Types and susceptibility profiles of Cryptococcus neoformans in environmental samples in Croatia and Kosovo

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This dissertation has been developed in collaboration with the Department for Parasitology and Mycology, Croatian Institute for Public Health, Zagreb, Croatia as well as with Laboratory Micologia Medica, Universita degli Studi di Milano, Milan, Italy and Department of Clinical and Molecular Microbiology, University Hospital Centre Zagreb, University of Zagreb School of Medicine, Zagreb, Croatia.

Mentor: Associate Professor Ivana Mareković, MD, PhD

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List of symbols and abbreviations

AIDS	Acquired immunodeficiency syndrome
AFLP	Amplified fragment length polymorphism
GPA	G protein alpha subunit
cAMP	Cyclic Adenosine monophosphate
CNS	Central nervous system
HIV	Human immunodeficiency virus
LHF	Heteroresistance to fluconazole
BMD	Broth microdilution
RPMI	Roswell Park Memorial Institute
ECMM	European Confederation of Medical Mycology
ECV	Epidemiological cut-off
CLSI	Clinical Laboratory Standard Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
MIC	Minimaly inhibitory concentration

1. INTRODUCTION AND BACKGROUND

Cryptococcus is an encapsulated basidiomycetous yeast that causes life-threatening infections in an immunocompromised host (Figure 1). Cryptococcosis is caused primarily by two species complex, *C. neoformans* and *C. gattii* species complex. Only in rare circumstances are other cryptococcal species found to cause the disease in humans. While *C. neoformans* species complex mainly affects patients with acquired immunodeficiency syndrome (AIDS) and those who are immunosuppressed (transplant patients, those on long-term corticosteroids, and those who are prescribed monoclonal antibodies), a quarter of patients with *C. gattii* species complex infections are immunocompetent and healthy.

Infection of the brain and meninges by *C. neoformans* species complex is the most important clinical form in immunosuppressed individuals as the organism has this unique and unexplained predisposition to establish an infection at this site (1, 2). Although the lung is most likely to have been the initial portal of entry and infection, the localized pulmonary form is found as a solitary or as multiple small nodules in an asymptomatic individual. Symptoms of acute pneumonia with cough, fever, and lobar pulmonary infiltrates affecting alveoli or a diffuse interstitial pattern, indistinguishable from *Pneumocystis jirovecii* infection occurs in patients with AIDS. Very rarely, it can present as an acute respiratory distress syndrome. Involvement of skin, eyes, bones, joints, heart and kidney is described (3).

Cryptococcosis caused by *C. gattii* species complex is significantly less frequent globally (<20%) than *C. neoformans* species complex (80%), and the major risk factors for *C. gattii* species complex infection remain unclear. In immunocompetent patients, *C. gattii* species complex infection is clinically characterized by pulmonary complications and pneumonia, with or without meningitis, with substantial mortality. Clinical presentation between regions may not be generalizable. More recent study from New Zealand and Australia revealed CNS involvement in 85% of 86 *C. gattii* species complex infected patients. By contrast, lung disease predominated in the outbreaks in North America with only 20-49% of patients having CNS disease (4). *C. gattii* species complex infections may involve skin, bone, joint, larynx and lymph nodes. Intra-abdominal infection is rare but may mimic malignant disease (5).

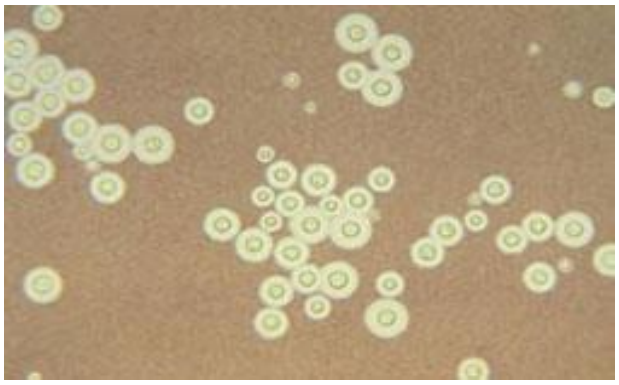


Figure 1. A photomicrograph of *Cryptococcus neoformans* species complex stained with India ink (6). According to CDC, Center for Disease Control Prevention, Fungal diseases. Internet image.

1.1 Taxonomy and classification

In 1894, pathologist Otto Busse and surgeon Abraham Buschke first described *C. neoformans* species complex as a human pathogen when they isolated a 'Saccharomyces-like' organism from a bone infection in a young woman. Later that year, Francesco Sanfelice reported the isolation from fermenting peach juice of a similar yeast, which he named *Saccharomyces neoformans* because of its unique colony form. Finally, in 1901, Jean-Paul Vuillemin renamed the organism "*C. neoformans"* because it did not produce ascospores, which is a defining characteristic of the genus *Saccharomyces* (7).

In 1970 Gatti and Eeckels described *C. gattii* species complex as an unusual variant of *C. neoformans* species complex causing meningitis in a 7-year-old boy with leukemia from the Congo (8). More recently, molecular analysis of a clinical isolate from France, reported by Curtis in 1896 as *Saccharomyces subcutaneus tumefaciens*, revealed that this was, in fact, the first reported case of human *C. gattii* species complex infection (9).

In 1950s, it was found that *C. neoformans* species complex could be divided into three groups based on capsular antigen properties, and these were named as serotypes A, B, and C. The fourth serotype, named serotype D, was identified in 1968 (10, 11).

Until recently, three varieties could be distinguished within *C. neoformans* species complex: variety *grubii* (serotype A; AFLP genotype 1), variety *neoformans* (serotype D; AFLP genotype 2), and a hybrid between these two varieties (serotype AD; AFLP genotype 3). A third variety, *C. neoformans* variety *gattii* (serotype B and C), was advanced to the species level in 2002 and was named *C. gattii* (12, 13).

Recent attempts to characterize the hybrid strains of *C.neoformans* species complex have led to the identification of a cryptic population of hybrid strains (H strain) with double DNA content but only a single mating type allele. Molecular typing of the strain collected in the European Confederation of Medical Mycology (ECMM) cryptococcosis survey revealed the presence of six H strain in the European population of *C.neoformans* species complex. H strain are subpopulation of *C.neoformans* species complex that originate from the hybridization of serotype A and D population, and can be isolated from clinical samples (14).

C. neoformans and *C. gattii* species complex are haploid yeasts that exist in two mating types, MATa and MATα. MATα type predominates in nature and among clinical isolates as well for unknown reasons (15).

The development of molecular techniques for microorganism typing has brought new possibilities to the fields of taxonomy identifications and diagnosis, as well as revealing more about microorganism phylogeny and evolution. Fungal taxonomy has been recently reconstructed on the basis of genome information, and nomenclatural rules recently proposed a rearrangement into seven species: *Cryptococcus neoformans (*var. *grubii), Cryptococcus deneoformans (*var. *neoformans), Cryptococcus gattii, Cryptococcus bacillisporus, Cryptococcus deuterogattii, Cryptococcus tetragattii, Cryptococcus decagattii* (16, 17, 18).

The taxonomy of *C. neoformans* species complex is still under major investigation. Since the seven new species are not known to be clinically distinguishable, universal adoption of the new system of nomenclature should be delayed until more-detailed studies employing a larger number of isolates reveal the clinical and biological relevance of the new species. Adoption of the proposed nomenclature at this juncture might separate taxonomy from clinical practice and in doing so inhibit the progress of both fields. Instead of "species," "species complex" would accommodate already-known cryptic species and those that might be discovered in the future.

At this moment, the term "species complex" is used to comprise all genetic, pathogenic, epidemiological, ecological, and clinical differences between the strains (18-22).

1.2 Pathogenesis of cryptococcosis

In the pathogenic fungi, a unique species cluster of the genera *Cryptococcus* exhibits novel virulence attributes and presents a similar example, enabling definition of virulence mechanisms in the fungal kingdom (23).

Humans are exposed to infectious agents via inhalation and cutaneous exposure and from mibrobiota (24).

C. neoformans species complex has the ability to grow in the filamentous and yeast forms, while morphological and genetics plasticity of *C. neoformans* species complex might contribute significantly to its survival from its environmental predators and propagation in animal hosts. The existence of the disease-associated yeast form in C*ryptococcus* strongly suggests that switching to yeast form and/or suppressing filamentous growth is important for this fungus to cause disease, which is similar to what has been proposed for dimorphic fungi. The reduction or loss of virulence associated with the filamentous form of *C. neoformans* species complex may be explained by the loss of expression of virulence associated hyphal specific genes, or a difference in the ability of the two morphotypes to defend host responses (1).

C. neoformans species complex has the ability to dramatically enlarge its size during infection to form "titan cells" that can reach up to 100 microns in cell body diameter, in contrast to typical cells' size of 5-7 microns. These titan cells pose a problem for the host because they contribute to fungal survival, dissemination to the central nervous system, and possibly even latency. Recent studies have clearly shown that titan cell production is a distinct morphological transition in *C. neoformans* species complex that promotes virulence of this important human fungal pathogen, suggesting a role for titan cells in the establishment and persistence of pulmonary infection (25).

Several studies have demonstrated that spores are capable of producing infection, serving as infectious propagules (24, 26).Spores isolated by microdissection were found to readily germinate even on water agarose medium. Humans are exposed to spores or dehydrated yeast cells of *Cryptococcus* by inhalation, leading to an initial pulmonary infection that can be asymptomatic, limited or can disseminate (24). It has been previously suggested that most people are exposed to *Cryptococcus* species complex early in childhood (27).

