunis (Tunisia). To test the capacity of this technique to differentiate between fluconazole sensitive and resistant strains, 58 *C. glabrata* strains were typed using extracted nucleic acid and nine Microsatellite loci. By MALDI-TOF mass spectra, Protein extraction protocol was performed recommended by Bruker Daltonik GmbH (Bremen, Germany). Genetic structure was analyzed based on isolates profiles of nine loci to investigate the genetic differentiation according to either geographic origin or fluconazole susceptibility.

Both Microsatellite length polymorphisms (MLP) and MALDI-TOF enabled differentiate among *C. glabrata* strains and separate them according to their French or Tunisian origin. Based on MLP results, High significant genetic differentiation (F_{ST} = 0.35,P< 10^{-5}) was found, and this differentiation was confirmed via phylogenetic tree, which showed that the isolates from French and Tunisian were grouped in separate clusters. Phylogenetic tree based on MALDI-TOF summary spectra also separated the strains according to geographic origin, and most of the isolates that were grouped within one MLP cluster were also grouped within the same MALDI-TOF cluster. The agreement between MLP and MALDI-TOF classification was statistically tested using kappa coefficient (0.37), which indicated slightly agreement between the classifications. Population genetic of MLP-based on fluconazole susceptibility results showed a significant differentiation and separately clustered the strains in phylogenetic tree according to fluconazole sensitive and resistant characters.

ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2015, 53, 462–469
doi: 10.1093/mmy/myv013
Advance Access Publication Date: 3 April 2015
Original Article

Original Article

MALDI-TOF typing highlights geographical and fluconazole resistance clusters in *Candida glabrata*

C. Dhieb[1], A.C. Normand[2], M. Al-Yasiri[3], E. Chaker[4], D. El Euch[5], K. Vranckx[6], M. Hendrickx[7], N. Sadfi[1], R. Piarroux[2,3] and S. Ranque[2,3,*]

[1]Laboratoire des Microorganismes et Biomolécules Actives, Faculté des Sciences de Tunis, 2092 Tunis, Tunisia, [2]Parasitolgy-Mycology, APHM, CHU Timone, Marseille, France, [3]Aix Marseille Université, IP-TPT UMR MD3, 13005, Marseille, France, [4]Laboratoire de Parasitologie, Hôpital La Rabta, Tunis, Tunisia, [5]Service de Dermatologie et de Vénéréologie, Hôpital La Rabta, Tunis, Tunisia, [6]Applied Maths NV, 9830, Sint-Martens-Latem, Belgium and [7]BCCM/IHEM: Scientific Institute of Public Health, Mycology and Aerobiology Section, Brussels, Belgium

*To whom correspondence should be addressed. Dr Stéphane Ranque. Laboratoire de Parasitologie-Mycologie, AP-HM Timone, F-13385 MARSEILLE CEDEX 5. Tel: +33 491 38 60 90; Fax: +33 491 38 49 58; E-mail: stephane.ranque@ap-hm.fr

Received 19 September 2014; Revised 23 December 2014; Accepted 9 February 2015

Abstract

Utilizing matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra for *Candida glabrata* typing would be a cost-effective and easy-to-use alternative to classical DNA-based typing methods. This study aimed to use MALDI-TOF for the typing of *C. glabrata* clinical isolates from various geographical origins and test its capacity to differentiate between fluconazole-sensitive and -resistant strains.

Both microsatellite length polymorphism (MLP) and MALDI-TOF mass spectra of 58 *C. glabrata* isolates originating from Marseilles (France) and Tunis (Tunisia) as well as collection strains from diverse geographic origins were analyzed. The same analysis was conducted on a subset of *C. glabrata* isolates that were either susceptible (MIC $\leq$ 8 mg/l) or resistant (MIC $\geq$ 64 mg/l) to fluconazole.

According to the seminal results, both MALDI-TOF and MLP classifications could highlight *C. glabrata* population structures associated with either geographical dispersal barriers ($p < 10^{-5}$) or the selection of antifungal drug resistance traits ($<10^{-5}$).

In conclusion, MALDI-TOF geographical clustering was congruent with MPL genotyping and highlighted a significant population genetic structure according to fluconazole susceptibility in *C. glabrata*. Furthermore, although MALDI-TOF and MLP resulted in distinct classifications, MALDI-TOF also classified the isolates with respect to their fluconazole susceptibility profile. Further prospective studies are required to evaluate the capacity of MALDI-TOF typing to investigate *C. glabrata* infection outbreaks and predict the antifungal susceptibility profile of clinical laboratory isolates.

Key words: *Candida glabrata*, MALDI-TOF, microsatellites, typing, fluconazole susceptibility, clinical microbiology.

Introduction

Second only to *Candida albicans*, the emerging human pathogenic yeast *C. glabrata* is characterized by a decreased susceptibility to azole antifungal drugs, especially fluconazole, compared with other *Candida* spp. [1–3]. This haploid yeast has the capacity to develop fluconazole resistance via several mechanisms, including alteration/overexpression of the CgErg11 target enzyme [4] and increased drug efflux triggered by ATP-binding cassette overexpression [5]. Therefore, molecular typing methods have been developed to trace *C. glabrata* strains during outbreak investigations and further understand the spread of fluconazole resistance within this species. Both multilocus locus sequence typing (MLST) [6,7] and microsatellite length polymorphism (MLP) analysis [8,9] are widely applied to trace strains and/or describe the genetic population structure for a number of *Candida* species. High-resolution MLP typing has enabled the tracing of *C. glabrata* strains [8,10]. In contrast, MLST has allowed for grouping of *C. glabrata* isolates in broad subpopulations; however, perhaps due to the highly clonal mode of reproduction of this species, this method displayed insufficient resolution for strain tracing [11,12].

Recently, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has emerged as a rapid, accurate, simple, and inexpensive method for microorganism identification in the clinical laboratory. Several publications have demonstrated the capacity of MALDI-TOF to differentiate bacterial subspecies [13,14] and detect antibiotic resistance-associated phenotypes [15]. With respect to yeasts, MALDI-TOF has displayed encouraging results for the typing of yeast within the *C. parapsilosis* complex [16,17]. Applying MALDI-TOF mass spectra for *C. glabrata* typing would be cost-effective and easy-to-use compared with DNA-based typing methods. The aim of this study was to apply MALDI-TOF mass spectra for the strain typing of *C. glabrata* clinical isolates derived from different geographical origins and test their capacity to differentiate between fluconazole sensitive and resistant strains.

Methods

Candida glabrata strains

Nine *C. glabrata* BCCM/IHEM reference strains, originating from various geographical areas and collection sites, and 49 clinical isolates collected from either Marseilles (France) or Tunis (Tunisia) university hospital patients, in whom *C. glabrata* had been sequentially isolated, were used in the geographical differentiation analysis (Table 1). Thirty-two unrelated *C. glabrata* clinical strains, which were isolated from different patients, in distinct hospital wards, including 19 fluconazole-resistant and 13 fluconazole-sensitive iso-

Table 1. Detail of the clinical isolates of *Candida glabrata* used in the geographical differentiation analysis.

Identification	Origin	Sampling date	Sampling site
Fr_P1_1	Marseille	11/01/2012	Lower respiratory tract
Fr_P1_2	Marseille	16/01/2012	Lower respiratory tract
Fr_P1_3	Marseille	26/01/2012	Lower respiratory tract
Fr_P1_4	Marseille	07/02/2012	Lower respiratory tract
Fr_P1_5	Marseille	06/06/2012	Lower respiratory tract
Fr_P1_6	Marseille	08/06/2012	Lower respiratory tract
Fr_P2_1	Marseille	07/03/2012	Lower respiratory tract
Fr_P2_2	Marseille	09/03/2012	Lower respiratory tract
Fr_P2_3	Marseille	09/03/2012	Digestive tract
Fr_P2_4	Marseille	09/03/2012	Upper respiratory tract
Fr_P2_5	Marseille	09/03/2012	Skin
Fr_P2_6	Marseille	14/03/2012	Blood
Fr_P2_7	Marseille	15/03/2012	Urine
Fr_P3_1	Marseille	21/05/2012	Urine
Fr_P3_2	Marseille	22/05/2012	Urine
Fr_P3_3	Marseille	23/05/2012	Vagina
Fr_P3_4	Marseille	23/05/2012	Vagina
Fr_P3_5	Marseille	25/05/2012	Vagina
Fr_P3_6	Marseille	08/06/2012	Vagina
Fr_P3_7	Marseille	25/05/2012	Urine
Fr_P4_1	Marseille	01/01/2012	Blood
Fr_P4_2	Marseille	01/01/2012	Blood
Fr_P4_3	Marseille	01/01/2012	Blood
Fr_P4_4	Marseille	02/01/2012	Blood
Fr_P5_1	Marseille	29/06/2012	Blood
Fr_P5_2	Marseille	29/06/2012	Blood
Fr_P5_3	Marseille	29/06/2012	Blood
Tu_P1_1	Tunis	09/01/2011	Urine
Tu_P1_2	Tunis	21/02/2011	Urine
Tu_P1_3	Tunis	26/02/2011	Urine
Tu_P1_4	Tunis	05/03/2011	Urine
Tu_P1_5	Tunis	13/03/2011	Digestive tract
Tu_P1_6	Tunis	09/01/2011	Upper respiratory tract
Tu_P2_1	Tunis	29/06/2012	Urine
Tu_P2_2	Tunis	29/06/2012	Digestive tract
Tu_P2_3	Tunis	14/09/2012	Digestive tract
Tu_P3_1	Tunis	11/06/2012	Digestive tract
Tu_P3_2	Tunis	21/062012	Digestive tract
Tu_P3_3	Tunis	26/06/2012	Upper respiratory tract
Tu_P3_4	Tunis	13/07/2012	Digestive tract
Tu_P4_1	Tunis	27/04/2012	Digestive tract
Tu_P4_2	Tunis	03/09/2012	Digestive tract
Tu_P4_3	Tunis	06/09/2012	Digestive tract
Tu_P4_4	Tunis	21/09/2012	Digestive tract
Tu_P5_1	Tunis	19/06/2011	Urine
Tu_P5_2	Tunis	23/06/2011	Biopsy
Tu_P5_3	Tunis	28/06/2011	Digestive tract
Tu_P6_1	Tunis	13/07/2012	Urine
Tu_P6_2	Tunis	13/07/2012	Digestive tract
Ref_1	French	IHEM22852	Urine
Ref_2	Peru	IHEM16180	Urine
Ref_3	Peru	IHEM16178	Urine
Ref_4	Bolivia	IHEM15478	Vagina
Ref_5	Belgium	IHEM6141	Lower respiratory tract

Table 1. continued.

Identification	Origin	Sampling date	Sampling site
Ref_6	USA	IHEM9556	Blood
Ref_7	Belgium	IHEM4141	Blood
Ref_8	Belgium	IHEM22819	Blood
Ref_9	Spain	IHEM4210	Vagina

Table 2. The collection of *Candida glabrata* strains used in the Fluconazole susceptibility differentiation analysis. The isolates were classified Fluconazole resistant (R): MIC ≥64 mg/L or Fluconazole sensitive (S): MIC ≤8 mg/l.

Identification	R/S	Fluconazole MIC (mg/l)	Geographical origin
Tu_1_R	R	64	Tunis
Tu_2_R	R	64	Tunis
Tu_3_R	R	128	Tunis
Tu_4_R	R	128	Tunis
Tu_5_R	R	64	Tunis
Tu_6_R	R	64	Tunis
Tu_7_R	R	64	Tunis
Tu_8_S	S	8	Tunis
Tu_9_S	S	8	Tunis
Tu_10_S	S	8	Tunis
Tu_11_S	S	4	Tunis
Fr_1_R	R	256	Marseille
Fr_2_R	R	256	Marseille
Fr_3_R	R	256	Marseille
Fr_4_R	R	256	Marseille
Fr_5_R	R	256	Marseille
Fr_6_R	R	256	Marseille
Fr_7_R	R	256	Marseille
Fr_8_R	R	256	Marseille
Fr_9_R	R	256	Marseille
Fr_10_R	R	256	Marseille
Fr-11_R	R	256	Marseille
Fr_12_R	R	256	Marseille
Fr_13_S	S	4	Marseille
Fr_14_S	S	1	Marseille
Fr_15_S	S	4	Marseille
Fr_16_S	S	4	Marseille
Fr_17_S	S	1,5	Marseille
Fr_18_S	S	4	Marseille
Fr_19_S	S	4	Marseille
Fr_20_S	S	4	Marseille
Fr_21_S	S	4	Marseille

lates, were selected for the fluconazole susceptibility analysis (Table 2). Each strain was cultured on Sabouraud chloramphenicol gentamicin (Oxoid, Dardilly, France) plates at 30°C for 48 h. The *C. glabrata* clinical strains were identified based on the MALDI-TOF MS results, as previously described [18]. All clinical isolates have been deposited in the BCCM/IHEM biomedical fungi and yeasts collection with accession numbers ranging from IHEM25889 to IHEM26007.

Susceptibility testing

Susceptibility to fluconazole was determined according to the minimum inhibitory concentration (MIC) measured via Sensititre™ YeastOne™ (Trek Diagnostic Systems, Ltd., UK) assay as recommended by the manufacturer. *C. glabrata* strains were then classified either fluconazole resistant (MIC ≥64 mg/l) or fluconazole susceptible (MIC ≤8 mg/L) [19].

MALDI-TOF MS typing assay

Proteins were extracted via the conventional extraction method recommended by Bruker Daltonik GmbH (Bremen, Germany), using formic acid and acetonitrile as previously described [18]. The supernatant of an extracted colony was deposited in quadruplicate on the MALDI plate, and MALDI-TOF MS analysis was performed using the Microflex LT™ system (Bruker Daltonik) with the default settings recommended by the manufacturer.

Microsatellite Length Polymorphism typing

DNA was extracted from a colony suspension in 800 μl of lysis buffer (bioMérieux, Craponne, France) using a NucliSENS™ easyMAG™ V2 (bioMérieux) for each isolate. Nine microsatellite loci were analyzed as described by Brisse *et al.* [9].

Population genetics analysis

The MLP typing results were analyzed using Arlequin ver. 3.5 software [20]. In particular, the genetic differentiation of *C. glabrata* strains according to either geographical origin (Marseilles, Tunis or the IHEM strain collection) or fluconazole *in vitro* susceptibility was tested. For this analysis, we assessed the fixation index (F_{ST}), which measures population differentiation due to genetic structure, the corrected average pairwise differences and the exact test of population differentiation based on genotype frequencies, which tests the nonrandom distribution of genotypes within population samples under the panmixia hypothesis [20].

MALDI-TOF phylogenetic tree

The BioNumerics ver. 7.0 software (Applied Maths NV, Sint-Martens-Latem, Belgium) was used to generate an UPGMA dendrogram based on the Pearson correlation coefficient using both microsatellite and MALDI-TOF summary spectra data.

MLP phylogenetic tree

The sequencer trace files of the different microsatellites were imported into BioNumerics version 7.0 (Applied Maths

NV). After normalization and band detection, a band matching analysis was performed with each band class corresponding to a possible repeat number. The tolerance for matching was set to ±1 bp to the band class position.

Statistical analysis

The strength of agreement between MLP and MALDI-TOF classifications was estimated via Cohen's kappa coefficient. The null hypothesis of random distribution of fluconazole resistance phenotype across MLP or MALDI-TOF clusters was tested using the likelihood ratio test. This analysis was performed with SAS version 9.2 (SAS Institute, Cary, NC, USA) using two-sided tests with alpha <0.05.

Results

Candida glabrata geographical differentiation

The first approach involved analyzing the 58 isolates according to their geographical origin using both MLP and MALDI-TOF assays.

MLP geographical differentiation

The population genetic analysis based on the MLP assay showed a highly significant genetic differentiation (F_{ST} = 0.35865, $p < 10^{-5}$) between strains from France and those from Tunisia and the collection strains (Table 3, part A). The differentiation between the isolates from Tunisia and the collection strains (F_{ST} = 0.20916, $p < 10^{-5}$) was of lesser magnitude (Table 3, part A). This differentiation is illustrated in the similarity dendrogram (Fig. 1), in which strains of French and Tunisian origin were grouped into separate clusters. The IHEM reference strains were dispersed throughout the dendrogram.

MALDI-TOF geographical differentiation

The MALDI-TOF summary spectra similarity dendrogram (Fig. 1) also separated the strains according to their French or Tunisian origin. The IHEM reference strains were grouped into two distinct clusters. The MALDI-TOF clusters were congruent with the MLP clusters. With the exception a few discrepancies, most of the isolates that were grouped within one MLP cluster were also grouped within the same MALDI-TOF cluster (Fig. 1). The kappa coefficient between MLP and MALDI classifications was 0.3795 (95% CI [0.2329 to 0.5261]), which indicates slight agreement between MLP and MALDI classifications. The null hypothesis of no agreement between the classifications can be rejected ($p < .0001$), which is further supported by the range of the 95% confidence interval, which suggests that the true kappa is greater than zero.

Candida glabrata fluconazole susceptibility differentiation

The strain characteristics and MIC results of the 19 fluconazole-resistant and 13 fluconazole-sensitive isolates are detailed in Table 2.

MLP fluconazole susceptibility differentiation

The MLP-based population genetic analysis showed a significant genetic differentiation (F_{ST} = 0.22091, $p < .009$) between *C. glabrata* strains that were either sensitive or resistant to fluconazole (Table 3, part B). This differentiation is highlighted in the MLP similarity dendrogram (Fig. 2, panel A), in which fluconazole sensitive and resistant strains clearly clustered separately. There was a statistically significant ($p = .0044$) association between MLP clusters and fluconazole resistance.

Table 3. Population genetic analysis between *Candida glabrata* strains from different origin and between the resistant and sensible strains to fluconazole showing the genetic differentiation according to the country of origin (A) and fluconazole susceptibility or resistance phenotype (B).

		Population pairwise F_{ST}*		Corrected average pairwise difference		Exact Test of Sample differentiation (*P* value)	
		F_{ST}	*P* value	PXY**	*P* value	Between all pairs	Global test of differentiation
A	Tunisia vs. France	0.35865	10^{-5}	1.77455	10^{-5}	$<10^{-5}$	$<10^{-5}$
	Tunisia vs. IHEM collection	0.06199	0.2959	0.13973	0.31965	0.01538	
	France vs. IHEM collection	0.20916	10^{-5}	1.05340	10^{-5}	$<10^{-5}$	
B	Resistant vs. sensitive	0.22091	0.00901	1.05839	0.00909	$<10^{-5}$	$<10^{-5}$

*F_{ST}: Fixation index. It is a measure of population differentiation due to genetic structure.
**PXY: The average number of pairwise differences among individuals in the sampled populations.