The interaction between macrophages and *C. neoformans* species complex is critical for extrapulmonary dissemination of this pathogenic yeast (28).Cryptococcosis begins with inhalation of desiccated airborne yeast cells, or possibly sexually produced basidiospores, into the lungs. Because the propagules are small (1.5–3.5 μm), they reach the distal airways and come into contact with alveolar macrophages. Activated alveolar macrophages recruit other immune cells through cytokines and chemokines and elicit a proper Th1 response and granulomatous inflammation (29).

A key feature of cryptococcal pathogenesis involves *C. neoformans* species complex leaving the lungs to enter the peripheral blood circulation and the central nervous system (CNS) compartment, causing meningoencephalitis ranging from progressive headache to serious neurological symptoms, including coma and death (24). In a normal host, an effective immune response eliminates most inhaled cryptococci. In contrast, in an immunocompromised host, the cryptococcal cells proliferate, hematogenously disseminate to the brain by crossing the blood–brain barrier and adapt to the suboptimal levels of oxygen and nutritional conditions of the brain to multiply and cause meningoencephalitis. Although virtually every organ in the body can be involved, infection of the central nervous system (CNS) is the most common clinical manifestation of cryptococcosis and the most common cause of death (29).

Another commonly occurring cryptococcal infection is the formation of a small lung–lymph complex where yeasts remain viable but dormant and these patients remain clinically asymptomatic until loss of local immunity. Upon this loss of immunity, the dormant yeast cells are activated and begin to multiply in the pulmonary–lymph node complex and disseminate into extrapulmonary sites (29).

Although this fungus is found primarily in the environment, it possesses features that allow survival facing multiple stress factors and proliferation within a human host. Virulence factors that ensure the infection ability to avoid the complex immune response and replicate are the following: metabolic adaptation to temperature, nutrients and metals, iron availability, adaptation to free radicals, melanin and antioxidant mechanisms, the polysaccharide capsule, capsular composition and capsule organization, the capsule as a protective structure, changes in capsular size and structure as mechanisms of immune evasion, exopolysaccharides, intracellular survival inside macrophages, recognition by macrophages, phagocytosis, survival and proliferation inside macrophages (30).

Synthesis of polysaccharide capsule is rather well investigated. It includes synthesis of activated sugar donors, transport of these compounds into the Golgi, polymerization of capsule components, export of product polysaccharides, and assembly at the cell surface. Enzymes required for synthesis of these proteins have been biochemically characterized. The lack of these proteins reduces capsule production and virulence in animal models. It is still not clear how capsule grows and how it is attached to cell wall. α -glucan, chitin and cations appears to have a role in association of capsule to the cell wall. The size and antigenicity of the *C. neoformans* species complex capsule is dynamic and regulated by the fungus in response to different environmental cues (29).

The capsule of *Cryptococcus neoformans* species complex is one of its major virulence factors as evidenced by the fact that capsular strains are virulent. The capsule contains three major components: glucoronoxylomannan (GXM), galactoxylomannan (Ga1XM) and mannoproteins (31). Cryptococcal mutants deficient in melanin or capsule production are less virulent than wild type strains in animal models of cryptococcal infection (32). All cells must be able to sense and respond to changes in the environment. There host specific signals often induce the production of microbial virulence factors that allow the pathogen to survive within new environment.GPA1 has a role in sensing diverse environmental signals required for mating and virulence by regulating cAMP metabolism in *C. neoformans* species complex (32).

In vitro, diverse signals like iron concentration, CO_2 level, pH, and nitrogen or glucose concentrations are important modulators of capsule size (29).

Melanin is produced by a wide variety of fungal species and the pigment deposited in the cell wall is known to play an important protective role against environmental stress. Numerous studies demonstrated role of cryptococcal melanin in protection from phagocytosis and killing by host cells, oxidants and microbicidal peptides. There are also reports about melanin protecting cryptococcal cell from antifungals amphotericin B and azoles (29).

The primary reason that both *C. neoformans* and *C. gattii* species complex are the only successful pathogens among *Cryptococcus* species is their ability to grow robustly at physiological temperatures. All the remaining cryptococcal species produce a polysaccharide capsule with or without melanin but fail to grow or grow poorly at 37°C (29).

Degradation enzymes produced by cryptococcal species are also important virulence factors. The most studied are urease and phospholipase B, both shown to have a role in cryptococcal pathogenicity. They promote intracellular survival of the yeast, hydrolysis of host cell membrane needed for penetration into the cell, immunomodulation and dissemination from lung to the brain (29).

Being an accidental, but a successful pathogen with a broad host range, advanced replicative cell aging of *C. neoformans* species complex is an emerging natural trait that contributes to virulence. The ‘old cell phenotype’ generated when *C. neoformans* species complex undergoes replicative aging, exhibits a thickened cell wall, inhibits phagocytosis and killing by antifungals *in vitro*. Together with the fact that old cells accumulate *in vivo*, this emergent characteristic could have significant impact on cryptococcal virulence and infection pathogenesis, and contribute to treatment failure (30).

It has been demonstrated that some infectious agents, such as *Mycobacterium tuberculosis*, *Trypanosoma cruzi*, *Leishmania chagasi*, *Klebsiella pneumoniae* and others can cause DNA cellular damages. Despite that, association between the infection and carcinogenesis has not been completely elucidated. One of the mechanisms is a generation of inflammatory response that culminates with the activation of the oxidative metabolism for the destruction of the pathogen. High NO production in peripheral blood mononuclear cells has been demonstrated only in association with *C. gattii* species complex (33).

C. gattii species complex expresses the same suite of major virulence determinants as *C. neoformans* species complex. Experimental data on the pathogenesis of *C. gattii* species complex are limited. In animal models, as with the much more intensively studied *C. neoformans* species complex, the outcome of exposure is determined by a complex interaction between pathogen-derived and host factors (34).

In human cryptococcosis due to *C. gattii* species complex, clinical and host differences associated with genotype have been observed. VGI infection typically presents with CNS disease, including cryptococcomas, often with concurrent lung lesions, whereas VGII infection more commonly presents with pulmonary disease. Both of these molecular types of *C. gattii* species complex have a predilection for immunocompetent hosts. In contrast, molecular type VGIII was present in a series of HIV-infected patients (7).

1.3 Epidemiology of cryptococcosis

Cryptococcosis is a potentially lethal disease of human/animals caused by *Cryptococcus neoformans* and *C. gatti* species complex.

An estimated 220,000 cases of cryptococcal meningitis occur among people with HIV/AIDS worldwide each year, resulting in nearly 181,000 deaths. Cryptococcosis is often fatal, even if treated. It is estimated that the three-month case fatality rate is 9% in high-income regions, 55% in low- and average-income regions, and 70% in Sub-Saharan Africa. Awareness and widespread availability of antiretroviral therapy in developed countries has helped improve the immune systems of many human immunodeficiency virus (HIV) positive patients so that they do not become susceptible to infection with *Cryptococcus* species complex. However, cryptococcal meningitis is still a major problem in resource-limited countries where HIV prevalence is high and access to healthcare limited (35,36).

C. gatti species complex (AFLP4/VGI), *Cryptococcus tetragttii* (AFLP/VGIV), *C. neoformans* and *C. deneoformans* have been isolated from both clinical and environmental sources in Europe. The study was conducted quantify the people in Europe and the entire Mediterranean area who are under risk associated with each of the three fungal pathogens in a spatially explicit way, generating a series of maps and population statistics per country. Niche modeling was applied to estimate the potential distribution of each fungal pathogen, then these models were overlapped with a map of population density to estimate risk levels. The potential number of people per risk level and per country was quantified using a map of population count per pixel. Prevalence of HIV per country was also included in the analysis to quantify the HIV-infected population under potential risk. People under risk associated with exposure to *C. gattii* species (*C. gattii* and *C. tetragattii*) reached 137.65 million, whereas those exposed to *C. neoformans* and *C. deneoformans* were 268.58 and 360.78 million people, respectively. More than a half million HIV-infected patients are exposed to each of the two species of the *C. neoformans* species complex, and more than 200,000 to the *C. gattii* species complex. The present results can be useful for public health planning by European governments, focusing on the provision of inputs for a "screen-and-treat" approach, availability of medical resources, and continuous monitoring programs in risk zones (37).

Brazilian studies show the highest prevalence of cryptococcosis in the age group between 12 and 39 years in both genders. Patients in this age group were born and raised in the period coinciding with the emergence and spread of HIV, which was accompanied by changes in sexual behaviour, including increased number of partners, thereby contributing to the spread of HIV and opportunistic infections such as cryptococcosis (38).

A study by Chan *et al.,* compared *C. neoformans* species complex with *C. gattii* species complex based on clinical and microbiological characteristics of cryptococcosis in Singapore. Of 62 patient with cryptococcosis, *C. neoformans* var g*rubii* was the predominant subtype affecting mainly younger immunocompromised hosts with HIV infection (39).

Differently, the majority of cryptococcosis in China were reported in the HIV uninfected patients especially immunocompetent hosts. Compared to immunocompromised patients, immunocompetent patients with cryptococcal meningitis often are presented with more intense inflammatory responses and more severe neurological complications, but less fungal burdens and disseminated infection (40).

Bovers *et al.*, described a novel *C. neoformans and C. gatti* species complex hybrid strain (serotype AB) that was previously described as *C. gatti* species complex and that caused a lethal infection in an AIDS patient from Canada (41). In 2003 Mirza *et al.,* examined trends in the incidence and epidemiology of cryptococcosis, of active population. This finding suggest that HIV infected persons who continue to develop cryptococcosis in the era of highly active antiretroviral therapy in the United States are those with limited access to health care (42).