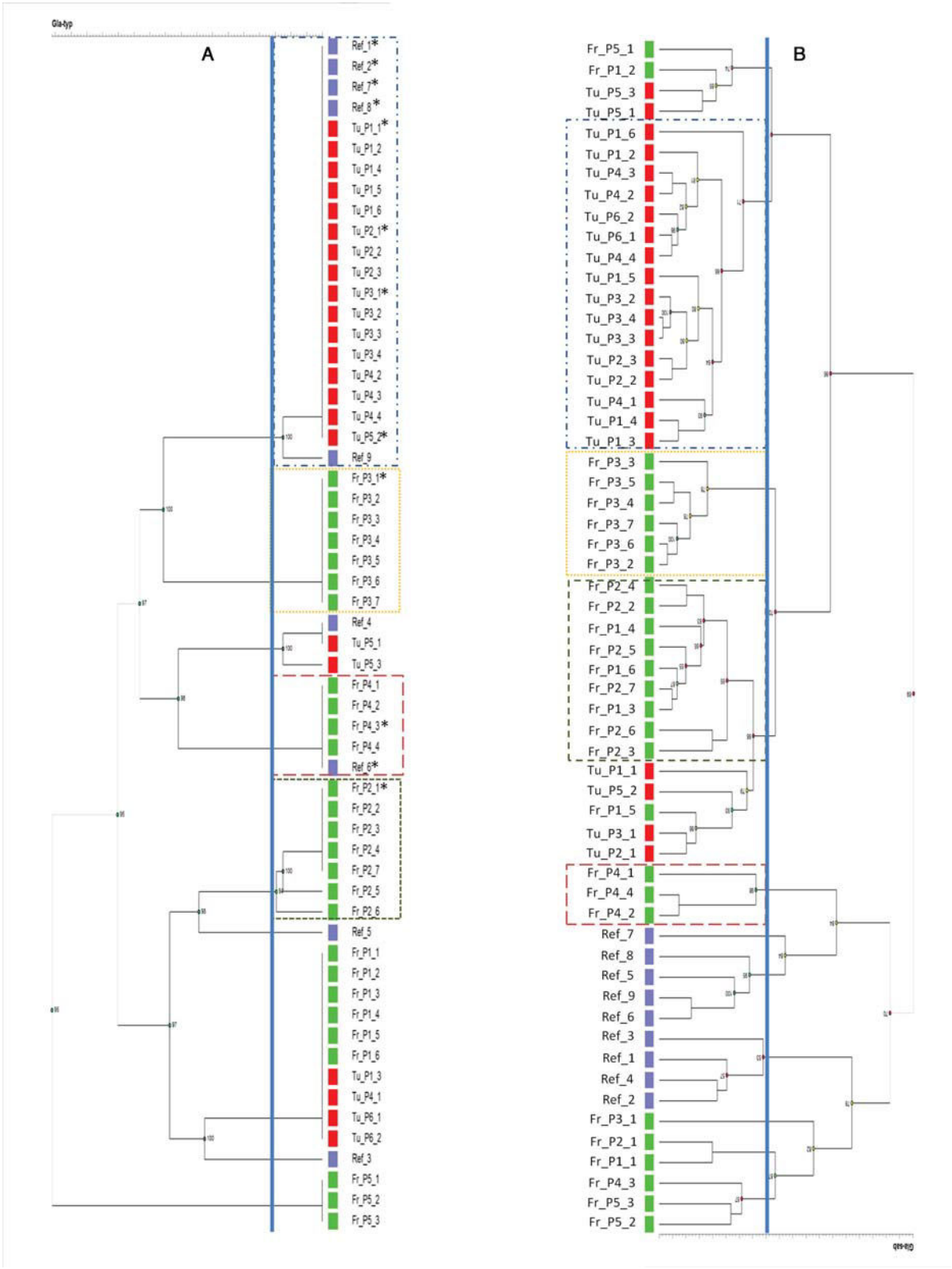

Figure 1. Comparison between microsatellite length polymorphism (MLP) (panel A) and MALDI-TOF (panel B) clusters. In panel A, the main MLP clusters are indicated with various dashed-line squares. The isolates corresponding to the MLP clusters are also indicated in the MALDI-TOF dendrogram with similar style dashed-line squares. The isolates within a MLP cluster that were not present in the corresponding MALDI-TOF cluster are marked with an asterisk. The color of the squares varies according to the origin of the strains. Ref, is for IHEM collection strain; Tu, is for Tunisia; Fr, is for France. The number preceded by the letter "P" identify the patients, either from Tunisia or France. This Figure is reproduced in color in the online version of *Medical Mycology*.

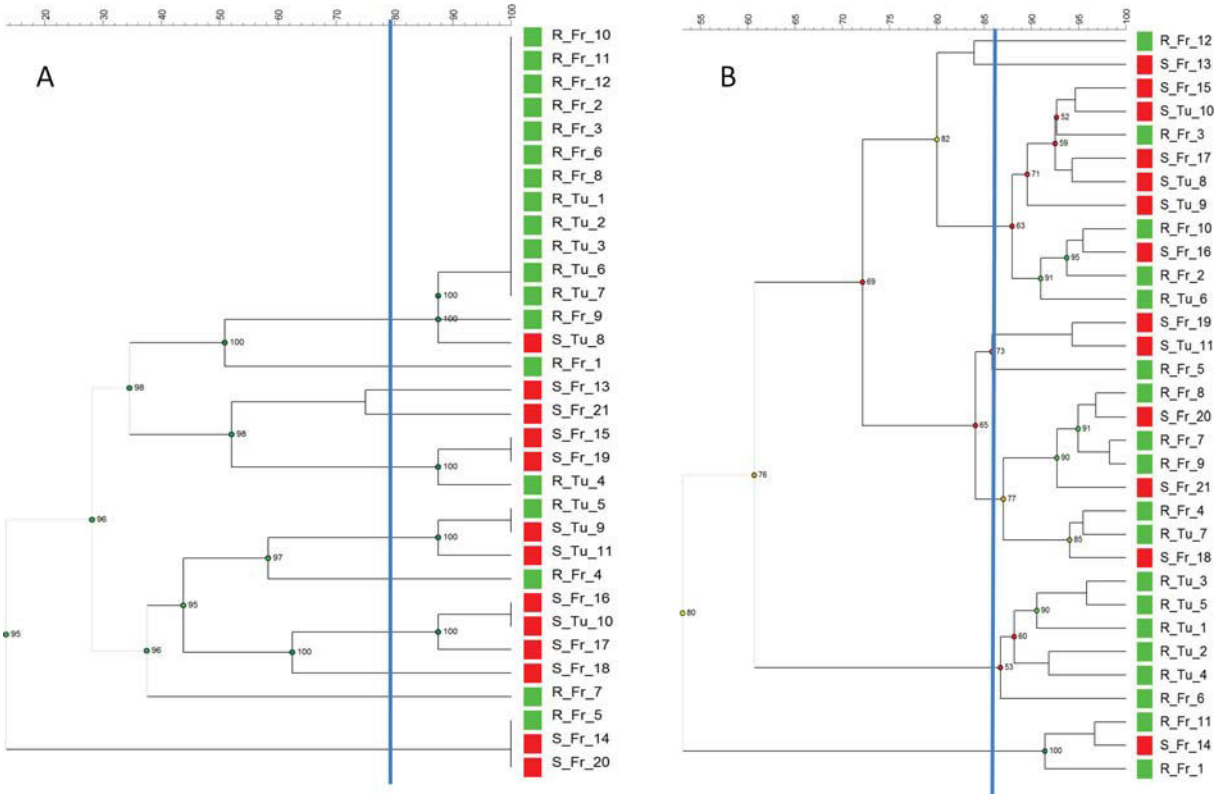

Figure 2. Distribution of fluconazole-resistant (R, light color square) or -sensitive (S, dark color square) *Candida glabrata* strains in microsatellite length polymorphism (panel A) and MALDI-TOF (panel B) dendrograms. Tu is for strains originating from Tunisia; Fr is for strains originating from France. This Figure is reproduced in color in the online version of *Medical Mycology*.

MALDI-TOF fluconazole susceptibility differentiation

The MALDI-TOF summary spectra similarity dendrogram (Fig. 2, panel B) also distinguished the fluconazole sensitive strains from the resistant strains. Of note, this MALDI-TOF classification was independent from both the MLP genotype and geographical origin of the isolates (Fig. 2). There was a statistically significant ($p = .0217$) association between MALDI-TOF clusters and fluconazole resistance.

Discussion

The seminal results described here illustrate that both MALDI-TOF MS and MLP typing were able to highlight *C. glabrata* population structures that were associated with either geographical dispersal barriers or antifungal drug resistance traits. Our observation of a geographical population structure of *C. glabrata* correlates with those of Brisse *et al.* [9] who highlighted, using the same MLP typing scheme, a significant population structure according to the continent of strain origin. Enache-Angoulvant *et al.* [12] have found a significant differentiation between strains involved in blood stream infections compared with those involved in gastrointestinal tract colonization. However, whether strains clustered according to their collection sample was not analyzed in the present study, as this was not an objective.

Several studies have evaluated the capacity of MALDI-TOF MS to rapidly discriminate between isolates within bacterial species. Nagy *et al.* [21] have used MALDI-TOF MS for the rapid identification and discrimination of *Propionibacterium acnes* phylotypes. Kuhns *et al.* [22] have demonstrated the capacity of MALDI-TOF MS to distinguish between typhoid and nontyphoid serotypes of *Salmonella enterica* subspecies *enterica*, while Stephan *et al.* [23] have applied this method for *Yersinia enterocolitica* subtyping. Regarding fungi, Pulcrano *et al.* have found that MALDI-TOF, similar to MLP typing, was useful to investigate the nosocomial transmission of a limited sample set of *C. parapsilosis* strains [16], which is in line with our results.

Interestingly, our MLP findings highlight, for the first time, the genetic differentiation of *C. glabrata* strains with respect to their *in vitro* fluconazole resistance phenotype. This population structure was independent of the geographical origin of the strains, thereby suggesting a selection pressure for resistance in *C. glabrata*, which might be due to the rise in fluconazole prescriptions over the last decades. Although this could not be tested in the present study, it is likely that this epidemiological shift might explain the

contrast with previous studies that found no significant association between antifungal resistance and *C. glabrata* genotypes [24–26]. This discrepancy may also be associated with the presence of confounding factors [24], the small number of isolates analyzed [25] and/or the insufficient discriminative power of the MLP typing scheme applied [26]. However, the present seminal findings indicating that although MALDI-TOF MS and MLP typing result in dissimilar classifications, MALDI-TOF mass spectra were associated with *in vitro* antifungal susceptibility in *C. glabrata* isolates, which is in line with several studies conducted on the bacterium *Staphyloccocus aureus*. Wolters *et al.* [27] have used MALDI-TOF MS to rapidly identify methicillin-resistant *S. aureus* (MRSA) isolates from various clinical specimens and showed its potential to rapidly type MRSA in infection control. Recently, Wang *et al.* [28] have identified specific biomarkers that differentiated MRSA from MSSA strains. Furthermore, Wybo *et al.* [29] have highlighted approximately 10 distinct MALDI-TOF peaks that could separate meropenem-resistant or -sensitive *Bacteroides fragilis* isolates.

However, sharing the same genotype is probably not the main factor to explain MALDI-TOF mass spectra similarity among *C. glabrata* isolates. Indeed, we observed that many fluconazole-resistant isolates with identical genotypes were grouped into separate MALDI-TOF clusters. In a recent study, we have also observed that MALDI-TOF failed to identify *C. albicans* clades [Dhieb *et al.*, Journal of Mass Spectrometry, *in press*: DOI 10.1002/jms.3538]. This supports that MALDI-TOF is more likely to detect phenotypic differences between isolates of the same species than intrinsic genetic variations which are better detected by nucleic acid-based methods. In addition, MALDI TOF spectra may be more dependent on a variety of technical issues, which is not the case for MLP. Further studies are warranted to gain more insight into the underlying reasons behind the similarity between MALDI-TOF spectra of strains according to their fluconazole susceptibility phenotype.

In conclusion, MALDI-TOF geographical clustering was congruent with MPL genotyping in a set of *C. glabrata* isolates. Moreover, MALDI-TOF classification, as observed with MLP genotyping, highlighted a significant population genetic structure according to fluconazole *in vitro* susceptibility. However, the reason behind the similarities between MALDI-TOF spectra remain unknown, and further studies are required to evaluate the application of MALDI-TOF typing to investigate outbreaks of hospital-acquired *C. glabrata* infection and predict the antifungal susceptibility profile of isolates in the clinical laboratory.

Acknowledgments

We thank the laboratory staffs at La Rabta and La Timone hospitals for skilled technical assistance.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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Annex 2: Distribution of Keratinophilic Fungi in Soil across Tunisia:

A Descriptive Study and Review of the Literature

Keratinophilic fungi decompose keratin, and some of which consider as potential infection for human and animals by invading keratinized tissues. There is no data on the distribution of this group of filamentous fungi in Tunisia. The aim of this study is to describe the frequency and distribution of keratinophilic fungi across Tunisia. A total of 354 soil samples were collected from various sites of 15 governorates of Tunisia. Vanbreuseghem's hair-baiting technique was used to isolate the keratinophilic fungi species, which identified according to the morphology characters with further nucleic acid and MALDI-TOF analysis when necessary. A total 164 of 354 soil samples were positive to keratinophilic fungi species, which belonged to 14 genera and 27 species. The predominant species was *Chrysosporium keratinophilum* followed by *Microsporum gypseum*. We reported that the soil samples of stables were 100% positive followed by garbage-cans (87.5 %), and the lowest positive rates were recorded in soil samples of beaches (5.6 %) and in parking (20 %). Keratinophilic fungi were more frequent in soils collected in rural (54.8 %) than in urban (41.1 %) areas. the highest positivity rate of keratinophilic fungi was recorded in clay soils (56.0 %) followed by sandy soils (39.9 %).

Mycopathologia
DOI 10.1007/s11046-015-9870-9

Distribution of Keratinophilic Fungi in Soil Across Tunisia: A Descriptive Study and Review of the Literature

Sonia Anane · Mohammed Hashim Yasir Al-Yasiri · Anne-Cécile Normand · Stéphane Ranque

Received: 17 July 2014 / Accepted: 3 February 2015

Abstract Data on the frequency and distribution of keratinophilic fungi in soil of Tunisia are scanty. The present survey aimed to describe the distribution of keratinophilic fungi in soils collected in Tunisia. Keratinophilic fungi were isolated using Vanbreuseghem's hair-baiting technique from 354 soil samples collected in 15 governorates of Tunisia and identified according to their morphology with further DNA and MALDI-TOF analysis when necessary. Keratinophilic fungi were isolated from 46.3 % of the samples from 14 governorates. *Chrysosporium keratinophilum* was the predominant species (30.5 %) followed by *Microsporum gypseum* (27.4 %). Other isolated species included *C. tropicum* (14.0 %), *C. indicum* (11.0 %), *Chaetomium* sp. (4.9 %), *Arthroderma curreyi, Arthroderma cuniculi* (3.7 % each), *C. merdarium* (3.1 %), *Anixiopsis stercoraria*, *C. parvum, Paecilomyces lilacinus*, *Auxarthron zuffianum* (2.4 % each), *Fusarium oxysporum*, *Aphanoascus verrucosus*, *Gymnascella dankaliensis* (1.2 % each) and 12 other species (0.6 % each). Two to five distinct fungal species were associated with 11.5 % of the positive samples. Keratinophilic fungi were more frequently isolated in rural (54.8 %) than in urban (41.1 %) areas ($p = 0.012$). The highest (100 %) positive culture rate was noted in soil collected in stables. Keratinophilic fungi are frequent throughout Tunisian territory, particularly in soils with a high organic matter content that should be regarded as humans and animals mycoses reservoir.

Keywords Keratinophilic fungi · Soil · Epidemiology · MALDI-TOF · DNA sequencing

S. Anane (✉)
Laboratory of Parasitology-Mycology, Faculty of Medicine, University of Tunisia El Manar, 15, Jabbari-Jebel Lakhdar Street, 1007 Tunis, Tunisia
e-mail: anane.sonia@planet.tn

M. H. Y. Al-Yasiri · S. Ranque
IP-TPT UMR MD3, Aix-Marseille Université, 13885 Marseille, France

A.-C. Normand · S. Ranque
Laboratory of Parasitology - Mycology, APHM CHU Timone, 13005 Marseille, France

Published online: 18 February 2015

Introduction

Keratinophilic fungi are an important group of filamentous fungi that decompose keratin; some of which cause diseases in human and animals by invading keratinized tissues. They include a variety of species mainly comprising Onygenales hyphomycetes, including dermatophytes and several other taxa. Dermatophytes are causative agents of human superficial fungal infections named dermatophytosis. Many of the non-dermatophytic hyphomycetes are closely related to dermatophytes with which they share the capacity to cause human and animal mycoses [1, 2]. The distribution of these keratinophilic fungi in soil has been studied in many

areas around the world [3–10]. Yet, data on the frequency and distribution of keratinophilic fungi in soil of Tunisia are scanty and limited to only two reports, in 1968 and 2011, in only one region (Djerba) [11, 12]. Thus, this study aimed at investigating the occurrence and distribution of keratinophilic fungi in soils collected throughout Tunisia and reviewing published studies in north of Africa.

Materials and Methods

In this prospective study, 354 soil samples were collected from various sites of 15 governorates of Tunisia. Before sampling, superficial vegetal matters and other debris were removed from the soil surface. The soil samples were collected with a sterile stainless steel spoon. Approximately 500 g of soil were taken from the surface layer to the depth of 3–5 cm and placed in sterile plastic bags. The samples were processed in the laboratory within a day or stored at 15 °C until processed within 2 days. The hair-baiting technique of Vanbreuseghem was used to isolate the keratinophilic fungi from the soil [13]. Briefly, a sterile Petri dish was half-filled with the soil sample, which was moistened with sterile distilled water, and then several pieces of sterile human hair were dispersed over their surface. The hair-baited soil Petri dishes were incubated at 25 °C for 2 months and re-moisturized when necessary. When fungal growth occurred on the hairs, some of them were stained with lactophenol cotton blue and examined microscopically and the others were inoculated onto Sabouraud's glucose agar with chloramphenicol and cycloheximide. The isolated colonies were identified mainly by morphologic feature based on the macro- and micromorphological characteristics [2, 4]. DNA sequence analysis and MALDI-TOF mass spectrometry were used, as described in [14–16] for some isolates for which the morphological identification was uncertain. Nucleotide sequences were deposited in GenBank under accession numbers: KP147957-KP147988 for the ITS rRNA region; KP172157-KP172183 for the D1-D2 variable region of the 28S rRNA; and KP202358 for the partial Calmodulin gene. The statistical analysis relied on the Chi-square test and Fisher exact test; a $p < 0.05$ was considered statistically significant.

We searched in the PubMed (NCBI) database, using the search terms: "Arthrodermataceae" [Mesh] AND "Soil Microbiology" [Mesh] AND "Africa, Northern" [Mesh]. We also reviewed the selected articles' reference lists to identify studies that had been missed by our search. No language restrictions and no publication date were used. Studies carried on the treatment of dermatophytosis in Africa were excluded.

Results

Keratinophilic fungi were isolated from a total of 164 (46.3 %) soil samples. The fungal isolates belonged to 14 genera and 27 species, as detailed in Table 1. *Chrysosporium keratinophilum* was the predominant species followed by *Microsporum gypseum*. An association of two to five fungi was noted in 11.5 % of the positive samples. The most frequent fungal association was *C. keratinophilum* and *M. gypseum* (3.7 %). *C. keratinophilum* was detected in the majority (87.5 %) of the sites. *M. gypseum* was most frequently (88.2 %) isolated from soils with high organic matter content. Among all biotopes, soils with a high organic matter content (including animal habitats, garbage-cans, gardens and agricultural fields) were particularly rich in keratinophilic fungi. The highest rate of positive cultures was reported in stables (100 %) followed by garbage-cans (87.5 %), whereas the lowest positive rates were recorded in beaches (5.6 %), then in parking (20 %), as detailed in Table 1.

In this study, keratinophilic fungi were statistically significantly ($p = 0.016$) more frequently isolated from soils collected in rural (54.8 %) than in urban (41.1 %) areas. We noted a higher positivity rate of Keratinophilic fungi from clay (56.0 %) than from sandy soil (39.9 %) with a statistically significant difference ($p = 0.003$). Nabeul was the governorate, which had the highest rate of positive culture (78.6 %) followed by Bizerte (68.8 %). The lowest rate (0.0 %) of positive culture was noted in Gabes.