Cases of cryptococcal infection in an immunocompetent host have been reported and primarily include pulmonary manifestations and cutaneous lesions, but there is also a case of crryptococcoma in an immunocompetent patient reported. It is important to recognize, however, that untreated cryptoccocal infection has resulted in severe pulmonary infection with respiratory failure, or systematic dissemination even in immunocompetent hosts. Cutaneous cryptococcosis lesions may be the first clinical manifestation of disseminated infection, particularly in subjects with cell immunity deficits (43,44). Direct inoculation into the skin as a result of traumatic injury can cause the condition of so called primary cutaneous cryptococcosis (43).

Knowledge regarding the global burden of *C. gattii* species complex infections is limited and incomplete. However, it is less frequent globally and the main risk factors remain unknown. Although more frequently encountered in tropical and subtropical parts of the world, lately it is also found in more temperate climate areas (43).

Hagen *et al*., reported activated dormant *C. gattii* species complex infection in a Dutch tourist who visited Vancouver Island (Canada). The case presented in this study shows that a latent infection caused by *C.gattii* species complex isolate which was identical to the Vancouver Island outbreak genotype AFLP6A/VGIIa, was activated during the period that the patient underwent a treatment with corticosteroids (prednisolone) in order to suppress the autoimmune hemolytic anemia that the patients developed five months after she visited Vancouver island (45).

Differences in ecological origin (AIDS) patients, non AIDS patient, animals or the environment were found to be statistically not significant (46).

1.4 Geographical distribution of *Cryptococcus neoformans* species complex in the environment.

The first direct evidence that *C. neoformans* species complex is present in the soil was demonstrated by Emmons in 1951 (47, 48). Numerous studies documented the presence of *C. neoformans* species complex in environmental samples from different geographical locations in the world. *C. neoformans* species complex was isolated in different parts of the world, like India, Korea, Venezuela, Singapore, China, Brazil from different environmental sources (38, 39, 49-52).

The first epidemiological survey in Spain was in year 1999 by Baro *et al.*, were *Cryptococcus neoformans* species complex was isolated from goats that suffered from pulmonary disease. This finding suggests that the autochthonous distribution of serotype B of *C. neoformans* species complex is not limited to tropical and subtropical areas but also includes areas with temperate climates, such as Spain, Portugal and Italy (53).

C. neoformans species complex primarily infect immunocompromised patient and occurs worldwide, whereas *C. gatti* species complex primarily infects healthy individuals and has been thought to occur in subtropical regions. However, the recent outbreak of infection with *C. gattii* species complex on Vancouver Island, British Columbia, Canada, expansion of this outbreak to mainland Canada and the Pacific Northwest region of the United States and identification of *C .gatti* species complex in Europe show that *C. gattii* species complex can also occur in temperate climates (54).

Research has demonstrated that *C. gattii* species complex is more than just an opportunistic pathogen because it harbours distinct mechanisms geared to evade, subvert, and manipulate the host immune system, while maintaining intracellular growth. *C. gattii* species complex is likely to have a wider geographical distribution than presently appreciated based on the ongoing expansion of clinical and environmental reports worldwide (55).

A limited number of studies were carried out in relation to the occurrence and identification of *C. neoformans* species complex from the environment in Europe. An extensive environmental survey was carried out during 2012-2015 by Cogliati *et al*. representing 12 countries with 6436 samples, which documented the distribution of *C. neoformans* species complex all around the Mediterranean basin, whereas *C. gattii* species complex was isolated in Greece, Southern Italy, and Spain. Croatia was represented with 18 environmental samples. The results showed that both *C. neoformans* and *C. gattii* species complex are present in the Mediterranean environment in association with carob and olive trees. *C. neoformans* was more prevalent than *C. gattii* species complex with a 13-fold higher percentage of colonized trees (56).

Two studies reported about isolates of *C. neoformans* species complex in clinical and environmental samples from Croatia. The first study by Mlinaric-Missoni *et al.* included 48 clinical *C. neoformans* species complex isolates from 15 patients in Croatia that were retrospectively investigated using *in vitro* antifungal susceptibility testing and molecular techniques to determine mating types and serotypes. PCR-based mating type and serotype determination revealed that cryptococcosis in 12 patients (80%) was caused by mating type α, whereas the remaining three (20.0%) patients were found to be infected by cryptococcal isolates with the mating types α and a. Combined with the serotype of these isolates, it was found that six (40.0%) patients were infected with αA, another six (40.0%) with αD, two (13.3%) with αA-aD, and one (6.7%) patient with αD-aA. The frequency of cryptococcosis caused by genotype AFLP1 and AFLP2 isolates was 40.0% each (six patients each), whereas genotype AFLP3 isolates were observed to have infected three patients (20.0%). AFLP fingerprinting revealed more diversity among isolates that were isolated from the same patient (57). The before mentioned study by Cogliati *et al*., involved 18 environmental samples, with no positive results for *C. neoformans* species complex in Croati (57).

Data are lacking on the presence of *C. neoformans* species complex in Kosovo, both in clinical and environmental samples.

1.5 Natural habitat of *Cryptococcus neoformans* species complex

Following the first clinical case mentioned previously, for the next 57 years this pathogenic yeast was known only from clinical cases, until the globally renowned medical mycologist Chester Emmons reported four incidental isolations of *C. neoformans* species complex in the United States during an investigation of 716 soil samples for *Histoplasma capsulatum*, using the mouse-inoculation techniques (27). The environmental isolation of the organism by Emmons later succeeded from pigeon excreta as well (28).

Chowdhary *et al.* declared that environmental isolates consisted of the same variety as clinical isolates, suggesting that environmental sources can contribute to the development of cryptococcosis among immunocompromised patients, and also that there may be an epidemiological relationship between clinical and environmental isolates (49).

Globally, the distribution of *Cryptococcus* species in nature is widespread and is particularly linked to decaying wood of certain tree species, fruits and bird droppings, in particular pigeon droppings (30, 33, 34).

Pigeon itself is not a reservoir of the pathogen but may serve as its mechanical carrier and disseminator in the environment (28, 35). After studying the role of birds as carriers and transmitters of *C. neoformans* species complex, Cafarchia *et al.*, published a study where it was documented that excreta are an enriched medium that permits the growth of yeasts. Also, it is worth mentioning that *C. neoformans* species complex is obtained from old withered pigeon droppings, not fresh droppings (58).

Several studies have shown that *C.neoformans* species complex remains viable in the dried excrement of bird, especially the excrement of species of the synanthropic pigeon, *Columbia livia*. These birds have found ideal conditions for survival in cities. Urban architecture associated with an abundance of food, generates large areas of potential habitation and consequently large concentration of excrement that can serve as a potential sources of infection. The population of domestic pigeons has significantly increased in many parts of the world, including Brazil, and has become an environmental problem that directly influences public health (58).

The pet birds may cause a hazard to human health, particularly to immune compromised patients, children and the elderly. Current periodical examinations of environmental factors related to birds as a water, excreta and air must be advised to devoid the zoonosis hazard infection to human health (59).

The real reservoir seems likely to be trees where the fungus could find favorable microhabitats inside trunk hollows or fissures to survive. This has been recently shown by the results obtained in a large environmental survey carried out in the Mediterranean area (56).

A retrospective study by Randhawa *et al.*, indicates that decayed wood is as good, if not a better natural habitat of *C. neoformans* species complex. A study from 2011 identified 22 tree species belonging to 17 genera and 9 families as hosts of the *C. neoformans* species complex. A decade-long study (2001-2011) revealed that the prevalence of *C. gattii* and *C. neoformans* species complex differs considerably not only from one host to another but also among trees of the same host species occurring in a given area or in different geographical regions (60).

Wood decay within living trees is distinct from decay in dead trunks, branches, logs and stumps. Probably the niche of *C. neoformans* species complex is not restricted to decay within living trees, but these biotopes represent a natural model to study *C. neoformans* species complex diversity and ecology. Hollows of living trees provide environments that are sheltered, dump and probably less exposed to changes in climatic conditions. The observation reinforces the recent evidence for decaying wood inside trunk hollows of some trees to be a new natural habitat of the variety *neoformans*. Nevertheless, the possibility of an endogenous source inside the tree, such a persistent inoculum in cracks and fissures, must be considered. An interconnecting system of hollowed out areas within the solid xylem cylinder of each tree may support prolonged survival and escape of *C. neoformans* species complex during fires. Moreover, under unfavorable conditions, different genotypes may shelter as a dormant fungal propagule for long periods (61).

The interaction between *C. neoformans* and *C. gatti* species complex with plants provide evidence that this pathogen can complete its sexual cycle in nature in association with plants. During pathogen plant interaction, plants activate distinct defense responses to combat microbial invasion. It is discovered a potential parasitic relationship between *Cryptococcus*, a human fungal pathogen and plants (62).

Also, it is found that *C. neoformans* and *C. gattii* species complex can share the same natural biotope, thus establishing a possible link between them in their life cycle in nature and suggesting the primary niche for the species. Until year 2000 has been considered to occur in a distinct habitat: one geographically restricted, associated with eucalyptus trees, and the other one cosmopolitan, associated with various organic substrata (61).

Seasonal variations in the prevalence of *C. neoformans* species complex in decayed wood inside trunk hollows occur during the year. The lowest prevalence of *C. neoformans* species complex was noted during rainy season followed by higher incidence in the spring. These environmental conditions, mainly humidity, temperature and solar radiation, can variably affect the occurrence of serotypes A, B, and C (60).

Probably the niche of *C.neoformans* species complex is not restricted to decay within living trees, but the biotypes represent a natural model to study *C.neoformans* species complex diversity and ecology (61).

The soil might be another important ecologic niche of *C. neoformans* species complex (27). It was previously considered that *C. neoformans* species complex is spread globally in the soil contaminated by pigeon droppings. Furthermore, exposure to colonized soil or trees enhances the possibility of inhalation of airborne propagules (yeast forms and basidiospores), and may cause pulmonary and central nervous system diseases (17, 47).

C. neoformans species complex can colonize variously in the different zones of a city, and its population's densities can be transmitted within the zones (48). The presence of pigeons and *Eucalyptus* trees in the vicinity to places such as rest homes and hospitals should be considered a risk factor for this vulnerable population (62).

Research conducted by Leite *et al.*, published in 2012 described the presence of *Cryptococcus* spp. on dust found on books in three libraries in the city of Cuiaba in Mato Grosso, Brazil. Of the 84 samples collected from the book dust, 18 (21.4%) were positive for *Cryptococcus* spp. The most frequently isolated species was *C. gattii*, followed by *C. terreus, C. luteolus, C. neoformans, C. uniguttulatus, C. albidus* and *C. humiculus*.The isolation and identification of the pathogenic species *C .laurentii* and *C. lutolus* demonstrates the importance of biodiversity and the identification of the environmental niches of pathogenic species because these species can increase the risk of infection of immunocompromised patients (63).

Signaling pathways that originally evolved to enable organisms to respond to nutritional starvation in the environment may have been co-opted during the evolution of pathogenic organisms to the similar regulatory functions in the often harsh environment of the infected host (32).

In a study by Montagna, it was described a great relation between clinical and environmental presence of *C. neoformans* species complex serotype A. In year 1995 the first autochthonous Italian case of meningitis caused by *C. neoformans* var *gattii* serotype B was diagnosed in Apulia (Southern Italy) in a women with AIDS. After confirming travelling status of the patient, in year 1996 an environmental study was carried out to determine if the *C. neoformans* var *gattii* serotype B type that had infected her had an ecological niche in the nearby area. Seventy samples were gathered from patient's garden and this samples yielded with *C. neoformans* var *neoformans* serotype A (62).

1.6 Antifungal susceptibility of *Cryptococcus neoformans* species complex isolates from environmental samples

The type of treatment usually depends on the severity of the infection and organ systems affected.

- For those with **mild-to-moderate pulmonary infections**, the treatment is usually fluconazole.

- For those with **severe lung infections or infections in the central nervous system** (brain and spinal cord), the recommended initial treatment is amphotericin B in combination with flucytosine. After that, patients usually need to take fluconazole for an extended time to clear the infection.

Resistance to antifungal drugs is rare among clinical isolates of *C. neoformans* species complex, but has been reported. Susceptibility profiles of medically important fungi in less developed countries remain uncharacterized. The use of antifungal agents, particularly in long-term suppressive regimens, has raised concerns about the development of drug resistance in *C. neoformans* species complex (65).

Molecular types of *C. neoformans* and *C. gatti* species complex are not equally distributed in the world, with VNIV being more often found in Europe, VGII the most common molecular type of *C. gattii* species complex recovered in the America's, and VGI prevailing as the primary type of *C. gattii* species complex in Oceania, Asia and Europe. Therefore, the differences in susceptibilities may have important implications in the choice of treatment, representing a new factor influencing clinical outcomes/treatment response, especially in regions where there is a high genetic diversity of the *C. gattii* VGII population (29).

The reported susceptibility profiles of clinical and environmental isolates of *C. neoformans* and *C. gattii* species complex vary in studies, particularly those in Europe and America. In 1999, heteroresistance to triazoles was reported in *Cryptococcus* strains isolated from an azole therapy failure case of cryptococcosis in an AIDS patient, as well as in strains from a non-AIDS patient. Sionov *et al.* analyzed 130 strains of *C. neoformans* species complex isolated from clinical and environmental sources. The pattern that they described as a heteroresistance was observed in *C. neoformans* species complex strains of serotypes A and D isolated from patients in Italy and Israel (66).

Fluconazole resistant strains of *C. neoformans* species complex have been increasingly reported from cases of treatment failure in AIDS patients undergoing long-term maintenance therapy (67). The level of heteroresistance was also associated with the ability of *C. neoformans* species complex yeast cells to survive in the medium containing toxins and antibiotics produced by soil microorganism. These findings indicate that the heteroresistance to fluconazole in *C. neoformans* species complex is intrinsic, and the mechanisms that control the level of heteroresistance to azoles in each *C. neoformans* species complex strain also contribute to the fungus ability to respond to various other environmental stresses (66, 67)

Sionov *et al.*, suggested that fluconazole resistance maybe due to chromosome duplication during prolonged azole therapy, a process that could be favored in certain molecular types (66).

In 1963, murine studies demonstrated that flucytosine was effective against *Candida albicans* and *C. neoformans* specie complex. Flucytosine was first used to treat human candidosis and cryptococcosis in 1968 and remains one of the oldest antifungal agents still in use. Rapid onset of resistance precludes the use of flucytosine monotherapy. Loyse *et al*., described two mechanism that can cause flucytosine resistance: (i) mutations leading to deficiencies in the enzyme required for cellular uptake or metabolism of flucytosine and (ii) increased synthesis of pyrimidines that compete with fluorinated antimetabolites of flucytosine (68).

Pfaller *et al.*, addressed this resistance in the data by monitoring a multi country antifungal susceptibility study of 1811 *C. neoformans* species complec isolates, obtained from 100 medical centers in five different geographic regions (Europe, Africa. North America, Latin Amerika and the Pacific region) over 15 years. North American isolates were considerably less susceptible to both flucytosine and fluconazole compared with other geographical regions (69).

For the flucytosine resistant isolate, the combination of amphotericin B with flucytosine was reported as a significantly better therapy than monotherapy for reducing fungal burden in the brain, and it could be beneficial for patients with disseminated cryptococcosis (70).

Varma and Kwon-Chung analyzed 71 clinical and environmental *C. gattii* species complex strains isolated before or after the advent of azole antifungals to determine their level of heteroresistance to fluconazole. Since all the clinical isolates that were not exposed to azole drugs, as well as the environmental strains, manifested heteroresistance to fluconazole, heteroresistance of *C. gattii* species complex to azoles is believed to be an intrinsic mechanism as in *C. neoformans* species complex, and is associated with the strain's virulence (71).

Two organizations, the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI), have standardized methods to perform antifungal susceptibility testing for yeasts. According to both guidelines, determination of broth microdilution minimal inhibitory concentrations is a referent method for susceptibility testing of yeasts. However, there are some unresolved issues regarding *Cryptoccocus* spp. *Cryptococcus* spp are non-fermentative yeasts. The lack of fermentation compromises growth in microdilution plates when done according to protocols suggested by both the CLSI and EUCAST. Nevertheless, as far as EUCAST standards are concerned, the current recommendation is that EUCAST methodology be also adopted for the testing of *Cryptococcus* species. Hence, it is recommended to use RPMI 2% glucose as growth medium, a final inoculum of 0.5×10^5 and 2.5×10^5 CFU/mL, incubation without shaking and read the plates when the OD value exceeds the background level by 0.2. In cases with insufficient growth, it is suggested that the test be repeated but with incubation of the plates at 30°C. EUCAST has established breakpoints for most yeast active compounds and *Candida* species. However, there are still no clinical breakpoints for interpretation of results for *Cryptococcus* spp. As far as CLSI standards are concerned, in comparison to *Candida* spp, reading time for *Cryptococcus* spp is longer (72 hours) and this standard, as well as EUCAST, still has not

established recommended breakpoints for interpretation of broth dilution results for *Cryptococcus* spp. (72,73).

The standardized broth microdilution methods of antifungal susceptibility testing are time-consuming and cumbersome for clinical laboratories. There are also other susceptibility methods that are less technically demanding and can be more easily introduced into the routine laboratory work but are not the referent methods. Some commercially available, including manual, semi-automated and automated methods, do not require complex handling and are cost-effective alternative methods to test antifungal agents *in vitro* against *Candida* isolates in routine usage, and *Cryptococcus* isolates and filamentous fungi for research purposes (74).

Sensititre YeastOne (ThermoFisher Scientific, USA) is a colorimetric microdilution panel for antifungal susceptibility testing of *Candida* spp, and includes anidulafungin, amphotericin B, micafungin, caspofungin, 5-flucytosine, posaconazole, voriconazole, itraconazole, and fluconazole. AlamarBlue™ agent provides a reliable, easy and consistent endpoint determination with visual read options (74).

Agar-based methods include disk tests that provide an ideal screening test. The disk diffusion method to test antifungals was developed and validated only in the case of azoles and echinocandins for *Candida* spp isolates. Although qualitative results provided by the disk diffusion method are useful in the clinical laboratory routine, quantitative minimal inhibitory concentrations (MIC) data are somewhat critical for the management of invasive infections. Commercially prepared strips are also available with predefined gradient of antifungal drug concentrations on a plastic strip that is used to determine the MIC (74).

The ATB FUNGUS 3 (bioMérieux, France) strips consist of 16 pairs of cupules including two growth control wells and five antifungal drugs at different concentrations: 5-flucytosine, amphotericin B, fluconazole, itraconazole, and voriconazole (74).

Vitek 2 (bioMérieux, France) is an automated method for yeast species identification and antifungal susceptibility testing through the analysis of yeast growth. It is a miniaturized version of the broth microdilution method in the form of a card with two-serial dilutions. The latest card version (VITEK®2 AST-YS08) comprises antifungals amphotericin B, caspofungin, micafungin, fluconazole, voriconazole, and flucytosine. An integrated software interprets susceptibility test results (74).