The literature review using the aforementioned search strategy yielded four references, and reviewing of related publication in the reference list yielded two additional references.

Discussion

Soil keratinophilic fungi are ecologically important; some of which constitute a potential infection source

Table 1 Distribution of keratinophilic fungi in different types of soil collected across Tunisia

Source of soil samples	Roadsides	Gardens	Fields	Beaches	Park + play ground	Schools + nursery schools	Bus stops	Industrial areas	Garbage-cans	Parking	Sahara	Stables	Other animal habitats	Total	Frequency of fungi (%)
Number of samples	49	41	28	36	47	7	43	15	8	15	5	5	55	354	
Number of positive samples (%)	23 (46.9)	28 (68.3)	17 (60.7)	2 (5.6)	21 (44.7)	3 (42,9)	16 (37.2)	4 (26.7)	7 (87.5)	3 (20)	2 (40)	5 (100)	33 (60)	164	(46.3)
Microsporum gypseum	3	7	6	0	7	1	5	0	1	2	0	3	10	45	(27.4)
Chrysosporium keratinophilum	9	6	5	1	4	1	6	1	3	2	0	1	11	50	(30.5)
Chrysosporium tropicum	3	10	2	0	1	0	1	2	0	0	2	0	2	23	(14)
Chrysosporium indicum	2	2	3	1	4	1	0	0	1	0	0	0	4	18	(11)
Chrysosporium anamorph of *Arthroderma cuniculi*	2	1	0	0	1	0	0	1	0	0	0	0	1	6	(3.7)
Chrysosporium anamorph of *Arthroderma curreyi*	0	1	0	0	3	0	2	0	0	0	0	0	0	6	(3.7)
Chrysosporium merdarium	0	1	1	0	0	0	0	0	1	1	0	0	1	5	(3.1)
Chrysosporium anamorph of *Anixiopsis stercoraria*	1	0	0	0	1	0	0	0	0	0	0	0	2	4	(2.4)
Chrysosporium parvum	2	0	0	0	0	0	1	0	1	0	0	0	0	4	(2.4)
Chrysosporium pannorum	1	0	0	0	0	0	0	0	0	0	0	0	0	1	(0.6)
Chaetomium sp.	2	2	0	0	1	0	1	0	0	0	0	0	2	8	(4.9)
Chaetomium jodhpurense	0	1	0	0	0	0	0	0	0	0	0	0	0	1	(0.6)
Paecilomyces lilacinus	0	1	0	0	1	0	1	0	0	0	0	0	1	4	(2.4)
Fusarium oxysporum	0	0	0	0	0	0	1	0	0	0	0	1	0	2	(1.2)

Table 1 continued

Source of soil samples	Roadsides	Gardens	Fields	Beaches	Park + play ground	Schools + nursery schools	Bus stops	Industrial areas	Garbage-cans	Parking	Sahara	Stables	Other animal habitats	Total	Frequency of fungi (%)
Fusarium solani	0	1	0	0	0	0	0	0	0	0	0	0	0	1	(0.6)
Geomyces asperulatus	0	0	0	0	1	0	0	0	0	0	0	0	0	1	(0.6)
Aspergillus flavus	1	0	0	0	0	0	0	0	0	0	0	0	0	1	(0.6)
Auxarthron zuffianum	1	1	1	0	1	0	0	0	0	0	0	0	0	4	(2.4)
Emericella violacea	0	1	0	0	0	0	0	0	0	0	0	0	0	1	(0.6)
Rasamsonia argillacea	0	1	0	0	0	0	0	0	0	0	0	0	0	1	(0.6)
Aphanoascus terreus	0	1	0	0	0	0	0	0	0	0	0	0	0	1	(0.6)
Aphanoascus															
verrucosus	0	2	0	0	0	0	0	0	0	0	0	0	0	2	(1.2)
Gymnascella aurantiaca	0	0	0	0	1	0	0	0	0	0	0	0	0	1	(0.6)
Gymnascella dankaliensis	0	0	1	0	1	0	0	0	0	0	0	0	0	2	(1.2)
Arachniotus flavoluteus	0	0	1	0	0	0	0	0	0	0	0	0	0	1	(0.6)
Arachniotus.															
littoralis	0	0	1	0	0	0	0	0	0	0	0	0	0	1	(0.6)
Metarhizium anisopliae	0	0	1	0	0	0	0	0	0	0	0	0	0	1	(0.6)

for humans and animals [1–8]. In this study, 41.8 % of the soil samples were contaminated with keratinophilic fungi. This frequency is similar to those reported in Italy, Bahrein and Egypt [6, 9, 17, 18], but clearly lower than in India, Iran and Australia, where the contamination rate reached 90 % [5, 8, 10, 19, 20]. In Djerba (an island south of Tunisia), a lowest (20.6 %) isolation rate was found [12]. This may be explained by Djerba's arid climate and the nature of its scarcely vegetated surface soils.

Only six studies focusing on North African soil keratinophilic fungi, which had been carried out in Tunisia and Egypt, could be identified through the literature review [11, 12, 17, 18, 21, 22]. Their main findings are summarized in Table 2. The comparison between our study and previous ones is hindered by the marked heterogeneity in the previous reports of the type of soil sample analyzed (Table 2). The more or less selectivity of the culture media used influenced each study's results (Table 2). Furthermore, a significant number of the fungi were identified via MALDI-TOF mass spectrometry and/or DNA sequencing in this study, which contrasts with the morphological identification only used in the previous studies (Table 2).

In keeping with previous studies, we found that *C. keratinophilum* was the predominant species [12, 18, 23, 24]. *C. keratinophilum* is a common soil fungus with a worldwide distribution [4, 5, 12, 18, 23–26]. It has been isolated occasionally from human onychomycosis [27]. Other *Chrysosporium* species are isolated from soils in our study including *C. tropicum* and *C. indicum.* These fungi were isolated with a diverse frequency from soils collected in many parts of the world [5, 6, 28, 29]. *C. indicum* which is the fourth fungal species in our study represents the most abundant keratinophilic fungus in soil surveys conducted in Australia with a 50 % positivity rate and in India with a positivity rate ranging from 12 to 25 %, possibly because it has adapted to the climatic conditions of these areas [5, 19, 20, 29, 30]. This species was not isolated in Djerba where, as mentioned above, the climate is completely different [12]. Zaki et al. [17] reported a predominance of *Chrysosporium zonatum* in Egyptian soils, a species that we did not find in our study. This discrepancy might be explained by the type of chosen soils (muddy soils) in Zaki's study.

Microsporum gypseum was the second more frequent keratinophilic fungi species isolated in our

Table 2 Studies on keratinophilic fungi in North Africa

Reference	Country and year	Origin of the soils	Number of soil samples	Used medium	Positivity rate	Number of genera	Predominant specie or genus
Galgoczy et al. [11]	Tunisia (1968)	Different sites	21	Sabouraud + chloramphenicol + cycloheximide	57.14 %	3	*Chrysosporium* sp.
Anane et al. [12]	Tunisia (2012)	Different sites	141	Sabouraud + chloramphenicol + cycloheximide	20.6 %	3	*Chrysosporium keratinophilum*
Zaki et al. [17]	Egypt (2005)	Muddy soils	100	Sabouraud + chloramphenicol +Streptomycin + penicillin	40 %	3	*Chrysosporium zonatum*
Bagy et al. [18]	Egypt (1992)	Desert and cultivated soils	40	Sabouraud's dextrose agar +Streptomycin + penicillin + cycloheximide	45 %	2	*Chrysosporium keratinophilum*
Abdel-Mallek et al. [21]	Egypt (1989)	NA	46	Agar + Streptomycin + penicillin	NA	18	*Aspergillus fumigatus*
Al-Doory [22]	Egypt (1967)	Tombs and public gardens	36	NA	55.55 %	2	*Trichophyton mentagrophytes*

NA not available

study. This geophilic dermatophyte was also reported as the first or second most common dermatophyte in soils in other studies [3, 5, 9, 19, 20, 28, 29, 31]. This high frequency of *M. gypseum* is noteworthy because this species causes dermatophytoses in both humans and animals. Like in other studies, this species was frequently present in soils with high organic matter content [1, 3]. *Trichophyton mentagrophytes* was dominant among the Egyptian soils [22]. The predominance of this zoophilic dermatophyte is due to the number and variety of animals inhabiting areas where soil samples were collected.

Among other fungal species, *Chaetomium* sp. was isolated from Tunisian soils as it has been reported in previous studies [24, 32]. This genus has been rarely involved in invasive mycosis or onychomycosis in humans; most of these cases have been reported within the last two decades [33]. *Fusarium solani* and *Paecilomyces lilacinus* have been occasionally recovered as keratinophilic fungus from soil, like the few isolates in the present study [8, 10, 24]. They can cause mainly keratitis and onychomycosis. They can be also the causal agents of invasive and cutaneous mycosis in immunocompromised subjects [34–36].

When more than two fungal species were grown in a soil sample, they were identified by MALDI-TOF MS or DNA sequence analysis, as described in [14–16]. Their identification was usually impossible according to their morphological features only. Numerous studies have shown that MALDI-TOF MS-based identification allows a highly discriminatory identification of yeasts and filamentous fungi starting from colonies [14, 15]. This approach allowed identifying species that were isolated for the first time from soils like *Arachniotus flavoluteus*, *Emericella violacea*, *Gymnascella aurantiaca* and *Rasamsonia argillacea*. Infections by *R. argillacea* previously known as *Geosmithia argillacea* have been recurrently reported in literature since 2010 [37, 38]. They are mainly involved in pneumonia and/or invasive infections in patients with chronic granulomatous disease. Given its thermotolerance and its resistance to various antifungals, especially to azole drugs, a special attention should be paid to the chronic colonization of the airways by this fungus in these patients [39].

MALDI-TOF MS also allows identification of some species that were rarely isolated in soils as *Aphanoascus terreus*, *Metarhizium anisopliae* and *Gymnascella dankaliensis* [40, 41]. *M. anisopliae* is an entomopathogenic fungus, which has rarely been involved in human keratitis and cutaneous infection [42]. *G. dankaliensis* was isolated in Italy, and it is incriminated in the genesis of keratitis and onychomycosis [43].

The distribution of keratinophilic fungal species in soil seems to largely depend on the amount of keratinized material deposited there by humans or animals [3, 6, 9, 19]. Our study shows that the highest rates of positive culture are noted in organic-rich soils, such as garbage-cans, animal habitat, garden and field. The spores of these fungi can survive in the soil for months or years and gain entrance into animal or human body through abrasion, implantation or inhalation and eventually produce disease. Thus, these areas could be considered as a source of human and animal fungal infections [3, 6, 9, 20]. However, in keeping with previous studies, our findings showed that beaches are not favorable to the development of fungi [25, 28]. Only one beach was positive for keratinophilic fungi. It was a beach located near the center of the city, which is highly frequented throughout the year.

Low positive culture rates were also noted along roadsides and in industrial areas. The high concentration of various pollutants in these areas may have inhibited the survival of these microorganisms [19, 25, 28]. This hypothesis may also explain the lower positive culture rate that we observed in urban areas compared with rural areas and the absence of keratinophilic fungi in the soil collected in the governorate of Gabes, which is an highly industrialized area.

More simply, the presence of agricultural activity and especially animals in rural areas may explain the high rate of positivity of keratinophilic fungi. Indeed, our study shows that the animal habitats are rich in fungi, especially horses and hens, which can release large amounts of keratinized matter in their environment. In contrast, Kachuei et al. [10] found a higher positivity rate in urban areas than in rural areas. This conflicting result might be explained by ecological heterogeneities between environments in Tunisia or Iran.

The governorates of Nabeul and Bizerte had the highest positive culture rate. They are both characterized by a high humidity rate, with important rainfall and an agricultural activity with livestock production, which are all known to favor fungal growth.

Furthermore, in these governorates, the soil is clay. Indeed, we found a particularly high frequency of keratinophilic fungi in clay soil, maybe because clay has the capacity to retain moisture and minerals, which both favor the growth of fungi.

In conclusion, the present study highlights the dispersal of keratinophilic fungi, predominantly two potential human pathogens *C. keratinophilum* and *M. gypseum*, throughout Tunisian territory and the critical influence of ecological factors, particularly humidity and organic matter content, on their distribution in soil.

Conflict of interest None.

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Annex 3: Antifungal activity of *Streptomyces* sp. against environmental and clinical *Cryptococcus* spp. isolates

The objectives of this study were to analyze the antifungal susceptibility of *Cryptococcus* spp that were isolated from environmental sources, and also to analyze the antifungal activity of *Actinomycetes* isolated from Mellah lack water against the *Cryptococcus* spp. Both, *C. albidus* and *C. diffluens* were isolated for the first time from pigeon droppings and atmospheric air sampled in Eastern Algeria. Those isolates showed relatively high antifungal resistance against most fungal drugs tested here compare with three clinical *Cryptococcus* isolates. Therefore, we attempted to use a new therapeutic compounds extracted from *Actinomycetes* (E96) isolated here. The secondary metabolites compounds produced by E96 isolate showed an important antifungal activity against both environmental and clinical *Cryptococcus* isolates, which were resistant to the fungal drugs especially, 5-fluorocytsine, azole and echinocandins.

Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(10): 1019-1027

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Antifungal activity of *Streptomyces* sp. against environmental and clinical *Cryptococcus* spp. isolates

M. Ouargli[1], D. Gacemi-Kirane[1], R. Mansouri[3], M. Al Yasiri[4,5], S. Ranque[4,5] and V. Roux[2*]

[1]*Laboratory of Genetic Improvement of Plants and Adaptation.Water, Soil, and Microorganisms Research team. Department of Biology, Badji Mokhtar University, Annaba, Algeria*
[2]*Research Unit on Emerging Infectious and Tropical Diseases, UMR CNRS 6236-IRD 198, Aix-Marseille University, France*
[3]*School of Medicine, University Badji Mokhtar Annaba, Algeria*
[4]*Parasitology & Mycology, APHM, CHU Timone, Marseille, France*
[5]*Aix-Marseille University, IP-TPT, UMRMD3, Marseille, France*

ABSTRACT

This study aimed to analyze the antifungal susceptibility profile of environmental Cryptococcus spp. and to determine the antifungal activity of bioactive molecules extracted from an actinomycete, strain E96, against these Cryptococcus spp. Cryptococcus strains were investigated from pigeon droppings, atmospheric air and living materials collected on Eucalyptus trees. The isolates were identified and compared for their sensitivity to antifungal agents to clinical strains of Cryptococcus neoformans. Actinomycetes were isolated from Mellah Lake water and screened for antimicrobial activity. Antimicrobial assays were performed on ISP2 agar. The taxonomic position of the strain E96 was determined regarding phenotypic and 16S DNA sequences features. It was tested for its antifungal activity against the Cryptococcus spp. isolates using both double layers and agar disc diffusion methods. Time course of E96 antifungal metabolites production was determined on ISP1, ISP2, GYEA and Bennett culture media. The actinomycete E96 strain showed a very promising activity against the Cryptococcus spp. strains, especially interesting when they are resistant to 5-fluorocytsine, azole or echinocandins.

Keywords : *Cryptococcus albidus*. *Cryptococcus diffluens.* Environment. *Streptomyces.* Antimicrobial activity.

INTRODUCTION

The genus *Cryptococcus* includes100 basidiomycetous fungal species characterized as variously encapsulated budding yeasts. *C. neoformans* and *C. gattii* are the major human and animal pathogens. However, other species such as, *C. laurentii*, *C. uniguttualus, C. gastricus, C. albidus, C. loteolus, C. adeliensis, C. humicola, C. magnus, C. diffluens, C. curvatus*, and others have been occasionally involved inmoderate-to-severe disease [1]. Cryptococcosis can result in serious meningitis, encephalitis, or meningoencephalitis and is acquired by inhalation of infective yeasts present in wood, fruits, rotting vegetables, soil, dairy products, and urban pigeon (*Columba livia*) droppings [2]. The drug of choice in the treatment of cryptococcosis is amphotericin B (AMB), which may or may not be combined with other drugs, such as fluconazole (FCZ) or 5-fluorocytosine [3, 4]. But it is responsible for many side effects and recent studies indicate that some patients remained resistant to new antifungal drugs [5]. Actinomycetes have been recognized as the potential producers of metabolites such as antibiotics, growth promoting substances for plants and animals, immunomodifiers, enzyme inhibitors and many other compounds of use to man [6].

In this study, we reported for the first time the isolation of *C. albidus* and *C. diffluens* from pigeon droppings and atmospheric air sampled near bird guanoin in Eastern Algeria. The antifungal susceptibility profile of these isolates was compared to the one of *C. neoformans* clinical strains. The relatively high antifungal resistance of these strains prompted us to search for other antifungal agents. Therefore, we attempted to identify *Actinomycetales* strains

isolated from Lake El Mellah that presented antifungal activity against these clinical and environmental strains of *Cryptococcus* spp.

EXPERIMENTAL SECTION

Collection and identification of yeast strains

Three clinical strains were used in this study: CN104 (isolated from the blood of a 46 year-old male in 2003), CN721 (isolated from the blood of a 19 year-old male in2007), CN954 (isolated from the CSF of a 22 year-old male in 2003). They were provided by the Parasitology-Mycology laboratory of the Marseille Timone Hospital, France.

The environmental samples were collected from public hospitals (inside and outside the buildings), private clinic, places of public affluence, as well as downtown streets in Algeria. We collected a total of 117 samples, 91 obtained from pigeon droppings, 16 from atmospheric air sampled near bird droppings and 10 from living materials collected on Eucalyptus trees, including flowers, fruits, leaves, bark, debris and soil [7] in the Annaba area (Figure 1). Air was sampled using the SAS AIR SAMPLER Duo 360 (Bioscience International, Rockville, USA). The device was placed in the sampling sites and programmed to aspirate 180l of air per minute for 2 min. Pigeon droppings and living materials from the Eucalyptus trees were collected in sterile bags and transferred immediately to the laboratory.

Pigeon dropping samples and Eucalyptus samples were suspended in sterile saline solution (NaCl 0.9%) at a ratio of 1:5 by vortexing and allowed to sediment for 20min. Supernatant aliquots of 0.5 ml were inoculated on Sabouraud Dextrose Agar (SDA) medium supplemented with (0.1 g/l) chloramphenicol. The plates were incubated at 26°C for 8 days and were daily examined to observe the appearance of the colonies and to select those corresponding to *Cryptococcus* species [8]. They were subcultured on malt extract agar at 30°C and were examined under the microscope, after India ink staining, to analyze their morphology and to visualize the presence of a capsule [9]. The phenoloxydase activity was tested using Niger (*Guizotia abyssinica*) agar. The biochemical identification relied on urease testing and ID32C auxanogram profile [8]. After morphological and biochemical identification, yeasts were conserved using the Protect Microorganism Preservation System (Technical Service Consultants Ltd, Heywood, Lancashire, UK) and maintained at -80°C until further subculture.

Fungal DNA was extracted using the EZ1 DNA Tissue Kit with the EZ1 Advanced XL instrument. Colonies were incubated in 200 µl Buffer G2 and 10 µl proteinase K at 56°C until total lysis. DNA was then purified as described by the manufacturer and eluted in100 µl of buffer. Primers D1 (AAC TTA AGC ATA TCA ATA AGC GGA GGA) and D2 (GGT CCG TGT TTC AAG ACG G), and primers ITS3 (GCA TCG ATG AAG AAC GCA GC) and ITS4 (TCC TCC GCT TAT TGA TAT GC) were used to amplify the D1-D2 variable region of the 26S and the internal transcribed spacer 2 of the ribosomal RNA gene, respectively, as described in Cassagne *et al.* [10]. The obtained sequences were queried against nucleotide sequences deposited in GenBank using the program BLAST through the NCBI server and the International Society for Human and Animal Mycology (ISHAM)-ITS reference DNA barcoding database [11].

Figure 1: Sample location and *Cryptococcus* spp. isolated in (A) Annaba area and (B) Annaba city (Algeria)
☆ : Environmental samples; △ : *Cryptococcu salbidus* ; ○ : *Cryptococcus diffluens.*

Isolation, characterization and antifungal activity of the Actinomycete strain

Water samples of the Mellah Lake were collected in sterile bottles kept at 4°C and transferred immediately to the laboratory. They were then inoculated onto seven different media: ISP1, ISP2, ISP4, ISP5, Gauss, Cross and Casein starch agar. Plates were incubated for 7, 14 and 21 days at 28°C. Characteristic colonies were subcultured on ISP2 agar and kept at 4°C [12]. Among these strains, we were interested in the E96 strain that displayed an antifungal activity against *Cryptococcus* spp. A taxonomic study based on morphological, biochemical and physiological characters was performed. The morphological characteristics and the production of melanoid pigment of the organism were determined by visual examination of 14-day-old cultures grown using the International Streptomyces Project (ISP) media (ISP1, ISP2, ISP3, ISP4, ISP5, ISP6, and ISP7) recommended by Shirling and Gottlieb [13] and casein starch medium. Advocated by Wink [14], the biochemical properties were detected using the available commercially API ZYM, API 50CH and API 20NE according to the manufacturer's instructions. A total of 55 tests have been performed to study the physiological characteristics, including the utilization of 12 carbohydrate compounds evaluated on ISP9 basal medium with a final concentration of 1% [13], degradation of valine, asparagine, proline, arginine, serine, paraffin and chitin according to Goodfellow's methods [15], hydrolysis of casein, urea, indole and starch [16] and hydrolysis of gelatin [17]. The strain was also tested for its ability to grow in the presence of 18 antibiotics, using the paper disc method of Goodfellow and Orchard [18] and inhibitory compounds including (w/v): sodium azide 0.001%, and phenol 0.1% on YEA medium as described by Goodfellow [15]. Growth at 15°C, 29°C, 37°Cand 43°C, and at pH4, pH5, pH6, pH7, pH8, pH9and pH10 was also tested.

Genomic DNA of the E96 strain was extracted from colonies grown after 48h at 30°C in COS medium by the method described by Roux *et al*. [19]. PCR amplification of 16S rDNA of Actinomycete strain was carried out using the universal primers Fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rp2 (5'-ACGGCTACCTTGTTACGACTT-3') [20]. The 3130 Genetic Analyzer automated sequencer was used for nucleotide sequence determination. The eight resulting sequences were assembled into a unique contig with ChromasPro version 1.5. The resulting nucleotide sequences were compared using the program BLAST via the NCBI server, with those included in a bank we constituted. The sequences included in the bank were those of the type strain of each species.

The antifungal activity was detected using two different methods: the double layers method described by Zitouni *& al.* [21] and the agar disc diffusion method described by Saadoun and Muhana [22]. The E96 strain production kinetics of metabolites with antifungal activity was determined using various media (ISP2, ISP1, Bennett and GYEA (glucose yeast extract agar)) for 14 days [23].

The extraction of the bioactive compounds was performed according to the technique detailed by Badji *& al.* [24]. The E96 strain was inoculated in very tight streaks onto the surface of the medium in a 90 mm diameter Petri dish. After 1 day of incubation at 30°C, the agar of one Petri dish was cut into small cubes which were then transferred into an Erlenmeyer flask containing 40 ml of one of the following four solvents: n-hexane, ethyl acetate, n-butanol or dichloromethane. The extraction was carried out with stirring for 2 h at room temperature. The organic extract obtained was filtered and then desiccated under vacuum at 45°C using a rotary evaporator. The dry residue was then suspended in 5 ml of methanol.

The in vitro antifungal susceptibility was determined using the SENSITITRE^{TM}YEASTONE^{TM} YO10 containing a serial dilutions of eight antifungal agents: anidulafungin (AND), micafungin (MF), caspofungin (CAS), 5-Flucytosine (FC), posaconazole (PZ), voriconazole (VOR), itraconazole (IZ), fluconazole (FZ) and amphotericin B (AB). This broth microdilution assay provides quantitative results of Minimum Inhibitory Concentration (MIC) for each tested antifungal. Yeast cells collected from a 48 hours culture were suspended in sterile water, turbidity was adjusted to 0.5 McFarland, and the suspension was inoculated into each well of the plate. The plates were incubated at 30°C and visually read after 48 h. Colorimetric MIC result was read as the first blue (no growth) well that showed a distinct color change as compared to the positive growth (red or purple) well [25, 26]. Because no clinical MIC breakpoints have been determined for *Cryptcoccus* spp., the susceptibility of the isolates was not categorized. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used for quality control [27].

The antifungal activity of the bioactive compound was tested using 6 mm diameter paper discs. The discs are soaked with 50 µl and 100 µl of methanol extract and placed onto Sabouraud agar previously inoculated with the yeast strain. The diameter of the inhibition zones was measured for each strain after 48h of incubation at 30°C.

RESULTS AND DISCUSSION

Collection and identification of yeast strains

Identification of clinical yeast strains showed that all isolates belonged to the species *C. neoformans* with 99-100 % homology with the sequences deposited in the Genbank database and the ISHAM-ITS reference DNA barcoding database.

We collected a total of 117 environmental samples, 91 pigeon droppings, 16 air samples and 10 from living materials collected on plant material or soil [7] in the Annaba area. Species belonging to the *Cryptococcus* genus were isolated from 5.5% of the pigeon dropping and 12.5% of the air samples (Table1). Many diverse fungal colonies were observed on the agar plate. The genera *Cryptococcus*, *Mucor, Cladosporium, Penicillium, Alternaria, Fusarium, Abisidia, Rhodotorula* and *Aspergillus* were identified according to morphological criteria. Thereafter, only the strains which morphology was compatible with *Cryptococcus* species, i.e. smooth colonies (creamy or mucous) with white beige or ocher color and a capsule observed at the microscopic examination of an India ink stain preparation, were kept. These isolates were further identified using biochemical and DNA-sequence comparison. All isolates belonged to the species *C. albidus* except one (F9) that was identified as *C. diffluens*. The 26S rRNA gene and ITS2 sequences of the *C. albidus* isolates were identical and showed 99-100 % homology with the sequences deposited in the Genbank database. The F9 isolate sequences showed 99-100 % homology with the *C. diffluens* sequences deposited in the GenBank database.

Table 1: Distribution of *Cryptococcus spp* isolated from environmental samples

Location	Samples (117)								
	Pigeon droppings (91)			Atmospheric air (16)			Eucalyptus trees (10)		
	Sample number	Positive samples	Isolates	Sample number	Positive samples	Isolates	Sample number	Positive samples	Isolates
Hospital	19	5	F1: *C. albidus* F2: *C. albidus* F6.5: *C. albidus* F8: *C. albidus* F9: *C. diffluens*	16	2	AF2: *C. albidus* AF10: *C. albidus*		0	
Clinic	31	0							
Mosque	12	0							
Annaba downtown	8	0							
Villages around Annaba	18	0					5		
El Kala's region	3	0					5		

In this study, we reported for the first time, the isolation of *Cryptococcus sp.* from pigeon droppings and atmospheric air sampled in Algeria. Only three studies have reported *Cryptococcus* spp. isolation in Algeria. Mounira *& al*. [28] isolated *Cryptococcus sp*. in patients hospitalized with neurological disorders. Akdouche *& al.* isolated *Cryptococcus sp* in the mammary glands of cows, and Bendjama *& al.* isolated *C. terreus*, *C. albidus* and *C. neoformans* from irrigation water samples from the Ouargla area (south of Algeria). One limitation of these studies was that their identification strains relied on morphological and biochemical criteria and not on nucleotide sequence analysis [29, 30]. In our study, *Cryptococcus albidus* was isolated from pigeon droppings and atmospheric air, whereas *Cryptococcus diffluens* was found only in pigeon droppings. It has been shown that the presence of birds in the environment was the main factor associated with *C. neoformans* infection and thus would be a source of human infection [29, 8, 7]. Our findings suggested that pigeon droppings offered suitable conditions for the survival, and might act as reservoir of various *Cryptococcus* species in the spore form which might infect humans via air borne transmission.

Identification and antifungal activity of the Actinomycete strain

The cultural characteristics of the Actinomycete strain were observed on various media after 14 days incubation at 30°C. The strain E96 showed good growth on ISP1, ISP2, ISP3, ISP4 and casein starch media and moderate on ISP5, ISP6 and ISP7. No soluble pigment was produced. Light microscopy observation indicated that the strain was Gram positive with a filamentous branched and no fragmented aerial mycelium. The spores of the strain were spirale chain (S), Rectus-Flexibilis (RF) and Retinaculum- Apertum (RA).

The physiological and biochemical proprieties of E96 strain are detailed in the Table 2. The E96 strain assimilates the following carbon sources: glucose, galactose, D-fructose, D-mannitol, sorbitol, mannose. It showed a moderate growth for L-rhamnose, but no growth on raffinose, saccharose, L-arabinose, D-xylose, D-mellibiose. It showed a good growth with valine, asparagine, proline**,** arginine, and no growth with serine. Good growth was observed at pH 5, 6, 7, 8, 9, 10 and at 15°C, 29°C, 37°C, but reduced growth was observed at 43°C and pH 4. E96 could growth in

the presence of several antibiotics such as ampicillin**,** nalidixic acid, amoxicillin, cefotaxime)**,** pefloxacin, cefixime, cefazolin, pipemidic acid, and cephalexin. E96 produced chitinase, caseinase, urease, amylase and gelatinase.

Table 2: Physiological and biochemical characteristics of the strain E96

Tests	Result
Utilization of carbohydrates	
Glucose	+
Galactose	+
Raffinose	-
Saccharose	-
L-Arabinose	-
D-Fructose	+
D-Mannitol	+
D-Xylose	-
Sorbitol	+
D-Mellibiose	-
Mannose	+
L-Rhamnose	+/-
Simmons citrate	+
Utilization of nitrogen sources	
Valine	+
Asparagine	+
Proline	+
Arginine	+
Serine	-
Degradation of	
Chitin	+
Casein	-
Urea	-
Indole	+
Starch	+
Gelatin	+
Growth at	
15°C	+
29°C	+
37°C	+
43°C	+/-
pH 4	+/-
pH5	+
pH6	+
pH7	+
pH8	+
pH9	+
pH10	+
Growth in presence of	
Phenol (0.1%)	-
Sodium azide (0.001%)	-
Paraffin	-
Growth in presence of antibiotics	
Ampicillin (25µg)	+
Nalidixic acid (30µg)	+
Kanamycin (30µg)	-
Erythromycin (15µg)	-
Amoxicillin (30µg)	+
Clindamycin (2µg)	-
Tobramycin (10µg)	-
Imipenem (10µg)	-
Cefotaxime (30µg)	+
Pefloxacin (5µg)	+
Nitroxoline (30µg)	-
Pristinamycin (15µg)	-
Spiramycin (100µg)	-
Cefixime (10µg)	+
Cefazolin (30µg)	+
Doxycycline (30µg)	-
Pipemidic acid	+
Cefalexin (30µg)	+

According to the Bergey's Manual of Systematic Bacteriology, the physiological and biochemical proprieties strongly suggested that the strain E96 belonged to the genus *Streptomyces* [31]. Molecular identification was based on the analysis of the 16S rDNA gene. A 1508pb fragment was obtained and compared with 16S rDNA genes sequences included in the bank we constituted. *Streptomyces celluloflavus* and *S. kasugaensis* sequences were

reported as the most similar with 98.8% identity. The position of E96 in the neighbor joining phylogenetic tree is shown in Figure 2.

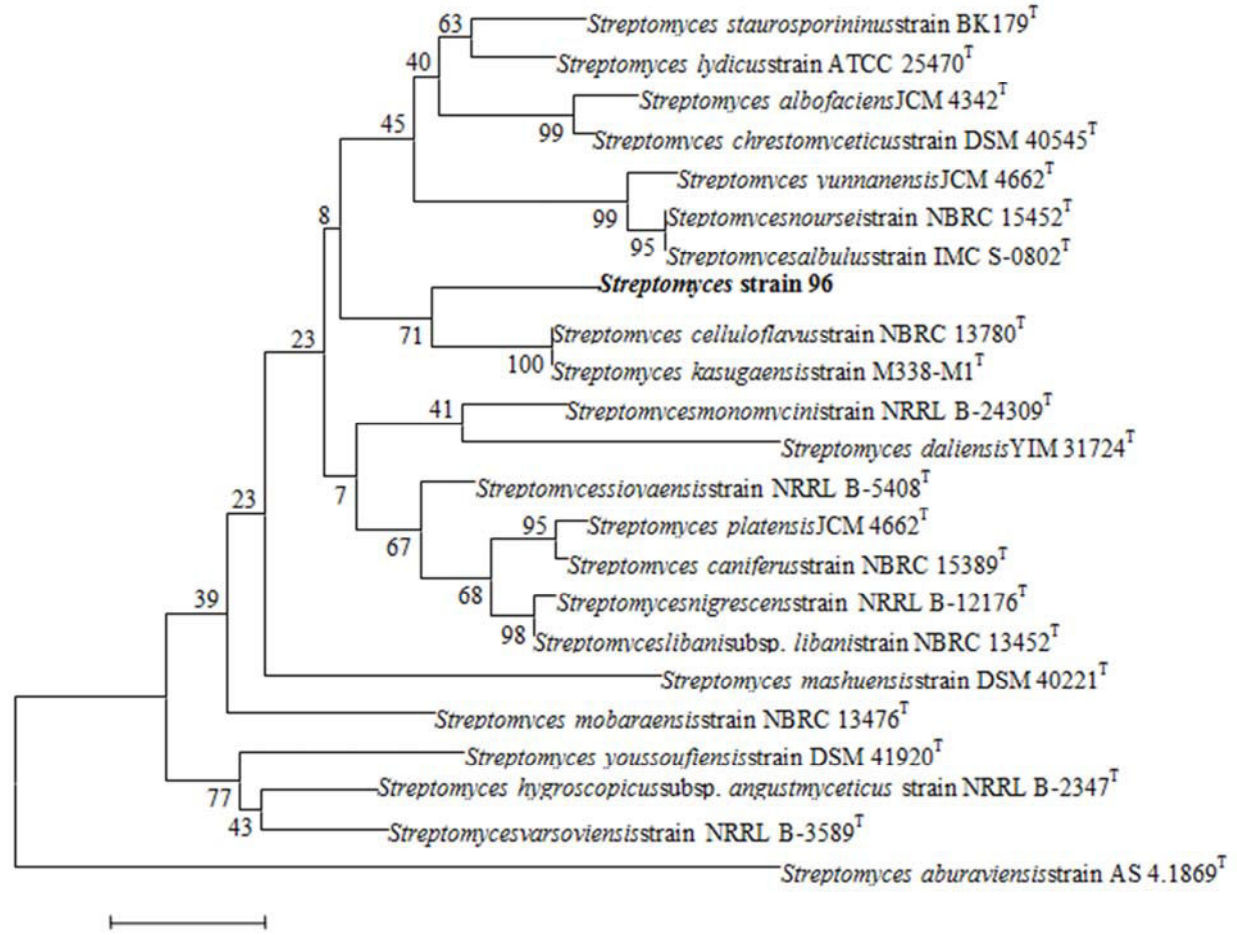

Figure 2: Neighbor-joining phylogenetic tree based on 16S rRNA sequence gene comparison showing relationships between *Streptomyces* E96 and related species

The scale bar indicates 0.005 substitutions

To evaluate the antifungal activity and to determine the best antifungal-active production medium, two techniques and four media respectively, were used. Antifungal activity was observed on all media tested (Figure 3). The ISP2 medium yielded the best production and was thus selected for antifungal compounds production. The antifungal-active metabolite production by the E96 strain on ISP1, ISP2, Bennett and GYEA started at the first day of culture on all media tested and reached a maximum inhibition zone (27 mm) on ISP2 medium (Figure4). Moreover, n-hexane solvent yielded the best secondary metabolites extraction (data not shown). The optimal antifungal extraction conditions were thus: at the first day of culture onISP2 medium using n-hexane organic solvent.

Regarding the *in vitro* susceptibility to the antifungal commercial agents (Table 3), ANI, MIC and CAS displayed elevated MIC against 100% of the tested isolates. 5FC displayed low MIC against 100% of the clinical *C. neoformans* isolates but in contrast it displayed elevated MIC against 67% of the environmental isolates. The azole antifungal agents (POS, VORI, ITRA, FLU) displayed low MIC against 71 % of all isolates. The antifungal active extract (EXT96) of the E96 strain showed a wide inhibition zone (23 mm) at 0.06mg/l. It was impossible to subculture isolate F2 and thus its susceptibility to antifungal agents could not be determined. In our study, only the strains isolated from droppings pigeon displayed a multi-antifungal resistance profile. Therefore azole resistance could be not intrinsic to the species *C. albidus* but to some isolates of this species. Clinical strains displayed a lower azole resistance level than environmental strains. Like for the other non-*C. neoformans* infections, the treatment of *C. albidus* infections is not well defined, which may explain the 50% mortality in *C. albidus* encephalitis despite antifungal therapy [32]. Increasing drug resistance in microbes, including fungi, prompted to search for new and enhanced therapeutic compounds from different sources, including the metabolites excreted by *Actinomycetes* [33]. At screening, our strain showed an important antifungal activity against both environmental and clinical strains of *Cryptococcus spp.* and displayed a higher inhibition zone than the tested commercially available antifungal agents.

Secondary metabolites production usually occurs during the stationary growth phase. However, we found that the antifungal compound produced by the E96 strain was highly correlated with the growth of strain (J1, Figure 4). Albeit, most of the antifungal antibiotics were extracted from liquid media and using ethyl acetate [6], those produced by E96 could not be extracted under these conditions but they were extracted from solid media using n-hexane solvent.