Li *et al.* conducted a comparative analysis between two different antifungal susceptibility testing's. One of them is a comparative analysis between E-test and broth microdilution method. Both methods are reliable alternatives and present good correlation for all evaluated drugs except amphotericin B (75, 76). Also, results obtained from the ATB FUNGUS 3 kit are similar to those obtained by the microdilution method (75).

Warnock *et al.* found that the MICs by E-test for amphotericin B are lower than the results obtained with the reference microdilution method, whereas the E-test MICs results for flucytosine, fluconazole, and itraconazole are higher than those obtained with the microdilution method (77). Other authors have reported that absolute azole MICs generated by agar-based techniques tend to be lower than those produced by broth assays (77).

A study from Malaysia tested 544 samples of birds' excreta collected from a local zoo, pet shops and public areas, where 20 strains of *C. neoformans* species complex were isolated. The antifungal susceptibility testing using agar diffusion method showed that all environmental strains were susceptible to amphotericin B, fluconazole, and itraconazole (78).

Another study by Choudhary *et al.* from 2011 tested 146 environmental strains collected from decayed wood trees and soil. *C. neoformans* var *grubii* and *C. gattii* species complex were found to be susceptible to the antifungals tested (79). However, a few studies reported on the antifungal susceptibility of clinical and environmental *C. neoformans* and *C. gattii* species complex strains. In an early study by Khan *et al*., there was no significant difference found between the antifungal susceptibility profiles from clinical and environmental isolates of *C. neoformans* and *C. gattii* species complex (80). On the contrary, Chowdhary *et al.* reported that environmental *C. neoformans* var *grubbii* in northwest India was significantly less susceptible to fluconazole and itraconazole than the clinical isolates (79). In 2015, Gutsch *et al.* reported that environmental isolates of *C. neoformans* var *grubbii* from soil contaminated with pigeon

excreta, pigeon droppings and trunk hollows of *Tamarinus indica* tree, exhibited lower susceptibility to fluconazole, to other environmental sources and clinical isolates (81).

Several studies have evaluated the relationship between antifungal susceptibility and serotypes of *C. neoformans* species complex strains. The study by Thomson *et al*. from 2009 shows no significant difference between the antifungal susceptibility values from the five antifungal compounds when compared to the serotype A, D, and AD background of the strains. In that study, a total of 128 *Cryptococcus* isolates were evaluated, including 86 isolates of *C. neoformans* species complex and 42 isolates of *C. gattii* species complex collected from both clinical and environmental sources from the United States, Australia, France, Denmark, Italy, Thailand, and the Democratic Republic of Congo (82).

There was no *in vitro* resistance observed to amphotericin B, 5-flucytosine, itraconazole, and voriconazole among the 48 clinical *C. neoformans* species complex strains from Croatia collected between 2005 and 2010. The method used to determine the antifungal susceptibility in Croatia was broth microdilution method according to CLSI. Results of the *in vitro* susceptibility against fluconazole and 5-flucytosine for the Croatian clinical *C. neoformans* species complex strains were in concordance with other studies (57).

A study by Pfaller *et al.* confirmed that *in vitro* resistance to standard antifungal agents used in the treatment of cryptococcosis remained uncommon among isolates of *C. neoformans* species complex from five broad geographical regions, and did not increase in the 15-year period, but despite these favorable findings a continuous surveillance of emerging resistance may be warranted given the global importance of these organisms as an opportunistic pathogen and the ever increasing use of antifungal agents in the immunocompromised patient population (69).

A continuous surveillance of antifungal susceptibility of environmental strains of *C. neoformans* and *C. gattii* species complex is desirable to monitor the emergence of any resistant strains in order to ensure more successful therapy of cryptococcosis (81).

1.7 Molecular characterization of *Cryptococcus neoformans* species complex

In year 1982 a paper published by Kwon-Chung described a improved diagnostic medium for separation of *C .neoformans* var *neoformans* (serotype A and D) and *C. neoformans* var *gattii* (serotype B and C) (83). Now, we can conclude that molecular typing techniques have become essential tools in tracking the epidemiology of *C. neoformans* species complex (84).

The accumulation of data generated by molecular methods may have a positive impact on monitoring resistant strains and treating diseases. The development of molecular techniques for microorganisms typing has brought new opportunities to the field of taxonomy, identification and diagnosis, as well as revealed more about microorganism's phylogeny and evolution (85).

The disease aspects of cryptococcal infection are defined better, while the life cycle of this fungus in the environment remains less determined. How this fungus spread worldwide, the nature of its population structure, and how it evolved to become a deadly pathogen are ongoing research subjects that are key to understanding of this environmental pathogen (85).

The determination of the molecular type of the causative agent in specific cases of cryptococcosis may provide important information that will allow for better guidance in the decision making for the best treatment choice, which subsequently would lead to a better disease outcome (86). PCR fingerprinting using $(GACA)_4$ as a single primer proved more stable than serology in discriminating among *C. neoformans* species complex serotypes A, D and AD and was able to distinguish among serotype AD strains (84).

Recently, more sensitive molecular subtyping methods have been employed as an alternative tool to serotyping methods for epidemiological studies.

Several molecular techniques have been applied for *Cryptococcus* spp. typing – multilocus enzyme electrophoresis (MLEE), DNA fingerprinting, random amplification of polymorphic DNA (RAPD), PCR fingerprinting, amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) of PLB1, GEF1, or URA5 genes, sequencing of ITS1-5.8S-ITS2 rDNA region or intergenic spacer region (IGS) and, more recently, multilocus sequence typing (MLST), multilocus microsatellite typing (MLMT), and matrix assisted laser desorption ionization – time of flight mass spectrometry-based (MALDI-TOF) analysis. Although numerous molecular techniques have been applied to subtype *C. neoformans* and *C. gattii* strains, only three methods have been shown to produce comparable results: PCR fingerprinting, AFLP, and MLST (85, 86).

The molecular types VNI, VNII, VNIII, and VNIV have been recognized among the *C. neoformans* species complex isolates and further four molecular types, VGI, VGII, VGIII, and VGIV, have been found among *C. gattii* species complex isolates. There is considerable genetic heterogeneity within the *C. neoformans* species complex. The accumulated epidemiological data suggest grouping the isolates into four major molecular types: VNI and VNII (*C. neoformans* var. *grubii,* serotype A, found worldwide), VNIV (C. *neoformans* var. *neoformans*, serotype D, mainly in Europe and South America), VNIII (serotype AD hybrids, mainly in Europe). VGI-IV (*C. gattii* serotypes B and C) are affecting immunocompetent and immunocompromised hosts (85).

A study by Ferreira *et al*. shows the presence of VNIV and VNI genotypes among environmental isolates in Northern Portugal (87).

In another study by Hagen *et al.,* most isolates from environmental samples were AFLP/VNI, and a small part belonged to the genotype AFLP1B/VNII, followed by other genotypes (88).

The large part of *C. neoformans* species complex isolates mirrors the observation made by the European epidemiological studies. Isolates collected during the period 1977-2007 in the Netherlands were found to be represented by 79.3% (n = 238) of genotype AFLP1/VNI (88).

Molecular typing techniques have also been used to compare clinical and environmental isolates of *C. neoformans* species complex. Often, isolates recovered from environmental sources and those isolated from patients have been shown to be closely related, presenting similar or even identical genetic profiles. However, in Mediterranean Europe, the number of *C. neoformans* species complex isolates in epidemiological surveys is often lower (56).

Cryptococcus neoformans species complex is a heterothallic basidiomycete which possesses a bipolar mating system based on two mating type allelles, *MAT a* and *MAT* α. Both mating types have been identified among serotype B, C and D strains, whereas only MAT α has been identified among serotype A isolates (89).

AFLP is a multilocus genotyping method combining universal applicability, high discriminative power and reproducibility for which only small amounts of DNA are required. The methods has been used for the genotyping of bacteria, plants, fungi and animals, and also for the construction of genetic maps. A large number of strain specific genetic characters is available, because of the large number of bands generated using AFLP compared with RAPD and PCR fingerprinting. This render AFLP a sensitive tool allowing differentiation of genetically related strains (46).

PCR has revolutionized the field of infectious disease diagnosis. During the past decade, advances in PCR technology and other DNA signal and target amplification techniques have resulted in these molecular diagnostics becoming key procedures. Such techniques are conceptually simple, highly specific, sensitive, and amenable to full automation. Multiplex PCR has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility. Since its introduction, multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics, including gene deletion analysis, mutation and polymorphism analysis, quantitative analysis, and RNA detection (90).

Molecular techniques can distinguish seven haploid genotypic groups within *C. neoformans* and *C. gatti* species complex. Recently, 3 serotype BD between *C. neoformans x C. gatti* hybrids were isolated from two HIV negative patients in Netherland and later a hybrid forma between *C. neoformans* species complex serotype A x *C.gattii species complex* serotype B was isolated from an HIV positive person. All *C. neoformans* x *C.gattii* species complex hybrids discovered have originated from clinical sources (41).

The occurrence of hybridization has consequences for the reproductive biology of the species, as new genotypes with altered virulence or susceptibility to antifungal drugs may arise through the exchange of genetic material (46).

Currently, studies using molecular biology techniques are in progress to establish the genomic DNA pattern of *C.neoformans* species complex and thus obtain a better understanding of the epidemiology of this pathogen.

So far, there are no studies on the molecular epidemiology of isolates of *C. neoformans* species complex in Croatia and Kosovo.

Considering the complete lack of data reported in the literature for the two states, the present study aimed to evaluate the possible environmental distribution of this pathogen in two different countries.