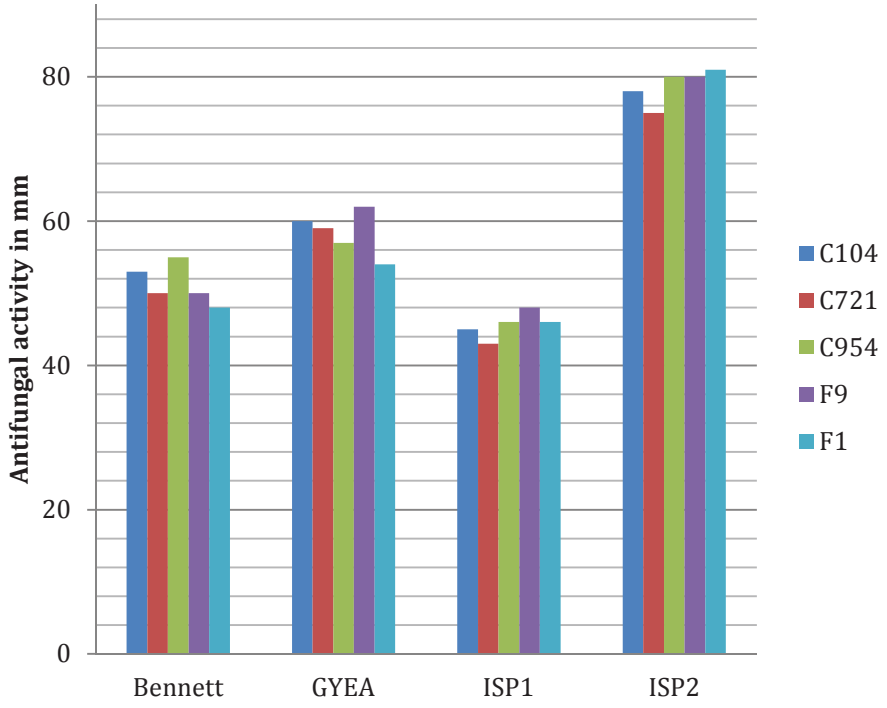

Figure 3: Antifungal activity determination of 5 *Cryptococcus* isolates on 4 different media by the double layer method

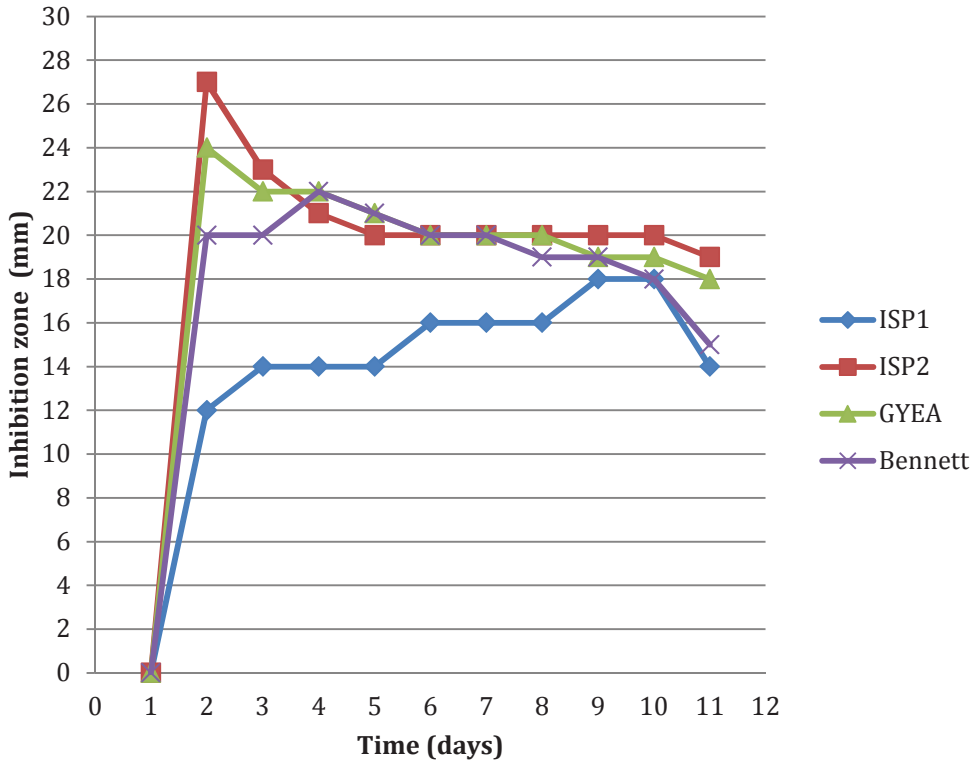

Figure 4: Time course of antifungal metabolites production by *Streptomyces* E96 in four culture media (ISP1, ISP2, GYEA, Bennett)

Table 3: Determination of the minimum inhibitory concentration (MIC, mg/l)ofenvironmental and clinical isolates
ANI: Anudulafungin, MICA: Micafungin, CAS: Caspofungin, 5FC: 5-flurocytosin, POS: Posaconazole, VORI: Voriconazole, ITRA: Itraconazole, FLU: Fluconazole, AMB: Amphotericin B. ND: not determined

Drugs	Range MIC(mg/l)	Samples (MIC/Susceptibility)									
		C. albidus F1	*C. albidus* F2	*C. albidus* F6.5	*C. albidus* F8	*C. diffluens* F9	*C. albidus* AF2	*C. albidus* AF10	*C. neoformans* CN104	*C. neoformans* CN721	*C. neoformans* CN954
ANI	8	>8	ND	>8	>8	>8	>8	>8	>8	>8	>8
MICA	8	>8	ND	>8	>8	>8	>8	>8	>8	>8	>8
CAS	8	>8	ND	>8	>8	>8	>8	>8	>8	>8	>8
5FC	4 – 64	>64	ND	>64	>64	>64	8	4	2	2	2
POS	0.25 – 1	1	ND	0.5	0.25	0.25	0.5	0.25	0.06	0.25	0.06
VORI	0.12 – 0.25	0.5	ND	0.25	0.12	0.12	0.25	0.12	0.03	0.25	0.03
ITRA	0.06 – 0.25	0.5	ND	0.25	0.5	0.06	0.12	0.12	0.06	0.12	0.06
FLU	8– 64	64	ND	16	16	32	16	8	8	8	4
AMB	0.12 – 0.25	0.25	ND	1	0.25	<0.12	<0.12	0.12	0.12	0.12	0.12
EXT96	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06

CONCLUSION

Our findings showed that *Cryptococcus* spp., which are potential human pathogens, are present in environments located in the close vicinity of human activities. We found that the E96 *Actinomycete* strain produces molecules that are highly active against *Cryptococcus* spp. clinical and environmental isolates and could be especially interesting when *Cryptococcus* spp. are resistant to 5-fluorocytsine, azole or echinocandins. The identification and the characterization of this molecule are ongoing.

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Annex 4: Environmental distribution of *Cryptococcus neoformans* and *Cryptococcus gattii* around the Mediterranean Basin

We took part in a wide environmental survey around Mediterranean basin about the distribution of two *Cryptococcus* species; *C. neoformans* and *C. gattii*. Human acquired the infection by these species from the environment. Therefore, it is important to saturate the subject of environmental sources of cryptococcal infections. A total of 6436 samples from 3765 trees were collected from 12 countries in Europe, and 5% of trees were positive. *C. neoformans* was isolated from 177 trees and *C. gattii* from 13. *C. neoformans* was distributed all around the Mediterranean basin whereas *C. gattii* was isolated in Spain, Southern Italy, and Greece. The results suggests that trees represent a fundamental niches to both *C. neoformans* and *C. gattii* in environment, and the secondary and temporary niche was bird excreta especially for *C. neoformans*.

Article status: accepted at journal of Federation of European Microbiology Society (FEMS) yeast research

ENVIRONMENTAL DISTRIBUTION OF *CRYPTOCOCCUS NEOFORMANS* AND *CRYPTOCOCCUS GATTII* AROUND THE MEDITERRANEAN BASIN

Massimo Cogliati[1], Roberta D'Amicis[1], Alberto Zani[1], Maria Teresa Montagna[3], Giuseppina Caggiano[3], Osvalda De Giglio[3], Stella Balbino[3], Antonella De Donno[4], Francesca Serio[4], Serdar Susever[5], Cagri Ergin[6], Aristea Velegraki[7], Mohamed S. Ellabib[8], Simona Nardoni[9], Cristina Macci[10], Salvatore Oliveri[11], Laura Trovato[11], Ludovico Dipineto[12], Volker Rickerts[13], Ilka McCormick-Smith[13], Sevim Akcaglar[14], Okan Tore[14], Emilija Mlinaric-Missoni[15], Sebastien Bertout[16], Michele Mallié[16], Maria da Luz Martins[17], Ana C.F. Vencà[17], Maria L. Vieira[17], Ana C. Sampaio[18], Cheila Pereira[18], Giuseppe Griseo[19], Orazio Romeo[19], Stéphane Ranque[20], Mohammed H. Y. Al-Yasiri[20], Meltem Kaya[21], Nilgun Cerikcioglu[21], Anna Marchese[22], Luigi Vezzulli[22], Macit Ilkit[23], Marie Desnos-Ollivier[24], Vincenzo Pasquale[25], Maya Korem[26], Itzhack Polacheck[26], Antonio Scopa[27], Wieland Meyer[28], Kennio Ferreira-Paim[28], Ferry Hagen[29], Bart Theelen[30], Teun Boekhout[30], Shawn R. Lockhart[31], Kathrin Tintelnot[13], Anna Maria Tortorano[1], Françoise Dromer[24], Ashok Varma[32], Kyung J. Kwon-Chung[32], João Inácio[33], Beatriz Alonso[33], Maria F. Colom[2]

1. Dip. Scienze Biomediche per la Salute, Università degli Studi di Milano, Milano, Italy
2. Universidad Miguel Hernández, Alicante, Spain
3. Università degli Studi di Bari "Aldo Moro", Bari, Italy
4. Università del Salento, Lecce, Italy
5. Cyprus Near East University, Nicosia, Cyprus
6. Pamukkale University, Denizli, Turkey
7. Medical School National and Kapodistrian University of Athens, Athens, Greece
8. Medical College University of Tripoli, Tripoli, Libya
9. Università di Pisa, Pisa, Italy
10. Istituto per lo Studio degli Ecosistemi (ISE), National Research Council (CNR), Pisa, Italy
11. Università degli Studi di Catania, Catania, Italy
12. University of Napoli Federico II, Napoli, Italy
13. Robert-Koch Institute, Berlin, Germany
14. Uludag University, School of Medicine, Bursa, Turkey
15. Croatian National Institute of Public Health, Zagreb, Croatia
16. Unité Mixte Internationale "Recherches Translationnelles sur l'infection à VIH et les Maladies Infectieuses", Montpellier, France
17. Instituto de Higiene e Medicina Tropical, Lisbon, Portugal
18. Universidade de Trás-os-Montes e Alto Douro, CITAB, Quinta dos Prados, Vila Real, Portugal
19. University of Messina, Messina, Italy
20. Aix-Marseille University, Marseille, France
21. Marmara University,School of Medicine, Istanbul, Turkey
22. Università di Genova, Genova, Italy
23. University of Çukurova Sarıçam, Adana, Turkey
24. Institut Pasteur, Unité de Mycologie Moléculaire, Paris, France
25. Università degli Studi di Napoli Parthenope, Napoli, Italy
26. Hadassah-Hebrew University Medical Center, Jerusalem, Israel
27. Università degli Studi della Basilicata, Potenza, Italy
28. Molecular Mycology Research Laboratory, CIDM, MBI, Sydney Medical School-Westmead Hospital, University of Sydney/Westmead Millennium Institute, Westmead, NSW, Australia
29. Canisius-Wilhelmina Hospital, Nijmegen, The Netherlands
30. CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands

31. Centers for Disease Control and Prevention , Atlanta, USA
32. National Institute of Allergy and Infectious Diseases, Bethesda, USA
33. School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, UK

Corresponding author

Massimo Cogliati
Lab. Micologia Medica
Dip. Scienze Biomediche per la Salute
Università degli Studi di Milano
Via Pascal 36, 20133 Milano, Italy
Phone: +39 0250315144
Fax: +39 0250315146
E-mail: massimo.cogliati@unimi.it

Running title:
Cryptococcus in the Mediterranean environment

Originality-Significance Statement

The present survey established a wide network that, for the first time, collected abundant information concerning the environmental distribution and ecology of *C. neoformans/C. gattii* species complex in Europe and the Mediterranean area. The results represent the basis for future studies and an important step towards the integration of the epidemiological data on *Cryptococcus* and cryptococcosis.

Abstract

In order to elucidate the distribution of *Cryptococcus neoformans* and *C. gattii* in this geographical area, an extensive environmental survey was carried out around the Mediterranean basin during 2012-15. A total of 302 sites located in 12 countries were sampled, 6436 samples from 3765 trees were collected, and 5% of trees were colonized by cryptococcal yeasts. *C. neoformans* was isolated from 177 trees and *C. gattii* from 13. *C. neoformans* colonized 27% of *Ceratonia*, 10% of olive trees, *Platanus* and *Prunus*, and a lower percentage of other tree species. The 13 *C. gattii* isolates were collected from five *Eucalyptus*, four *Ceratonia*, two pines, and two olive trees. *C. neoformans* was distributed all around the Mediterranean basin, whereas *C. gattii* was isolated in Spain, Southern Italy, and Greece, in agreement with previous findings both from clinical and environmental sources. Among *C. neoformans* isolates VNI was the prevalent molecular type but VNII, VNIV and VNIII hybrid strains were also isolated. With the exception of a single VGIV isolate, all *C. gattii* isolates were VGI. The results confirmed the presence of both *Cryptococcus* species in the Mediterranean environment, and showed that both carob and olive trees represent an important niche for these yeasts.

Introduction

Cryptococcosis is a life-threatening fungal infection caused by the encapsulated basidiomycetous yeasts *Cryptococcus neoformans* and *C. gattii*. The infection is likely acquired from the environment by inhalation of spores or dehydrated yeast cells that are able to penetrate the pulmonary alveoli and then disseminate through the bloodstream causing soft tissue infections, pneumonia and most often meningoencephalitis (Lin, 2009).

Cryptococcosis caused by *C. neoformans* is a major cause of mortality in AIDS patients. An estimated one million cases of cryptococcal meningitis occur among people with HIV infection worldwide each year, resulting in nearly 625,000 deaths (Park *et al.*, 2009). Before antiretroviral therapy was established, cryptococcosis and other opportunistic infections were a major problem for people with advanced AIDS. Since then, the cases of fungal infections and and the number of deaths due to fungal infections in people with advanced HIV infection have decreased substantially in developed countries. However, fungal diseases, particularly cryptococcosis, are still a major concern for people living with HIV infection worldwide (Park *et al.*, 2009). While cryptococcosis cases in HIV infected patients have been decreasing, an increase in the number of cases has been reported in non-HIV patients due to the rising number of susceptible patients such as patients with hematological malignancies, organ transplant recipients, and patients affected by autoimmune diseases (Bratton *et al.*, 2012; Henao-Martinez and Beckham, 2015; Sanchini *et al.*, 2014).

The real incidence of cryptococcosis is difficult to establish because the disease is reportable in only a few states. Results from active, population-based surveillance in two US locations in the year 2000 indicated that the annual incidence of cryptococcosis among persons with AIDS was between 2 and 7 cases per 1,000 PWA, and the overall incidence was 0.4 to 1.3 cases per 100,000 population; the case-fatality ratio was approximately 12% (Mirza *et al.*, 2003). More recently, an analysis of stored serum samples from HIV-infected persons with low CD4 counts enrolled in studies in the US during 1986–2012 found the prevalence of cryptococcal antigenemia to be 2.9%,

indicating that the prevalence of cryptococcal infection among HIV patients is largely underestimated (McKenney *et al.*, 2014).

In Europe, the epidemiology of cryptococcosis is even more obscure for two reasons: There are only a few out-dated reports on epidemiology of cryptococcosis from a limited number of countries and the lack of coordination to collect epidemiological data among EU countries. The epidemiological data thus far available on cryptococcosis are from UK, France, Italy, Germany and Spain. The data from the rest of the EU countries are either scarce of completely lacking especially from Eastern European countries where a higher incidence of cryptococcosis is expected due to a heavier burden of HIV infection compared to Western Europe (de Colombani *et al.*, 2004).

A unique attempt for a prospective European survey was performed from 1997 to 1999 (Viviani *et al.*, 2006). During the survey 655 cases from 17 countries were reported and 311 cryptococcal isolates were collected for molecular typing. Although the survey represents a milestone in the elucidation of the European epidemiology of cryptococcosis, the results underestimate the burden of the disease since many countries did not participate in the study. At the national level, a recent study carried out in France reported 1850 cases of cryptococcosis from 2001 to 2010 and an incidence of 0.3 per 100,000 population/year with a fatality rate of 15% (Bitar *et al.*, 2014), while 129 cases were recorded in a study carried out in Germany from 2004 to 2010 (Sanchini *et al.*, 2014).

Due to its geographical location, Europe is also subjected to extensive immigration of people from both Asia and Africa where cryptococcosis represents the third highest cause of death in HIV infected patients (Assogba *et al.*, 2015; Park *et al.*, 2009). This inevitably favors the spread of new genotypes through the European continent and thus the potential for geographic distribution of virulent strains. Furthermore, the high flow of people to and from Europe for business and tourism allows the emergence of cryptococcosis cases acquired in endemic areas easier (Dromer *et al.*, 1992; Hagen *et al.*, 2010). The recent cryptococcosis outbreaks occurring on Vancouver Island (Canada) and the Pacific Northwest of North America showed how this fungal menace could spread

rapidly in the environment once it has found a niche favorable to survival (Bartlett *et al.*, 2012; Byrnes and Marr, 2011; Hagen *et al.*, 2012). The Centers for Disease Control and Prevention in the USA worked with local public health authorities to implement a plan to monitor the epidemiology of *C. gattii* in the states of Washington and Oregon where the report of this fungal disease is now mandatory. Unfortunately, the coordination of such actions is very slow in Europe and thus European countries are not prepared for the occurrence of unexpected outbreaks.

Few studies to assess the occurrence of *C. neoformans/C. gattii* species complex in the environment have been performed in Europe. *C. neoformans* was mainly reported to be associated with bird excreta (Colom *et al.*, 1997; Garcia-Hermoso *et al.*, 1997; Pernice *et al.*, 1998; Montagna *et al.*, 2003, Boekhout *et al.*, 2001; Lagrou *et al.*, 2005; Cafarchia *et al.*, 2006) and only few isolates were recovered from arboreal sources (Criseo *et al.*, 1995; Criseo and Gallo, 1997: Lo Passo *et al.*, 1997; Campisi *et al.*, 2003; Bauwens *et al.*, 2004, Chowdhary *et al.*, 2012). *C. gattii* was recovered for the first time from the European environment in Southern Italy (Montagna *et al.*, 1997, Romeo *et al.*, 2011) and then also in The Netherlands (Chowdhary *et al.*, 2012) and Spain (Colom *et al.*, 2012). However, these surveys were limited to a restricted territory and carried out in different period of time. The results are geographically and temporally fragmented data.

The present study represents a first collaborative study aimed to understand the environmental distribution of *C. neoformans* and *C. gattii* on trees around the Mediterranean basin and in continental Europe. In addition, data and isolates collected during the survey represent an important source for comparison and correlation with European and global clinical data.

Materials and methods

Network and study design

The ISHAM Working Group for Genotyping of *Cryptococcus neoformans* and *C. gattii* (http://www.isham.org/WorkingGroups/Genotyping_neoformans_gattii) established a network to

survey the distribution of *C. neoformans* and *C. gattii* in the environment focusing the samplings around the Mediterranean basin. Thirty-two centers from nine European countries and three extra-European countries (Turkey, Israel, and Libya) participated in the study. Each participating center was required to collect samples from trees and soil in urban and rural areas, and to record the information about the site of sampling, the type of sample, the tree species, the date of sampling, the daily mean temperature, the number of collected samples, and the number of positive samples. Both information and isolate were sent to the coordinator center at the Medical Mycology Laboratory, Università degli Studi di Milano (Italy). All isolates were coded and stocked, and then processed for molecular analyses. Three additional extra-European centers, two from the USA and one from Australia, joined the study and contributed to the molecular strain typing.

Environmental samplings

Samples were collected mainly from public gardens in urban areas but also from some rural areas. The sources of samples were hollows and fissures of trees, flowers, leaves, bark, fruits, decaying wood, and soil near the trees. All the sampled trees were identified at the species level when possible.