2. HYPOTHESIS

- Serotype A and mating type α are predominant types in Croatia and Kosovo;
- All environmental isolates of *C. neoformans* and *C. gattii* will be susceptible to antifungals tested.

3. AIMS OF THE RESEARCH

General aims:

- To isolate *C. neoformans* species complex from environmental samples collected from public places in different geographical locations in Croatia and Kosovo;
- To perform *in vitro* antifungal susceptibility testing of *C. neoformans* species complex isolates from environmental samples in different geographical locations in Croatia and Kosovo;
- To determine molecular characteristics of environmental *C. neoformans* species complex isolates from different geographical locations in Croatia and Kosovo.

Specific aims:

- To compare the frequency of *C. neoformans* species complex isolation between environmental samples of birds' excreta and tree hollows;
- To investigate the frequency of isolation of *Cryptococcus* spp. in different geographical locations in Croatia and Kosovo;
- To compare antifungal susceptibility profiles of environmental isolates of *C. neoformans* species complex from Croatia and Kosovo;
- To compare serotypes, mating types and genotypes of environmental isolates of *C. neoformans* species complex from Croatia and Kosovo.

4. MATERIALS AND METHODS

4.1. Geographical characteristics of Croatia and Kosovo

The geography of Croatia is defined by its location – it is described as being a part of centraleastern Europe (Figure 2). Croatia's territory covers 56,594 km^2, making it the 127th largest country in the world (91). Most of Croatia has a moderately warm and rainy continental climate as defined by the Köppen climate classification with a mean monthly temperature in the coldest month of the year above –3 °C and below 18 °C. The country is among the most biodiverse in Europe, because of its climate and geomorphology. According to the 2011 census, the permanent population of Croatia reached 4.29 million. The largest city and the nation's capital is Zagreb, with an urban population of 686,568 in the city itself, and a metropolitan area population of 978,161 (92).

Figure 2. Map of Croatia. Cities in Croatia included Zagreb, Split, Osijek, Pula, Krk, Rijeka, Šibenik, Varaždin, Korčula, Makarska, Dubrovnik, and Rovinj. According to CroatiaTraveller, Internet image, 2005-2019 (91).

Kosovo is a small landlocked country in southeastern Europe, in the center of the Balkan Peninsula (93). (Figure 3). With an area of 10,908 km^2, it is one of the smallest countries in Europe. Its geographical location gives the country a large annual temperature range. The highest temperatures in the summer can reach $+30^0$C, the winter temperature could be as low as -10^0C. According to the Strahler classification map, the climate in Kosovo is considered moist continental. It can be stated that the Kosovo territory is characterized by a sunny climate with variable temperature and humidity conditions (94).

Figure 3. Map of Kosovo. Cities included in Kosovo are Prishtina, Peja, Mitrovica, Prizren, and Podujevo. According to Wikipedia, the free encyclopedia, 2008 - Geography of Kosovo, up-dated 18.04.2019 (93).

4.2. Samples

Samples in a form of swabs were collected during the summer and autumn season from 2013 to 2016, from cities in different geographical locations in Croatia and Kosovo. The samples were collected from different urban public places including squares, parks, gardens, hospital areas, and school playgrounds. Swab samples were taken from tree hollows and birds' excreta in the soil beneath the trees. Swab samples from tree hollows were taken from old trees and from large trunk hollows.

Sampling of decayed wood in the trunk hollows and birds' excreta in the soil beneath trees was done by rubbing the cotton-tipped swab that had been dipped in sterile physiological saline solution containing gentamycin (25mg/L) against the surface. Two swabs were used for sampling of decayed wood in each trunk hollow. The excreta sampled were desiccated, not fresh and moist.

4.3 Sample processing and isolation

The samples collected in Zagreb were processed within 2 hours after collection. The samples collected from other geographical locations were stored at 4^0C until processing at the Department of Mycology, Croatian Institute of Public Health, Zagreb, Croatia, where identification and antifungal susceptibility testing were performed.

Swabs from tree hollows and birds' excreta in the soil beneath trees were processed for this research. Swabs from tree hollows and birds' excreta in the soil beneath trees were suspended in 9 ml of sterile saline solution (NaCl 0.9%) containing 1000 µg/ml streptomycin and 500 IU penicillin/ml. Swabs were removed and the suspension was left to sediment for 15 minutes to allow the material to sink. A total of 0.1 ml of the supernatant and 0.1ml of the diluted supernatant (1:10 in sterile distilled water) were inoculated separately into two Sabouraud's dextrose agar plates each with chloramphenicol (0.5 g/ml) and biphenyl (0.1%).

The plates were incubated at 27^0C and 37^0C for seven days and were monitored daily. The dark brown colonies suggestive of *C. neoformans* species complex were subcultured on Sabouraud's agar peptone dextrose agar plates.

The number of *C. neoformans* species complex colonies appearing on each culture plate was counted and recorded.

4.4. Identification of *Cryptococcus* species complex

Identification of *C. neoformans* species complex included conventional mycological methods comprising the growth on corn meal agar and presence of capsule in India ink preparations as well as commercial identification systems ID 32C (BioMérieux, France) based on assimilation of carbohydrates and nitrate.

ID 32C system consists of a single-use disposable plastic strip with 32 wells containing substrates for 29 assimilation tests (carbohydrates, organic acids, and amino acids), one susceptibility test (cycloheximide), one colorimetric test (esculin), and a negative control. The yeast identification procedures were conducted in accordance with the manufacturer's instructions as follows:

- a portion of growth from well-isolated colonies of each isolate was aseptically transferred from a freshly inoculated stock culture to sterile distilled water to prepare a suspension with a final turbidity equivalent to McFarland standard #2. Five drops of this suspension were then dispensed to an ampule of C medium provided by the manufacturer and homogenized to prepare an even dispersion of inoculum. After homogenizing, the inoculum suspension was used to inoculate the wells in the strip, the lid of the strip was replaced, and the system was incubated at 30°C for 48 h;
- the strips were then visually examined, and growth was determined to be positive or negative depending on the presence or absence of turbidity in the wells. The results were transformed into numerical bio codes, and the isolates were identified through the use of the ID 32C Analytical Profile Index;
- Quality control. Each system was tested with the manufacturer's recommended quality control test organisms before starting the formal evaluation, which included *Candida guilliermondii* (ATCC 6260).

Up to ten colonies of *Cryptococcus* species obtained from both sample types collected from all locations were stored in glycine buffer at -26^{0}C until antifungal susceptibility testing and molecular analysis were conducted.

4.5 *In vitro* antifungal susceptibility testing of *Cryptococcus* species complex isolates

Antifungal susceptibility profiles of *Cryptococcus* species isolates were determined by ATB FUNGUS 3 (BioMérieux, France) microdilution method using strips containing 16 pairs of cupules including two growth control wells and five antifungal drugs at different concentrations: 5-flucytosine (4–16 µg/mL), amphotericin B (0.5–16 µg/mL), fluconazole (1–128 µg/mL), itraconazole (0.125–4 µg/mL), and voriconazole (0.06–8 µg/mL). Following the manufacturer's instructions, a suspension with a turbidity of 2 McFarland was prepared and 20 µL of this suspension was transferred to an ampule of ATB FUNGUS 3 medium. Then, 135 µL of the inoculated medium was transferred into each cupule.

After incubation at 35°C for 24 h, the strips were read both visually and automatically on the ATB Expression Bacteriology Analyzer automatic system (BioMérieux, La Balme-les-Grottes, France). According to the manufacturer's instructions, the minimal inhibitory concentrations (MIC) were determined by the growth score for each of the cupules compared with the control cupules. For amphotericin B, the MIC corresponds to the lowest concentration enabling complete growth inhibition (score = ’0’). For 5-flucytosine, fluconazole, itraconazole, and voriconazole, the MIC corresponds to the lowest concentration of the antifungal agent enabling partial inhibition of growth with which a score of ‘2’, ‘1’, and ‘0’ is obtained. MICs to flucytosine, amphotericin B, fluconazole, itraconazole, and voriconazole were determined at the recommended endpoints and time intervals by the manufacturer.

Isolates were also tested with a standard broth microdilution method according to the Clinical Laboratory Standards Institute (CLSI) for amphotericin B, fluconazole, and voriconazole (88). The testing was done at the University Hospital Centre Zagreb, Department of Clinical and Molecular Microbiology. Since there are no clinical breakpoints defined by the CLSI for *C. neoformans* species complex, epidemiological cutoff values (ECV) were used as reference values to define a strain as wild type or non-wildtype. Isolates were considered wild type when the minimal inhibitory concentration (MIC) value for amphotericin B was ≤0.5 µg/mL, for flucytosine ≤8 µg/mL, for fluconazole ≤8 µg/mL, for itraconazole ≤0.25 µg/mL,

for voriconazole ≤0.25 μg/mL. Susceptibility testing according to CLSI was done in 2019, two years after other investigations were already finished (95).

Unlike *C. neoformans* species complex, *C. gattii* species complex has not been included in CLSI guidelines for testing of yeasts and was tested only with ATB FUNGUS 3. The ECVs were used as reference values to define a strain as wild type or non-wildtype regardless of genotype: for amphotericin B was ≤1.00 μg/mL, for flucytosine ≤4 μg/mL, for fluconazole ≤8 μg/mL, for itraconazole ≤0.5 μg/mL, for voriconazole ≤0.25 μg/mL (96, 97).

4.6 Molecular analysis of *Cryptococcus* species isolates

Molecular analysis of *C. neoformans* species complex isolates was done in Laboratory Micologia Medica, Universita degli Studi di Milano, Milan, Italy.