Hollows and fissures on the tree trunk

Samples were collected by rubbing the inner of the hollows or fissures of the trees with a sterile cotton-tipped swab moistened in a solution of sterile distilled water supplemented with chloramphenicol (10 mg/L). The swab was placed in a tube with 3 ml of the solution and the tube was shaken for 5 min without removing the swab. The swab was removed and the suspension was left to sediment at least for 10 min. One hundred µl of the supernatant and 100 µl of the diluted supernatant (1:10 in sterile distilled water) were inoculated onto two different plates containing Niger seed agar medium (Kwon Chung and Bennett, 1992). The plates were incubated at 37°C for at least 10 days. All brown colonies grown on the plates were isolated for species identification.

Flowers and leaves

About 10-20 g of the sample were collected and sealed in a zip-lock bag. A portion of the sample (5 g) was transferred in a sterile mortar and fragmented with a pestle. The fragments were suspended in 50 ml sterile distilled water and mixed by vortexing for about 2 min. Sediment was allowed to settle for 15-20 min. Two ml of the supernatant was mixed with 8 ml of sterile distilled water containing chloramphenicol (10 mg/L). 100 µl of the supernatant and 100 µl of the diluted supernatant were inoculated in two different plates containing Niger seed agar medium. The plates were incubated at 37°C for at least 10 days. All brown colonies grown on the plates were collected for identification.

Bark and decaying wood

Samples were obtained by scraping the surface of the wood with a scalpel and sealing the shavings in a zip-lock bag. Following vigorous grinding with a mortar, one g of the sample was suspended in 50 mL of sterile distilled water containing chloramphenicol at 10 mg/L, shaken for 2 min and allowed to settle for 30 min. 100 µl of the supernatant and 100 µl of the diluted supernatant (1:10 in sterile distilled water) were inoculated on two different Niger seed agar plates. The plates were incubated at 37°C for at least for 10 days. All brown colonies grown on the plates were collected for identification.

Soil

Approximately 10-20 g of soil was collected and sealed in a zip-lock bag. Part of the soil (5 g) was suspended in 50 ml sterile distilled water and mixed by vortexing for about 2 min. Sediment was allowed to settle for 15-20 min. Two ml of supernatant was mixed with 8 ml of sterile distilled water containing chloramphenicol (10 mg/L). One hundred µl of the supernatant and 100 µl of the diluted supernatant were inoculated on two different Niger seed agar plates. The plates were

incubated at 37°C for at least for 10 days. All brown colonies grown on the plates were collected for identification.

Isolation, species identification, coding and storage

Brown colonies were streaked for isolation on a fresh plate containing Niger seed agar in order to collect single pure colonies. Isolates were then examined under a microscope for the yeast morphology and capsule presence, tested for urease activity, and the ability to grow at 37^{o} C and assimilate inositol as a carbon source.

The species was identified by inoculating onto glycine-canavanine-bromothymol blue (GCB) agar differential medium (Kwon-Chung and Bennett, 1992).

A code identifying the country, the place of origin, the type of sample and the tree code number was assigned to each isolate, which were then suspended and stored in a vial containing 3 ml of sterile distilled water at room temperature.

Molecular analyses

Genomic DNA was extracted as previously reported (Viviani *et al.,* 1997). Molecular type and mating type of all isolates was determined by four multiplex PCRs specific for both *C. neoformans* and *C. gattii* as described elsewhere (Cogliati *et al.,* 2000; Esposto *et al.,* 2004; Feng *et al.,* 2013; Cogliati *et al.,* 2015). Molecular types were assigned according to the standard nomenclature of the ISHAM working group for genotyping of *C. neoformans* and *C. gattii* (Meyer *et al.,* 2009). Strains H99 (VNI-αA), JEC20 (VNIV-**a**D), JEC21 (VNIV-αD), IUM 96-2828 (VNI-**a**A), WM 626 (VNII-αA), WM779 (VGIV-αC), NIH312 (VGIII-αB), NIH191 (VGIII-**a**C), WM201 (VGI-αB), IUM 00-5363 (VGII-**a**B) were used as reference strains.

Mating assay

Mating assay was performed to test the fertility of some environmental isolates collected during the survey. The isolate was streaked onto a 90-mm Petri dish containing 20 ml Murashige-Skoog agar medium (0.44% Murashige-Skoog basal medium, Sigma-Aldrich, and 4% agar in distilled water) and then mixed with a tester strain of an opposite mating type. Co-cultures were incubated at 25 °C in the dark for at least 3-4 weeks and checked periodically for the formation of hyphae and basidiospores. Basidiosporespores were collected by cutting a square of the agar on which hypha were produced and transferring it to a tube containing two ml of sterile distilled water. After the tube was gently stirred, the supernatant was transferred to a new tube and checked microscopically for the presence of basidiospores. A 100 µl volume of the spores suspension was plated on Sabouraud dextrose agar and incubated at 37°C for 48 h. Ten single colonies grown on the plate were collected and processed for molecular typing. Nearly 1:1 ratio of MATa and MATalpha spores confirmed the successful mating between the two tested strains. The strain pairs of JEC20 (VNIV-**a**D), JEC21 (VNIV-αD), and H99 (VNI-αA), and IUM 96-2828 (VNII-**a**A) were used as control strains.

Taxonomy

Although recently a new taxonomic classification of *Cryptococcus neoformans*/*C. gattii* species complex has been proposed (Hagen *et al.,* 2015), a complete consensus of the scientific community has not yet been reached. Therefore, in the present study we continue to adopt the classical taxonomy which classify the agents of cryptococcosis in two species, *C. neoformans* and *C. gattii*, and *C. neoformans* into two varieties, var. *grubii* and var. *neoformans.*

Results

Sampling distribution

The survey was performed in the period 2012-2015. A total of 302 different sites from 12 countries were sampled. Samples were collected primarily in Italy (152 sites) followed by Spain (47 sites), France (27 sites), Turkey (19 sites), Croatia (18 sites), Portugal (11 sites), Germany (10 sites), Greece (7 sites), Cyprus (6 sites), Libya (3 sites), and Israel and The Netherlands with one site each. A map with the distribution of samplings is shown in Fig. 1.

Trees

The samples were collected from 3765 trees representing more than 100 different genera (Tab. S1). Most of the trees were *Eucalyptus* (37%), olive trees (14%), pines (13%), oaks (5%), carobs (3%), *Prunus* (2%), and *Platanus* (2%). The highest percentage of sampled trees was in Italy (43%), followed by Turkey (12%), Cyprus (10%), Libya (9%), Spain (6.5%), and Greece (6%). A total of 188 trees (5%) were colonized by *C. neoformans* or *C. gattii.* Colonized trees were found in Spain, Italy, France, Cyprus, Libya, Portugal, Greece, and Turkey with the highest percentage of positive trees recorded in Spain and Greece (16.7% and 16.8% respectively). The percentage of colonized trees relative to each kind of tree was the following: carobs (27.7%), *Platanus* (10.1%), *Prunus* (9.4%), olive trees (9.3%), pines (4.7%), *Eucalyptus* (3.7%), and oaks (1.1%). Sporadic positive samples were also found in *Aesculus hippocastanum*, *Carpinus betulus*, *Juglans nigra*, *Juniperus* spp., *Gleditsia triacanthos*, and *Pyrus communis*.

Samples

A total of 6436 samples were collected from trunk hollows (62%), bark (11.5%), leaves (8%), flowers (1.3%), soil under the tree (16%), and other samples (1.2%), such as fruits, decaying wood, debris near the tree, or bird excreta on the tree. The majority of the samples, 44.4% and 9.6%, were collected in Italy and Greece, respectively. A total of 3.4% of the samples were positive. *Cryptococcus neoformans* or *C. gattii* were recovered from cultures of trunk hollow swabs, bark scrapings, soil, and decaying wood, whereas no isolates were recovered from leaves and flowers.

Trunk hollows had a percentage of positivity of 4.1% (n=3986), bark of 3% (n=738), and soil of 2.4% (n=1039). In addition, nine samples from debris of decaying wood were positive.

Distribution of C. neoformans and C. gattii

During the survey 512 isolates from 188 trees were recovered representing 474 *C. neoformans* and 38 *C. gattii*. The percentage of colonized trees relative to the two species was 4.6% for *C. neoformans* and 0.4% for *C. gattii* with a ratio of 13.5:1, respectively. *C. neoformans* was isolated from 177 trees belonging mainly to the genera *Eucalyptus*, *Olea* (olive trees), *Ceratonia* (carobs), and *Pinus*, but also from *Quercus*, *Prunus*, *Platanus*, *Aesculus*, *Carpinus*, *Juglans*, *Juniperus*, *Gleditsia*, and *Pyrus* (Fig. 2). The trees colonized by *C. neoformans* strains were found in Portugal, Spain, France, Italy, Greece, Cyprus, Turkey, and Libya.

Cryptococcus gattii was isolated from 13 trees belonging to four different genera: five *Eucalyptus*, four *Ceratonia* (carobs), two *Olea* (olive trees), and two *Pinus pinea* (Fig. 2). The trees were distributed in Spain (Alicante, Tarragona, and Mendivil), Italy (Bari and Catania), and Greece (Athens and Salamina island). One carob tree in Spain and one olive tree in Italy were colonized by both *C. neoformans* and *C. gattii* isolates.

Prevalence and distribution of molecular types

The map in Fig. 3 shows the distribution of molecular types around the Mediterranean basin. The prevalent molecular type was VNI with 330 isolates from 129 trees distributed in all seven countries that yielded positive samples. Molecular type VNII was only isolated from two trees in Tripoli, Libya, whereas 107 VNIV isolates were recovered from 27 trees located in Spain, Italy, Greece, and Turkey. In addition, 35 AD-hybrids (molecular type VNIII) were isolated from 26 trees in Greece, Libya, and Turkey.

VGI was the prevalent molecular type for *C. gattii* with 37 isolates from 12 trees. One isolate from a Spanish carob tree was identified as VGIV. Trees colonized by both VNI and VGI (two trees), or both VNI and VNIV (four trees), or both VNI and VNIII (three trees) were also found.

Prevalence and distribution of mating types

Both mating type **a** and mating type α were found among *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii* isolates collected during the survey. Mating type allelic pattern αA was the prevalent mating type (327 isolates) among *C. neoformans* var. *grubii* whereas the opposite mating type **a**A was identified in seven isolates, six in Spain and one in Italy. *C. neoformans* var. *neoformans* αD mating type was recovered in Italy (71 isolates), Spain (one isolate), and Turkey (6 isolates), whereas mating type **a**D was found only in Greece (29 isolates). The 35 AD-hybrid isolates presented two different mating-type allelic patterns, three were heterozygous αAD**a** and 32 were homozygous αAAα. One olive tree in Italy and four carob trees in Spain were colonized by both αA and **a**A strains whereas two *Eucalyptus* trees in Greece were colonized by both αA and **a**D strains. The co-existence of αA and αD as well as αA and AD-hybrids was also observed in Italy (two trees) and Turkey (three trees), respectively.

Regarding *C. gattii*, 10 trees were colonized by mating type αB strains, two trees in Italy by mating type **a**B, and one in Spain by mating type αC. One of the two Italian **a**B isolates shared the same olive tree with *C. neoformans* **a**A and αA strains. Similarly, in Spain one *C. gattii* αB strain co-existed with one *C. neoformans* αA strain in a carob tree.

Seasonality and daily mean temperature

Data of seasonality are reported in Fig. 4. Samples were collected during all 12 months of the year. The highest number of samples was recorded in July (19.9%) followed by September (19.4%) and October (14.4%) and the lowest was February (0.8%) and January (1.6%). The percentage of trees

colonized by *C. neoformans* showed a significant increase in March (22%), April (6.5%), September (9.2%) and December (8.1%) when compared to the mean value of 5% colonization. In the other months the value was lower with a minimum recorded in February (no positive trees) and August (1.1%). *C. gattii* colonization of trees was higher than the mean of 0.4% in March (1.7%), April (3.3%), and May (0.8%). No *C. gattii* positive trees were found in August, October, December, January, and February.

Samples were collected within a range of daily mean temperatures from 7°C to 32°C. Depending on the mean temperature of the sampling day, the data relative to every sampling were included in one of five temperature ranges: <10°C, 11-15°C, 16-20°C, 21-26°C, and >26°C. The percentage of colonized trees relative to every range was calculated for both the *Cryptococcus* species and displayed in a histogram (Fig. 5). The results showed that, although *C. neoformans* was recovered equally in all ranges of temperature, *C. gattii* was recovered only when mean daily temperature was higher than 10°C.

Results of mating assays

Seven mating assays were performed to test the fertility of the isolates originated from the trees where two opposite mating type strains shared the same niche. Five assays were intra-variety mating assays between **a**A and αA isolates whereas the other two were inter-variety assays between αA and **a**D isolates. All intra-variety assays produced filaments and basidiospores. In addition, molecular analysis showed that the progeny included both **a**A and αA mating types in a Mendelian ratio (Fig. 6). In contrast, none of the inter-varietal matings was fertile during four weeks of observation.

Discussion

The present study is the first European collaborative prospective environmental survey of the distribution of *C. neoformans*/*C. gattii* species complex. It covered a wide territory of Europe

including a large part of the Mediterranean coast as well as some continental areas. This first survey produced an immense quantity of data which should be followed by detailed analysis in future studies.

The results showed that both *C. neoformans* and *C. gattii* are present in the Mediterranean environment in association with trees. *C. neoformans* is more prevalent than *C. gattii* with a percentage of colonized trees 13 fold higher. Our findings suggests that trees may be primary niches for both species in the environment, and that bird excreta could represent a secondary and temporary niche especially for *C. neoformans.* The association of trees with cryptococci is also implicated by the findings of the yeasts as infectious or colonizing agents in animals such as koalas, squirrels, monkeys and parrots whose lives revolve around trees. Goats have the habit of eating tree barks and they are also victimized by cryptococcosis (Roussilhon *et al.*, 1987; López-Martinez *et al.*, 1995; Torres-Rodriguez *et al.*, 1999; Krockenberger *et al.*, 2003; Stilwell and Pissarra, 2014; Iatta *et al.*, 2015; Maestrale *et al.*, 2015).

This study reveals a difference in the association of the two *Cryptococcus* species with specific trees. In particular, *Ceratonia* (carob tree) and *Olea* (olive tree), two trees typical of the Mediterranean region, together with *Eucalyptus*, produced the highest number of positive samples. However, when the values were normalized as percentage of positive tree genus and stratified for *Cryptococcus* species and varieties, the results showed that *Ceratonia* is an important niche for both *C. gattii* and *C. neoformans* var. *grubii* but not for *C. neoformans* var. *neoformans* and AD-hybrids since they were not recovered in these trees. In addition, *C. gattii* was recovered only from trees typical of the Mediterranean climate (*Ceratonia*, *Olea*, *Eucalyptus* and *Pinus pinea*), whereas *C. neoformans* var. *grubii* colonized 12 different genera of trees confirming the great ability of this pathogen to adapt in different environments and, hence, its global distribution. The importance of *Ceratonia siliqua* as a niche for *C. gattii*, shown in the present survey, is in agreement with the data reported by a previous environmental study carried out in Spain (Colom *et al.*, 2012). In contrast, *C. neoformans* var. *neoformans* and AD-hybrids showed a preference to colonize trees typical of the

sub-continental climate such as *Platanus*, *Prunus* and *Quercus.* This could reflect the ability of var. *neoformans* to tolerate lower temperature better than var. *grubii* and *C. gattii* as previously showed by other authors (Martinez *et al.*, 2001). Although the two *C. neoformans* varieties and *C. gattii* have differences in tree preference, the climatic zones do not have sharp boundaries and they overlap along all the Mediterranean basin. Therefore, in the Mediterranean environment, the different populations are continuously in contact with each other. This is confirmed by the finding that isolates belonging to different species or varieties shared the niche in the same tree, as well as by the presence of hybrids in the same area.

Hybridization between var. *grubii* and var. *neoformans* is well documented in Europe by the identification of numerous AD-hybrids in clinical isolates which have a prevalence of about 30% (Viviani *et al.*, 2006). In contrast, only a few hybrids have been isolates from pigeon droppings (Baró *et al.*, 1999; Ferreira *et al.*, 2014) due to the paucity of environmental studies carried out in Europe. Our study reports for the first time the association of AD-hybrids and trees and their presence in the same area where the putative var. *grubii* and var. *neoformans* parental strains may co-exist. This finding suggests that hybridization between the two *C. neoformans* varieties is occurring in the European environment and it may play an important role as a mechanism of evolution of this species (Cogliati *et al.*, 2009).

The presence of *C. gattii* in Greece, Southern Italy and Spain confirms the previous results obtained in these geographical areas where the pathogen was isolated from both clinical and environmental sources (Montagna *et al.*, 1997; Torres-Rodriguez *et al.*, 1999; Velegraki *et al.*, 2001; Colom *et al.*, 2005; Viviani *et al.*, 2006; Solla *et al.*, 2008; Ropstad *et al.*, 2011; Colom *et al.*, 2012; Romeo *et al.*, 2012; Iatta *et al.*, 2012). The survey also confirmed that VGI is the prevalent *C. gattii* molecular type in agreement with a previous analysis carried out by Hagen *et al.* (2012) that identified an endemic VGI cluster in Europe. However, one isolate was identified as VGIV-alphaC (Linares 2015) suggesting that other genotypes are also able to colonize the Mediterranean basin. Further

genotyping of the isolates collected during this survey by MLST will elucidate the relationships and the clusters present in the European environment.

C. neoformans VNI was the prevalent molecular type distributed all around the Mediterranean basin from Portugal to Libya confirming the ubiquitous presence of this pathogen worldwide (Cogliati, 2013). In this study, a high prevalence of the VNIV molecular type was found in Greece, where it represented the most prevalent molecular type, and Northern Turkey, where it was the only molecular type present, and less frequently in Italy and Spain. On the basis of these results it could be speculated that this molecular type is spreading from sub-continental areas of South-Eastern Mediterranean towards the Western part of Europe. Further sampling and an accurate niche modeling analysis is in progress to corroborate the above hypothesis.

Our results showed that mating type **a** and α are present in the Mediterranean environment for both *C. neoformans* varieties and *C. gattii*. Interestingly, we found that the occurrence of two strains with different mating types in the same tree is not rare and, therefore, trees are likely the optimal niche to complete the sexual cycle. This hypothesis is supported by the observation that most of the isolates with different mating types sharing the same niche were able to produce filaments and recombinant basidiospores in mating assays.

These findings suggest that the clonal population structure observed for both *C. neoformans* and *C. gattii* could be due to the genotyping results mainly on clinical isolates and the relatively low number of available environmental isolates (Cogliati, 2013). The analysis of a larger number of environmental isolates might show a more relevant involvement of sexual reproduction in *C. neoformans*/*C. gattii* species complex evolution. Our data are corroborated by previous studies reporting recombination among *C. neoformans* and *C. gattii* populations isolated from the environment (Litvintseva *et al.*, 2003; Saul *et al.*, 2008; Carriconde *et al.*, 2011).

A different trend can be observed with respect of seasonality depending on the *Cryptococcus* species considered. Sampling during different seasons did not greatly influence the recovery of *C. neoformans* although a peak of positive trees was observed during spring. Similarly, *C. gattii*

recovery was more probable during spring and early summer, but was absent in the colder seasons. Both species presented a decreased positivity in August, which is the hottest and driest month in the Mediterranean area, suggesting the difficulty of these yeasts to survive in such climatic conditions. When daily mean temperatures were considered, the results confirmed that *C. neoformans* can be recovered during a wide range of temperatures whereas *C. gattii* seems to suffer at temperatures below 10 °C.

In conclusion, the present survey established a wide network that, for the first time, collected abundant information concerning the environmental distribution and ecology of *C. neoformans/C. gattii* species complex in Europe and the Mediterranean area. The results represent the basis for future studies and an important step towards the integration of the epidemiological data on *Cryptococcus* and cryptococcosis.