The molecular type and mating type of the isolates was determined by multiplex PCR, as previously described (46, 98, 99). Strains H99 (VNI), WM626 (VNII), JEC21 (VNIV), and CBS 132 (VNIII) were included as reference strains (100).

Multiplex PCR method, using primers specific for each of the mating type and serotype combination, was applied to determine the mating type (a or α) and serotype of the *Cryptococcus* species isolates.
Primers that were used were:
800F1 GTCTCTTAATTACTCAGC, 800R1 GCTTTTTTTTCTGTCTCC,
500F1 ACGGCAACCTTCTTATG, 500R1CGGTAGTGCGTTTTAAG,
470F2 ACACAAAGGAAACTCGCT, 470R3ACAAACCCCCCTCTTC,
420F2 ATTGTCGGTGCTTGCAT, B420R AACATCTCCCATTCCTC (100).

4.7 Statistical analysis

Statistical analysis included descriptive frequency tables, and Fisher's exact test. Statistical significance was determined when $p \leq 0.05$ was observed

5. RESULTS

Total of 709 samples were collected from both countries, 509 (71.8%) in Croatia and 200 (28.2%) in Kosovo (Table 1).

Table 1. The number of samples collected in Croatia and Kosovo

		N	%
Country	Croatia	509	71.8
	Kosovo	200	28.2
	Total	709	100.0

The number and percentage of samples based on the monthly collection in Croatia and Kosovo is shown in Table 2. The highest number of samples were collected in June (N=387 54.6%) and the lowest in December (N=2, 0.3%).

Table 2. Distribution of samples collection in Croatia and Kosovo by month and year.

	Month, year	**N**	**%**
	June, 2013	21	3.0
	July, 2013	15	2.1
	August, 2013	20	2.8
	October, 2013	36	5.1
	May, 2014	23	3.2
	June, 2014	181	25.5
	September, 2014	10	1.4
	October, 2014	5	0.7
	November, 2014	79	11.1
	December, 2014	2	0.3
	May, 2015	82	11.6
	June, 2016	185	26.1
	August, 2016	50	7.1
	TOTAL	**709**	**100,0**

The samples were collected through all seasons of the year. The highest number of samples was collected in the summer (N=472, 66.57%), and the lowest in the winter (N=2, 0.3%).

Table 3. Distribution of samples collected throughout seasons of the year.

Month	Spring	Summer	Autumn	Winter	Total
May	105				105
Jun		387			387
Jul		15			15
Aug		70			70
Sep			10		10
Oct			41		41
Nov			79		79
Dec				2	2
Total	105	472	130	2	**709**

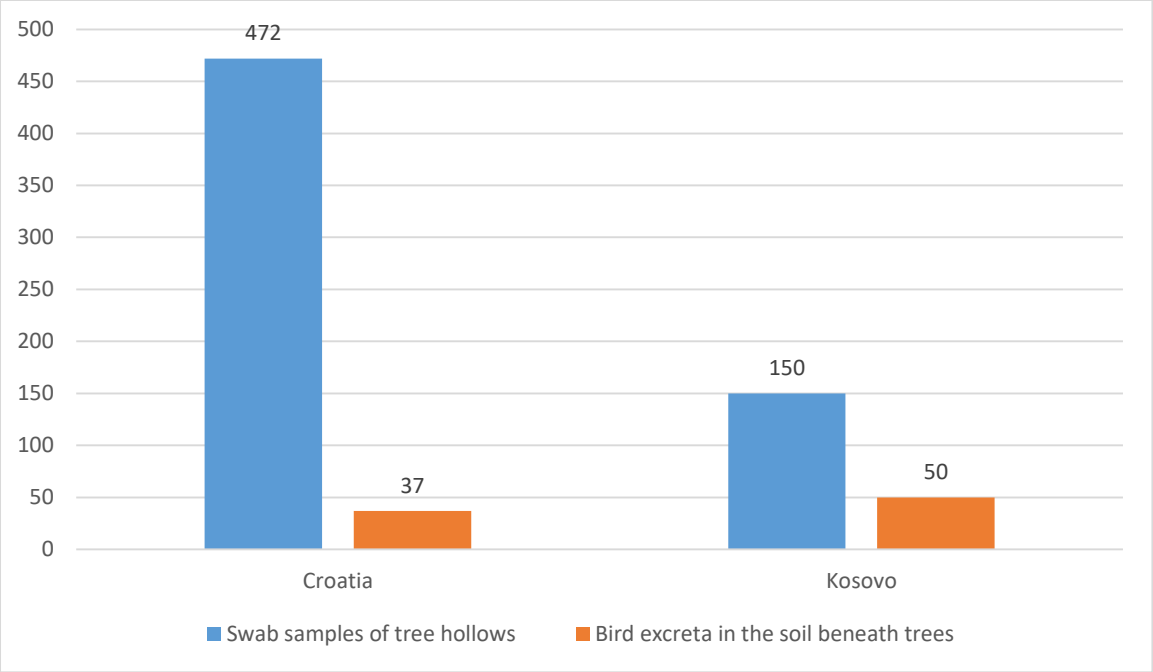
The total number of samples collected in the two countries by sample type is shown in Table 4. In both countries, 622 (87.7%) samples were taken from trunk hollows and 87 (12.3%) samples from birds' excreta in the soil beneath the trees.

Table 4. Number and percentage of samples by sample type in two countries.

	N	%
Swab sample from tree hollows	622	87.7
Swab samples from birds' excreta in the soil beneath trees	87	12.3
Total	709	100.0

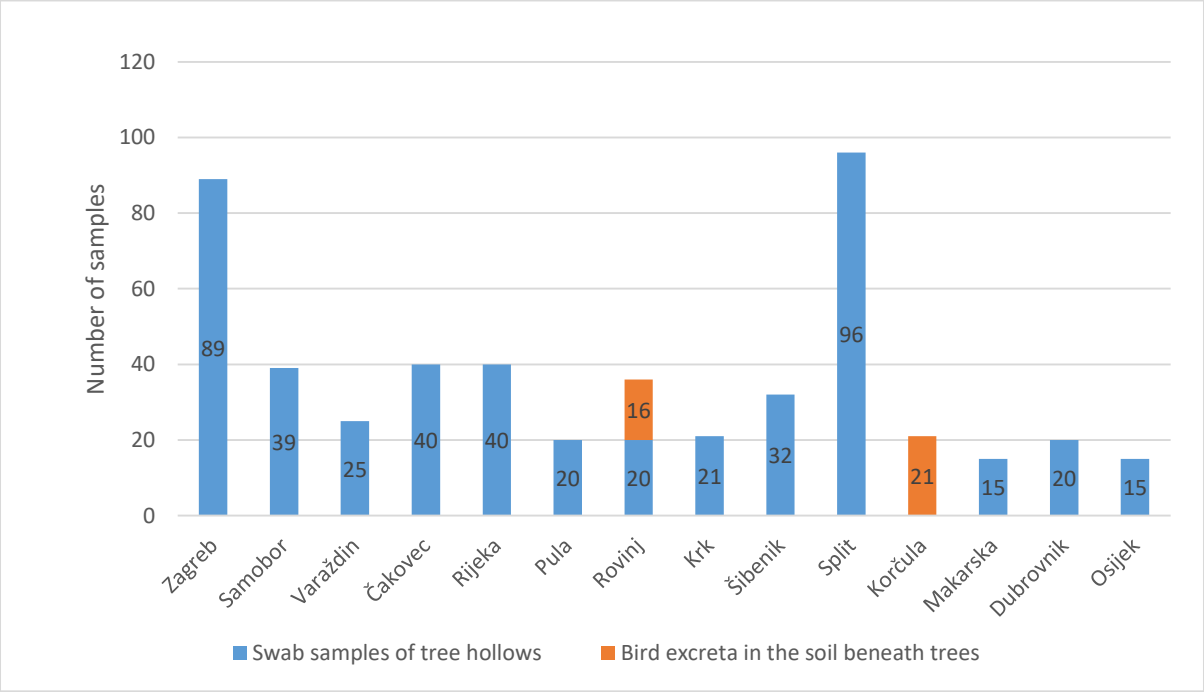
Distribution of samples according to type and two countries is shown in Figure 4. In Croatia 472 (92.7%) samples were collected from tree hollows and 37 (7.3%) swabs of bird excreta in the soil beneath trees, while in Kosovo 150 (75.0%) samples were collected from tree hollows and 50 (25.0%) from swabs of bird excreta in the soil beneath trees.

Figure 4. Number of samples by sample type in Croatia and Kosovo.