Acknowledgements

The work by K.J. Kwon-Chung and A. Varma was supported by funds from the intramural program of the National Institute of Allergy and Infectious Diseases, NIH.

This work was in part supported by CITAB under the project UID/AGR/04033/2013.

Disclosures

The findings and conclusions of this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention

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Figure legends

Fig. 1. A map of the Mediterranean basin showing the sampling sites (red dots). The coordinates for each site were recorded and then plotted on the map using GoogleMaps (www.google.com).

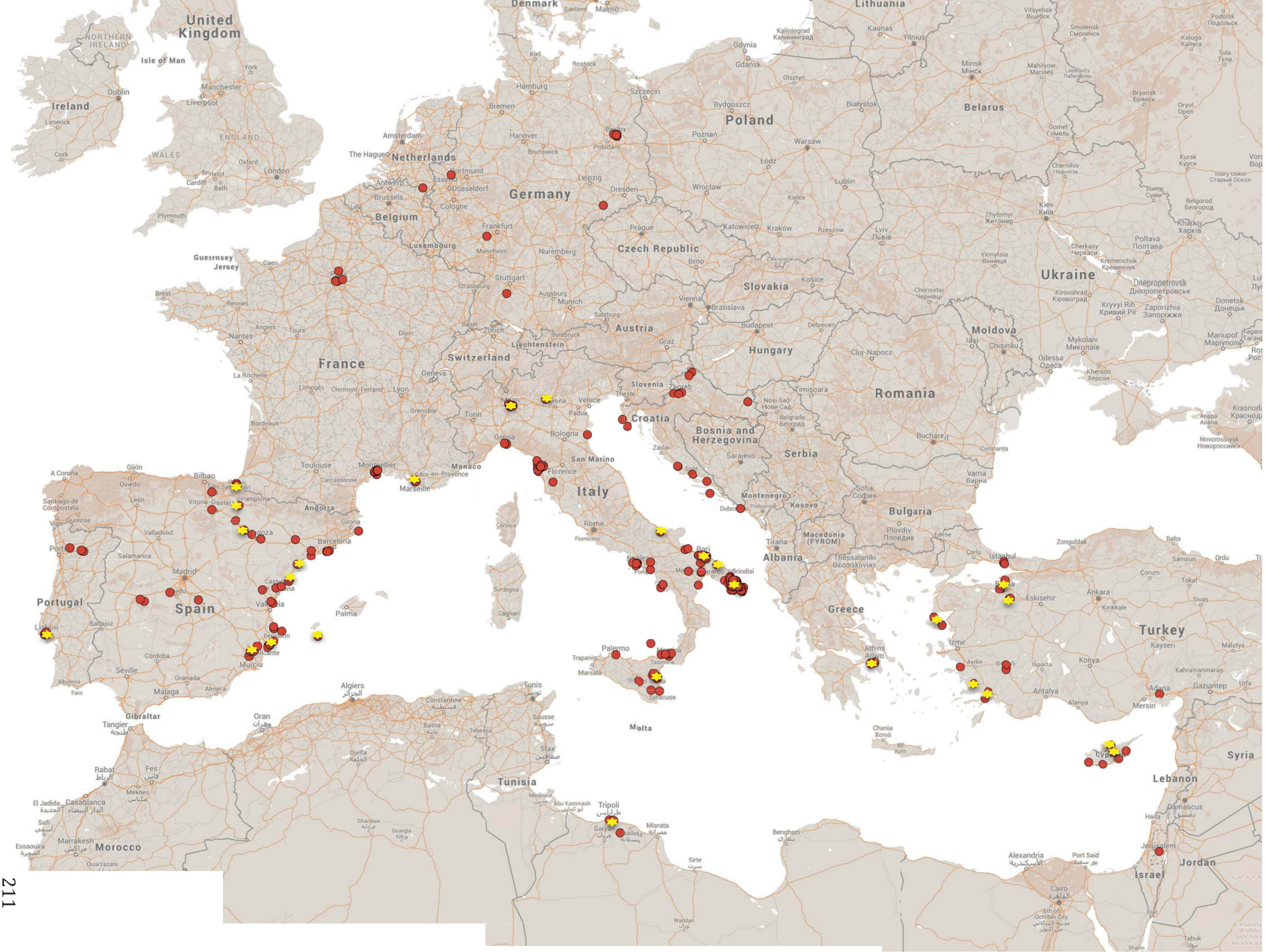
Fig. 2. Percentage of colonized trees in the different tree genera. In the histogram, only the tree genera that are represented at least 2% of the total sampled trees have been included.

Fig. 3. Distribution and prevalence of *C. neoformans* and *C. gattii* molecular types around the Mediterranean basin. Numbers inside the circles indicate the numbers of positive trees. Participating countries are presented in yellow.

Fig. 4. Percentage of trees colonized by *C. neoformans* and *C. gattii* recorded in each month of the year. The blue and red dashed lines represent the mean values for *C. neoformans* and *C. gattii* respectively.

Fig. 5. Percentage of trees colonized by *C. neoformans* and *C. gattii* in relation to the daily average temperature during the sampling days.

Fig. 6. Results of one representative mating assays performed on Murashige Skoog agar medium. One VNI-αA isolate and one VNI-**a**A isolate which shared the same niche in the same tree was used for crossing. A. VNI-αA culture. B. VNI-**a**A culture. C. Filament production in the mixed culture (VNI-αA x VNI-**a**A). The inset (upper-right) shows a basidium with long chains of basidiospores . D. Multiplex PCR to determine the mating type allelic pattern of the progeny. Each lane represents DNA from a single basidiospore cultures.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Kosovo
Bulgaria
Macedonia (FYROM)
Albania
Greece
Turkey
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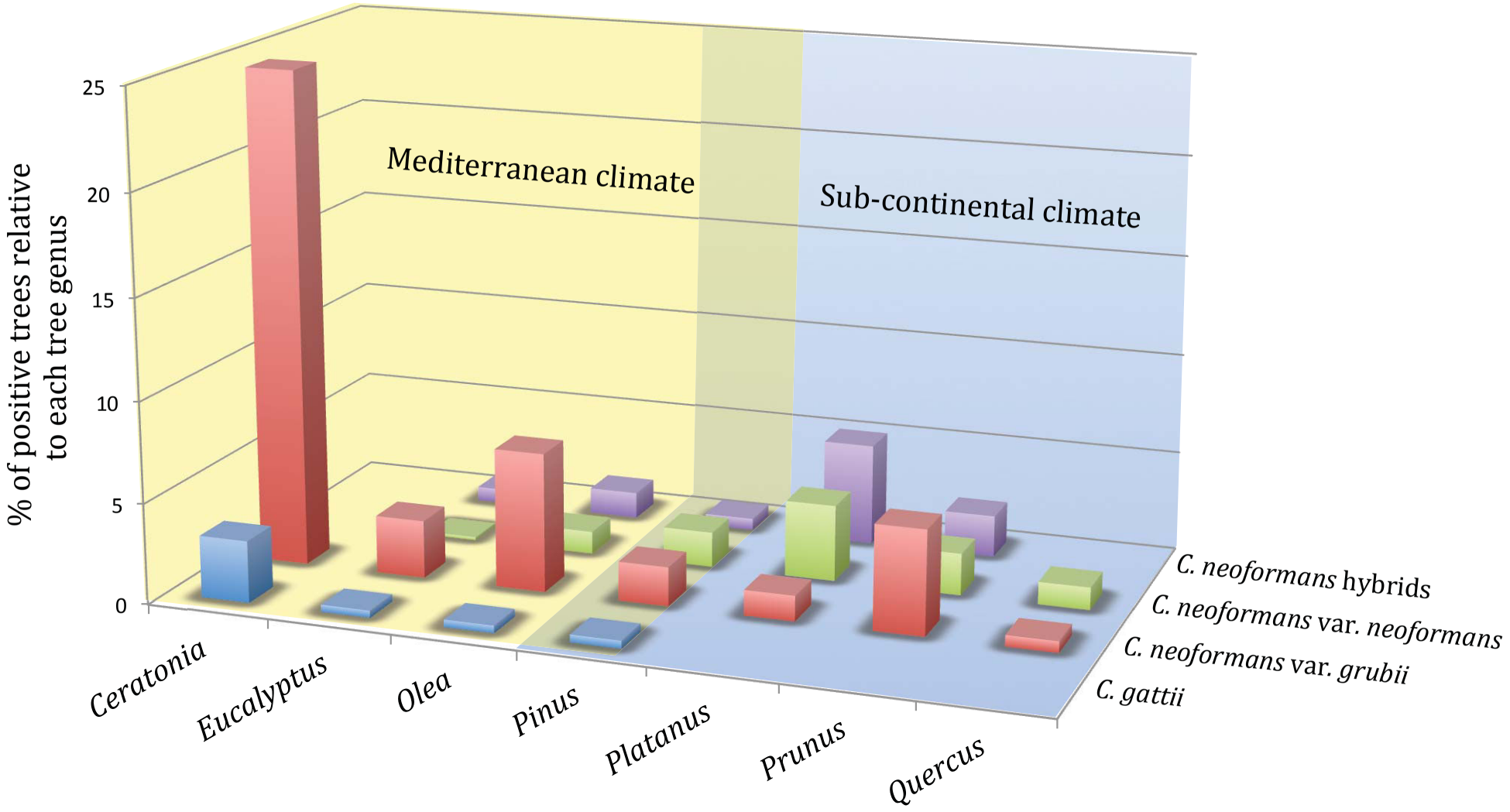
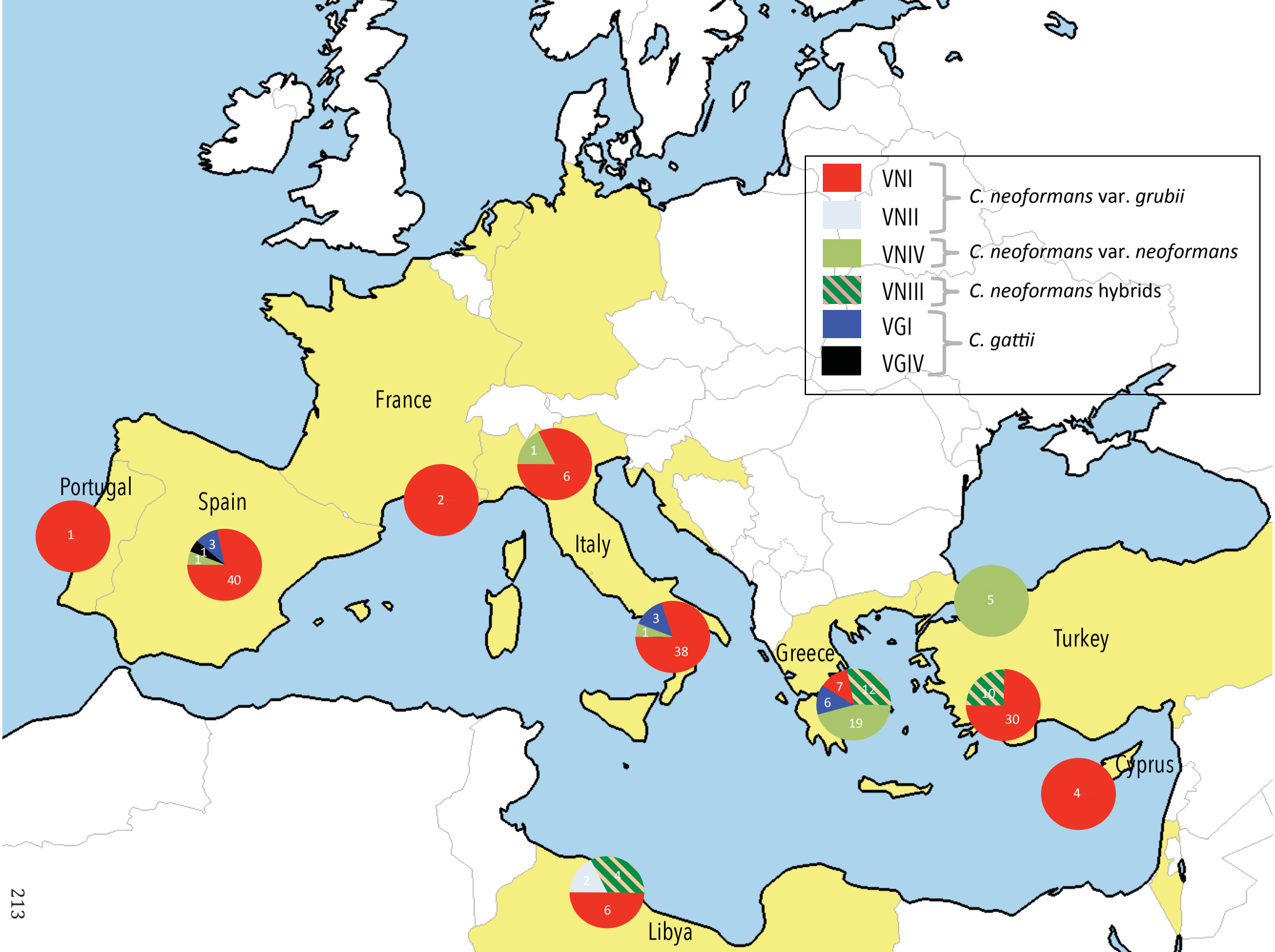

% of positive trees relative to each tree genus
25
20
15
10
5
0
Mediterranean climate
Sub-continental climate
Ceratonia
Eucalyptus
Olea
Pinus
Platanus
Prunus
Quercus
C. neoformans hybrids
C. neoformans var. neoformans
C. neoformans var. grubii
C. gattii


VNI
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VNIII
VGI
VGIV
C. neoformans var. grubii
C. neoformans var. neoformans
C. neoformans hybrids
C. gattii
Portugal
Spain
France
Italy
Greece
Turkey
Cyprus
Libya
1
40
1
3
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2
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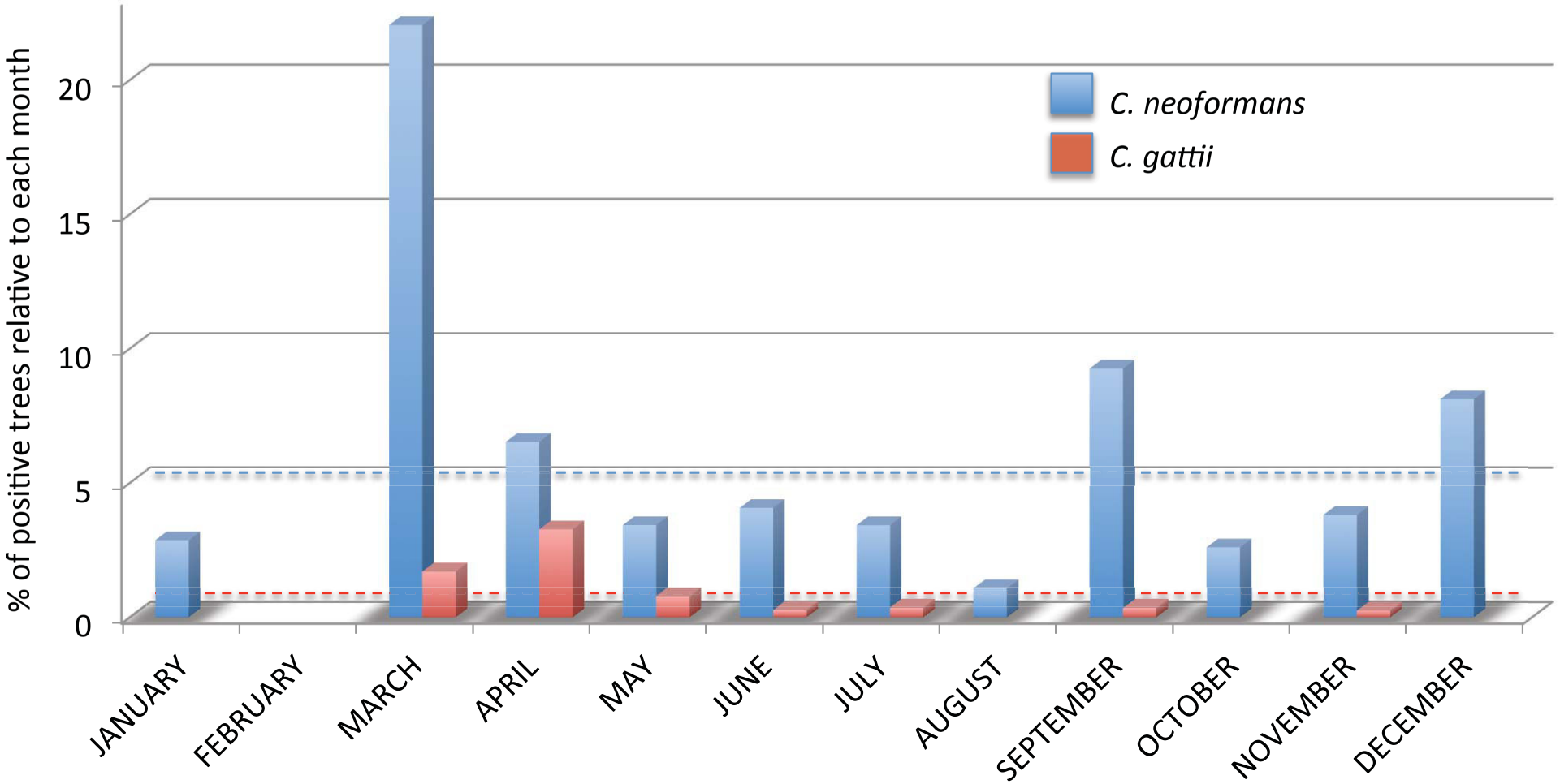
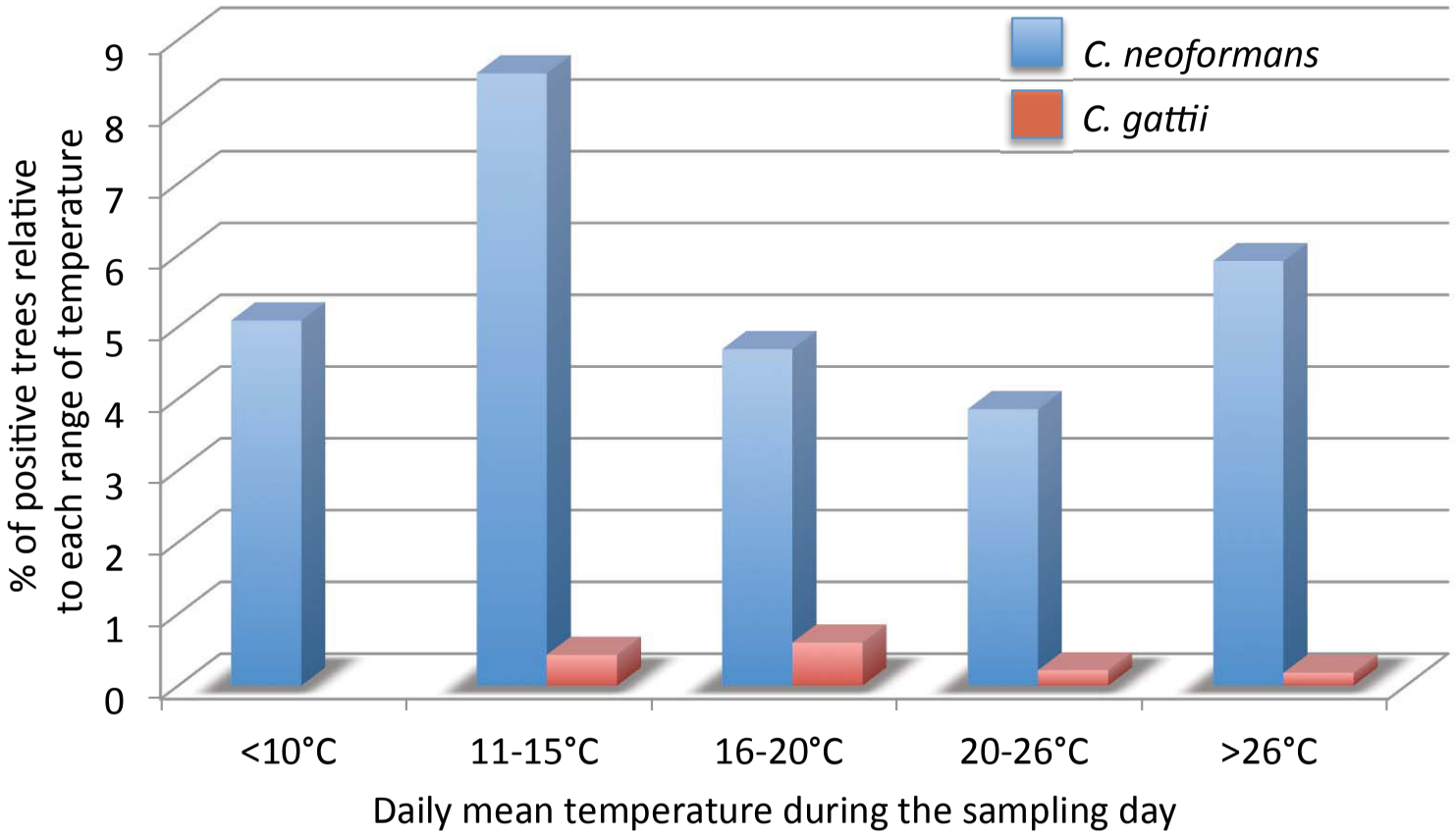
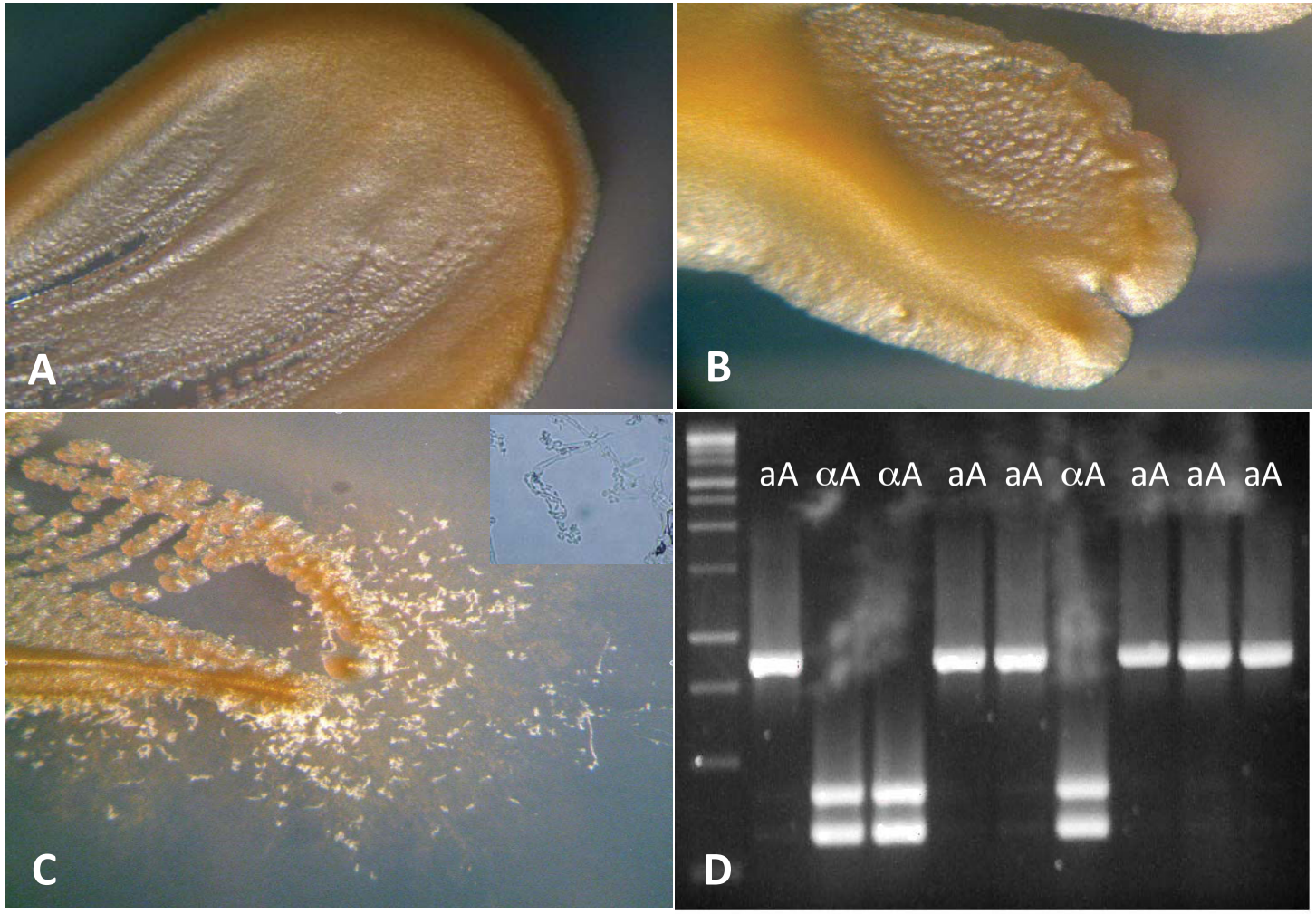

% of positive trees relative to each month
0
5
10
15
20
C. neoformans
C. gattii
JANUARY
FEBRUARY
MARCH
APRIL
MAY
JUNE
JULY
AUGUST
SEPTEMBER
OCTOBER
NOVEMBER
DECEMBER


C. neoformans
C. gattii
% of positive trees relative to each range of temperature
0
1
2
3
4
5
6
7
8
9
<10°C
11-15°C
16-20°C
20-26°C
>26°C
Daily mean temperature during the sampling day


A
B
C
D
aA αA αA aA aA αA aA aA aA

TREE SPECIES	N° SAMPLED TREE	N° POSITIVE TREES	N° EXAMINED SAMPLES	LEAVES	SOIL	BARK	FLOWERS	TRUNK HOLLOW	OTHER
Abies spp.	18		18	0	0	8	0	10	0
Abies procera	8		8	0	0	0	0	8	0
Acacia baileyana	3		6	3	0	0	3	0	0
Acacia dealbata	5		5	0	0	0	0	5	0
Acacia spp.	2		2	0	0	1	0	1	0
Acer campestre	15		15	0	0	0	0	15	0
Acer monspessulanum	2		4	0	0	0	0	4	0
Acer negundo	5		8	0	0	0	0	8	0
Acer nigrum	1		5	0	0	0	0	5	0
Acer platanoides	9		9	0	0	4	0	5	0
Acer pseudoplatanus	2		2	0	0	0	0	2	0
Acer saccharinum	4		6	0	0	0	0	6	0
Acer spp.	14		15	0	1	1	0	13	0
Aesculus ippocastanum	48	1	89	2	20	13	0	50	4
Ailanthus altissima	1		1	0	0	1	0	0	0
Albizia julibrissin	1		1	0	0	0	0	1	0
Albizia spp.	1		2	0	0	0	0	2	0
Amygdalus communis	3		6	3	3	0	0	0	0
Amygdalus webbii	1		2	1	1	0	0	0	0
Araucaria bidwillii	1		1	0	0	0	0	1	0
Arbutus unedo	11		19	3	0	0	0	15	1
Betula pendula	15		46	0	2	4	0	40	0
Calycanthus praecox	1		1	0	0	0	0	1	0
Carpinus betulus	24	1	78	6	21	7	0	33	11
Castanea sativa	24		46	2	4	6	2	32	0
Catalpa bignonioides	4		6	0	0	0	0	6	0
Cedrus atlantica	7		13	4	2	1	0	6	0
Cedrus deodara	2		3	1	1	0	0	1	0
Cedrus libani	10		10	0	0	0	0	10	0
Cedrus spp.	1		1	0	0	0	0	1	0
Celtis australis	16		25	0	0	1	0	24	0
Ceratonia siliqua	130	36	234	9	28	6	7	177	7
Cercis siliquastrum	3		5	0	1	1	0	3	0
Chamaecyparis lawsoniana	1		4	1	1	1	0	1	0
Chamaerops humilis	1		4	1	1	1	0	1	0
Cinnamonum camphora	1		1	0	0	0	0	1	0
Cinnamonum glanduliferum	1		1	0	0	0	0	1	0
Citrus limon	6		11	4	1	0	0	6	0
Citrus reticulata	2		10	0	0	0	0	10	0
Citrus sinensis	1		1	0	0	0	0	1	0
Colocedrus decurrens	2		2	2	0	0	0	0	0
Corylus avellana	4		5	2	1	0	0	2	0
Cotinus coggygira	1		4	1	1	1	0	1	0
Crataegus laevigata	2		2	0	1	0	0	1	0
Crataegus spp.	8		8	0	0	0	0	8	0
Crocus longiflorus	1		1	0	0	0	1	0	0
Cupressus arizonica	2		2	0	0	0	0	2	0
Cupressus macrocarpa	1		1	0	0	1	0	0	0
Cupressus sempervirens	33		53	3	6	6	0	38	0
Cupressus spp.	14		25	2	2	4	2	13	2
Cycas revoluta	1		4	1	1	1	0	1	0
Cydonia oblonga	1		3	0	1	1	0	1	0
Dendrocalamus giganteus	3		6	3	0	0	0	0	3
Diospyros kaki	2		4	0	1	1	0	2	0
Diospyros virginiana	1		3	0	1	1	0	1	0
Douglas fir	12		15	0	0	0	0	15	0
Elaeagnus angustifolia	3		3	0	0	3	0	0	0
Eriobothrya japonica	2		3	3	0	0	0	0	0
Eucalyptus cornuta	11		11	0	0	0	0	11	0
Eucalyptus odorata	15		18	0	0	0	0	18	0
Eucalyptus accedens	1		2	1	1	0	0	0	0
Eucalyptus astringens	27		32	2	3	0	0	27	0
Eucalyptus brockwayii	25		29	3	2	0	0	24	0
Eucalyptus calycogona	1		2	0	0	0	1	1	0
Eucalyptus camaldulensis	1069	52	1671	238	274	274	35	848	2
Eucalyptus dawsonii	1		4	1	1	0	1	1	0
Eucalyptus globulus	6		13	2	2	2	0	7	0
Eucalyptus gomphasephala	85		93	10	6	0	0	77	0
Eucalyptus intertexta	5		5	0	0	0	0	5	0
Eucalyptus leucoxylon	4		5	1	1	0	0	3	0

Eucalyptus melliodora	21		26	4	4	0	0	18	0
Eucalyptus nicholii	3		8	3	1	0	0	0	4
Eucalyptus occidentalis	45		54	5	0	5	0	44	0
Eucalyptus platypus	1		2	1	1	0	0	0	0
Eucalyptus pruinosa	1		2	0	0	0	1	1	0
Eucalyptus salmaniphloia	8		10	0	0	0	0	10	0
Eucalyptus sargenti	46		52	5	5	0	0	42	0
Eucalyptus toreliana	1		2	0	1	0	0	1	0
Eucalyptus torquata	1		2	0	1	0	0	1	0
Eucalyptus spp.	15		61	9	8	22	0	18	4
Fagus sylvatica	30		41	1	2	2	0	36	0
Ficus benjamin	18		25	0	0	0	0	25	0
Ficus carica	39		67	4	3	5	0	54	1
Ficus elastica	3		3	0	0	0	0	3	0
Ficus macrophylla	2		4	0	0	0	0	4	0
Ficus spp.	2		3	0	0	0	1	2	0
Fraxinus angustifolia	1		3	0	0	0	0	3	0
Fraxinus excelsior	1		1	0	0	0	0	1	0
Fraxinus ornus	14		22	0	0	0	0	22	0
Fraxinus spp.	3		3	0	0	0	0	3	0
Ginkgo biloba	3		10	1	1	1	0	7	0
Gleditsia triacanthos	3	1	30	3	14	3	0	8	0
Grevillea robusta	1		1	0	0	0	0	1	0
Hedera helix	4		16	0	0	0	0	16	0
Ilex aquifolium	2		5	1	0	0	0	4	0
Jacaranda ovalifolia	1		1	0	0	0	0	1	0
Jacaranda spp.	1		1	0	0	0	0	1	0
Juglans nigra	9	1	30	0	0	0	0	30	0
Juglans regia	5		9	2	0	1	0	5	1
Juglans spp.	1		1	0	0	0	0	1	0
Juniperus oxycedrus	1		2	0	0	1	0	1	0
Juniperus spp.	10	2	10	0	0	0	0	10	0
Lagunaria patersonii	1		1	0	0	0	0	1	0
Lantana spp.	1		1	0	1	0	0	0	0
Laurus nobilis	9		22	2	1	2	0	17	0
Lavandula sp.	1		2	2	0	0	0	0	0
Ligustrum vulgare	2		7	1	2	2	0	2	0
Liquidambar orientalis	1		3	0	1	1	0	1	0
Lupinus luteus	2		6	2	0	0	2	0	2
Magnolia grandiflora	45		64	0	17	0	1	46	0
Magnolia soulangeana	3		5	4	1	0	0	0	0
Magnolia spp.	3		3	0	0	1	0	2	0
Malus domestica	2		10	0	0	0	0	10	0
Mimosa pudica	1		1	0	0	0	0	1	0
Morus alba	7		14	2	2	8	0	2	0
Morus bombycis	2		2	0	0	0	0	2	0
Morus nigra	2		6	0	0	0	0	6	0
Morus spp.	10		16	0	4	5	1	6	0
Nerium oleander	8		14	2	1	1	2	8	0
Olea europaea	527	48	976	29	230	79	4	633	1
Olea sylvestris	9	2	14	0	9	0	0	5	0
Ostrya carpinifolia	4		5	0	0	0	0	5	0
Palm	3		3	0	1	0	0	2	0
Paulownia tomentosa	2		2	0	0	0	0	2	0
Persea gratissima	1		1	0	0	0	0	1	0
Phillyrea angustifolia	1		1	0	0	0	0	1	0
Phillyrea latifolia	1		1	0	0	0	0	1	0
Phoenix canariensis	7		9	0	0	2	0	5	2
Phoenix dactilyfera	3		4	0	0	0	0	4	0
Phytolacca dioica	2		2	0	0	0	0	2	0
Picea abies	1		1	0	0	0	0	1	0
Picea sitchensis	1		1	0	0	0	0	1	0
Pinus halepensis	26	3	51	4	10	16	0	20	1
Pinus pinaster	70		131	19	33	14	0	60	5
Pinus pinea	296	17	495	21	91	44	4	329	6
Pinus radiata	1		4	1	1	1	0	1	0
Pinus spinosa	1		2	2	0	0	0	0	0
Pinus sylvestris	10		30	0	2	0	0	28	0
Pinus spp.	109	4	177	8	18	27	2	118	4
Pistacia lentiscus	2		2	0	0	0	0	2	0
Pistacia terebinthu	1		1	0	0	0	0	1	0
Pittosporum tobira	3		4	0	1	0	0	3	0

Platanus acerifolia	3		6	0	0	0	0	6	0
Platanus hispanica	3	1	7	2	2	0	0	2	1
Platanus occidentalis	2		10	0	0	0	0	10	0
Platanus orientalis	34	7	164	3	59	29	0	73	0
Platanus x hybrida	19		33	2	4	2	0	23	2
Platanus spp.	18		28	0	6	7	0	15	0
Platycladus orientalis	2		2	0	0	0	0	2	0
Populus alba	6		7	0	0	2	0	5	0
Populus nigra	5		8	0	0	0	0	8	0
Populus tremula	2		2	0	0	0	0	2	0
Prunus armeniaca	3	1	4	3	0	0	0	0	1
Prunus avium	7	2	14	0	3	1	0	10	0
Prunus cerasifera	14		17	0	0	3	0	14	0
Prunus cerasus	3		11	0	0	0	0	11	0
Prunus dulcis	42	4	108	3	22	29	1	52	1
Prunus persica	2		2	2	0	0	0	0	0
Prunus serrulata	2		2	0	0	0	0	2	0
Prunus spinosa	13		20	3	5	0	0	12	0
Prunus spp.	10	2	10	0	0	0	0	10	0
Pterocarya fraxinifolia	1		1	0	0	0	0	1	0
Punica granatum	5		5	1	0	1	0	3	0
Pyrus communis	2	1	4	0	1	1	0	2	0
Quercus cerris	1		1	0	0	0	0	1	0
Quercus coccifera	2	1	3	0	0	0	1	2	0
Quercus ilex	98		162	11	25	15	0	106	5
Quercus palustris	2		2	0	0	2	0	0	0
Quercus pubescens	5		7	0	0	0	0	7	0
Quercus pubescens	1		3	0	0	0	0	3	0
Quercus pubescens	4		5	0	1	0	0	4	0
Quercus pubescens	6		6	0	0	0	0	6	0
Quercus robur	32	1	86	9	9	2	4	59	3
Quercus rubra	6		6	0	0	0	0	6	0
Quercus suber	3		7	2	1	2	0	2	0
Quercus spp.	19		20	0	0	1	0	19	0
Rapidophilum hystrix	1		5	0	0	0	0	5	0
Robinia pseudoacacia	7		18	1	1	1	0	15	0
Rosmarinus officinalis	1		5	0	0	0	0	5	0
Rosmarinus officinalis	1		2	1	1	0	0	0	0
Salix alba	1		2	0	1	1	0	0	0
Salix babylonica	5		11	2	2	2	0	5	0
Sambucus nigra	5		9	3	0	0	2	4	0
Schinus molle	4		6	0	1	2	0	3	0
Sophora japonica	1		1	0	0	0	0	1	0
Sorbus aucuparia	1		1	0	0	0	0	1	0
Sorbus domestica	4		20	0	0	0	0	20	0
Sterculia platanifolia	1		1	0	0	0	0	1	0
Tamarix gallica	1		2	0	0	0	0	2	0
Tamarix spp.	5		7	0	2	5	0	0	0
Taxodium distichum	1		1	0	0	0	0	1	0
Taxus baccata	8		15	1	2	2	0	10	0
Thuja occidentalis	1		1	0	0	0	0	1	0
Tilia cordata	15		23	0	5	0	0	18	0
Tilia grandifolia	2		10	0	0	0	0	10	0
Tilia platyphyllos	17		26	3	3	3	0	17	0
Tilia spp.	21		27	0	4	12	0	11	0
Tipua tipus	1		2	0	0	2	0	0	0
Ulex minor	1		1	1	0	0	0	0	0
Ulmus glabra	1		1	0	0	0	0	1	0
Ulmus minor	2		2	0	0	0	0	2	0
Ulmus spp.	1		1	0	0	0	0	1	0
Vitis vinifera	5		11	2	1	1	0	7	0
Ziziphus jujuba	2		2	0	0	0	0	2	0
Ziziphus zizyphus	1		5	0	0	0	0	5	0
Ziziphus spp.	1		1	0	0	0	0	1	0
Decaying wood	5		17	0	11	4	0	1	1
Unidentified	40		73	6	8	12	4	41	2

Total	**3765**	**188**	**6436**	**514**	**1039**	**738**	**82**	**3984**	**77**