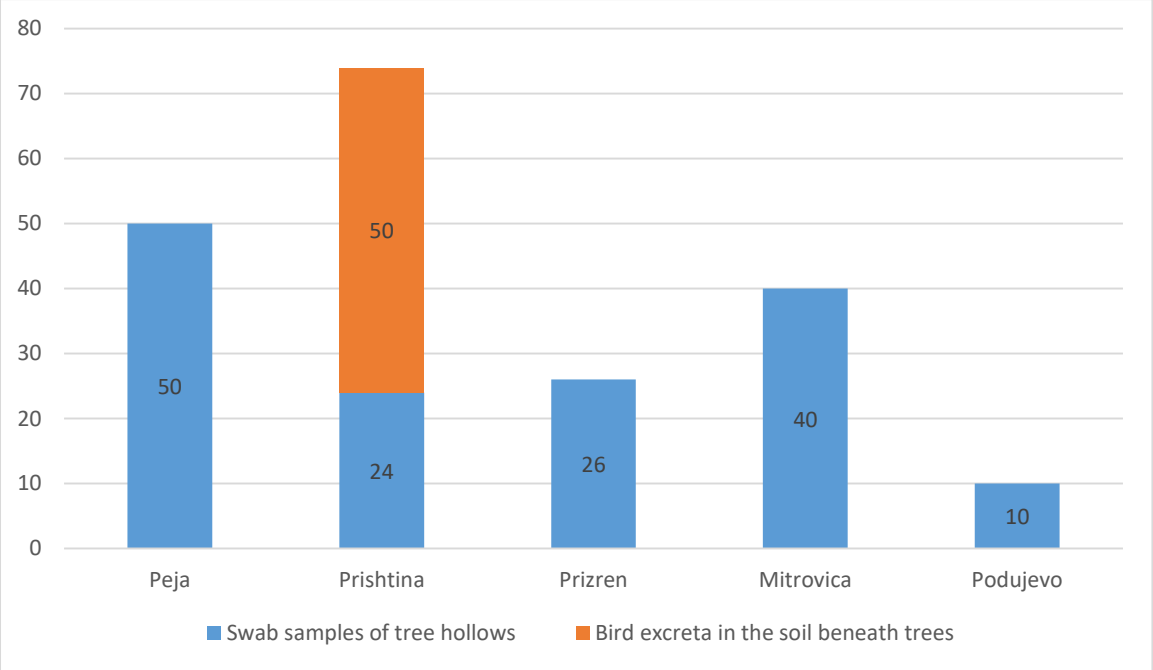
The number and type of samples collected in different cities in Croatia are shown in Figure 5. Split was the city with the highest percentage of collected samples (96/509; 18.9%), whereas Osijek and Makarska had the lowest percentage (15/509; 2.9%). Samples of bird excreta in the soil beneath the trees were collected only in two locations: Rovinj and Korčula.

Figure 5. The number and type of sample collected in each of the 14 sampling locations in Croatia.



The number and type of samples collected in different cities in Kosovo are shown in Figure 6. Prishtina was the city with the highest percentage of collected samples (74/200; 37.0 %), whereas Podujevo had the lowest percentage 10/200; 2.9%). Samples of bird excreta in the soil beneath the trees were collected only in Prishtina.

Figure 6. The number and type of sample collected in different cities in Kosovo.



In total, there were 53 different tree species from which samples were collected in Croatia and Kosovo. The higher numbers of samples were taken from tree species *Olea europea* (olive tree), followed by *Ceratonia silique* (carob tree) and *Pinus sylvestris* (scots pine) (101).

In Croatia a total of 46 different tree species were sampled (Table 5). Most of the trees were *Olea europea* (olive tree, 25.5%), *Ceratonia siliqua* (carob tree, 5.3%), *Pinus sylvestris* (scots pine, 5.1%), *Betula pendula* (silver birch, 4.5%), *Castanea* spp. (chestnut tree, 4.3%) and *Prunus dulcis* (almond tree, 3.9%).

Table 5. Number of samples collected from different tree species in Croatia

Tree species	**Number of samples**	**Percentage (%)**
Olea europea	130	25.5
Ceratonia siliqua	27	5.3
Pinus sylvestris	26	5.1
Betula pendula	23	4.5
Castanea spp.	22	4.3
Prunus dulcis	20	3.9
Sorbus domestica	20	3.9
Juglans nigra	18	3.5
Ficus carica	16	3.1
Hedera helix	15	2.9
Stone Pine tree	15	2.9
Robinia pseudoacacia	11	2.7
Laurus nobilis	12	2.4
Malus domestica	11	2.2
Prunus cesarus	11	2.2
Quercus robur	11	2.2
Citrus reticulata	10	2.0
Fagus sylvatica	10	2.0
Fraxinus ornus	10	2.0
Tilia grandifolia	10	2.0
Morus spp.	8	1.6
Platanus occidentalis	6	1.2
Acer nigrum	5	1.0

Carpinus betulus	5	1.0
Citrus limon	5	1.0
Cupressus sempervirens	5	1.0
Morus nigra	5	1.0
Arecaceae	5	1.0
Rapidophilum hystrix	5	1.0
Rosmarinus officinalis	5	1.0
Vitis vinifera	5	1.0
Prunus laurocerasus	4	0.8
Celtis australis	2	0.4
Cercis siliquastrum	2	0.4
Magnolia grandiflora	2	0.4
Acer spp.	1	0.2
Corylus avellana	1	0.2
Ginkgo biloba	1	0.2
Ligustrum	1	0.2
Nerium oleander	1	0.2
Paulownia elongate	1	0.2
Pittosporium spp.	1	0.2
Quercus cerris	1	0.2
Siringa vulgaris	1	0.2
Viburnurn spp.	1	0.2
Ziziphus ziziphus	1	0.2

In Kosovo a total of 11 different tree species were sampled (Table 6). Most of the trees were *P. sylvestris (*45/150; 30.0%), followed by *Tilia grandifolia* (linden; 21/150; 14.0%) and *Quercus robur (*oak tree, 18/150; 12.0%).

Table 6. Number of samples collected from different tree species in Kosovo.

Tree species	**Number of samples**	**Percentage (%)**
Pinus sylvestris	45	30.0
Tilia grandifolia	21	14.0
Betula pendula	8	5.3
Castanea sativa	14	9.3
Poplar tree	12	8.0
Platanus occidentalis	10	6.7
Robinia pseudoacacia	12	8.0
Willow tree	4	2.7
Paulownia tree	2	1.3
Maple tree	4	2.7
Quercus robur	18	12.0
TOTAL	150	100.0

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Results from the culture of supernatant and diluted supernatant incubated at 27ºC are shown in table 7. Supernatant and diluted supernatant had the same culture results when incubated at 27ºC regarding the yield of yeast isolation. Out of 709 collected samples, 10 (1.4%) samples resulted in growth of fungi from both supernatant and diluted supernatant incubated at 27ºC on Sabouraud agar, and 699 (98.6%) had no growth.

Table 7. Culture results of supernatant and diluted supernatant incubated at 27ºC on Sabouraud's dextrose agar.

Culture results	N	%
no growth	699	98,6
growth	10	1.4
Total	709	100.0

Culture results from supernatant and diluted supernatant incubated at 37ºC are shown in Table 8. Supernatant and diluted supernatant had the same culture results when incubated at 37ºC regarding the yield of yeast isolation. Out of 709 collected samples, 92(13.0%) samples resulted in growth of yeasts from supernatant incubated at 37ºC, and 617 (87.0%) had no growth.

Table 8. Culture results of supernatant and diluted supernatant incubated at 37ºC on Sabouraud's dextrose agar.

Culture results	N	%
no growth	617	87.0
growth	92	13.0
Total	709	100.0

Total number of culture-positive and culture-negative samples in Croatia and Kosovo is shown in Table 9. In comparison to Croatia, the percentage of culture positive samples was significantly lower in Kosovo (15% vs 7.5%) at 95% probability $X^2 = 6.48$; Degree of freedom = 1, N = 709, P = *0.011 (<0.05).*

Table 9. The number of culture-positive and culture-negative samples in Croatia and Kosovo.

		Culture-negative samples	Culture-positive samples	Total
Croatia	N	432	77	509
	%	84.9%	15.1%	100.0%
Kosovo	N	185	15	200
	%	92.5%	7.5%	100.0%
TOTAL	N	617	92	709
	%	87.0%	13.0%	100.0%

Table 10 shows the number of fungal isolates in different cities of Croatia and Kosovo. Some cities like Rijeka (N=8), Krk (N=9), and Prishtina (N=7) showed the higher number of different isolated species. In Kosovo, microorganisms were isolated only from samples obtained from Prishtina, Mitrovica and Prizren. There were total of 92 isolates from different locations in Croatia and Kosovo. The most frequent isolates belonged to *Candida* species (41/92; 44.6%) and *Cryptococcus* species (38/92; 41.3%) followed by *Rhodotorula mucilaginosa* (8/92; 8.7 %), *Trichosporon mucoides* (4/92;4.3 %) and *Pseudomycelium* (1/92; 1.1%).

Table 10. Fungal isolates in different cities in Croatia and Kosovo

Country	City	Isolate	N	%
CROATIA	Zagreb	*Candida formata*	2	2.2
		Candida guilliermondii	1	1.1
	Samobor	*Candida tropicalis*	5	5.4
	Čakovec	*Candida krusei*	1	1.1
		Candida sphaerica	1	1.1
	Rijeka	*Candida albicans*	1	1.1
		Candida colliculosa	3	3.2
		Candida famata	2	2.2
		Candida glabrata	1	1.1
		Candida guilliermondii	5	5.4
		Candida parapsilosis	1	1.1
		Candida tropicalis	1	1.1
		Cryptococcus neoformans	1	1.1
	Pula	*Cryptococcus albidus*	1	1.1
		Cryptococcus laurentii	2	2.2
	Rovinj	*Cryptococcus laurentii*	5	5.4
	Krk	*Candida albicans*	1	1.1
		Candida famata	1	1.1
		Candida sake	1	1.1
		Candida tropicalis	2	2.2
		Cryptococcus albidus	1	1.1
		Cryptococcus gattii	2	2.2
		Cryptococcus laurentii	6	6.4
		Cryptococcus neoformans	3	3.2
		Trichosporon mucoides	2	2.2
	Šibenik	*Cryptococcus laurentii*	1	1.1
		Cryptococcus terreus	1	1.1
		Cryptococcus uniguttulatus	2	2.2
	Split	*Candida guilliermondii*	3	3.2
		Candida rugose	1	1.1
		Candida utilis	1	1.1
		Cryptococcus albidus	3	3.2
		Cryptococcus laurentii	6	6.4
		Rhodotorula mucilaginosa	4	4.3
		Trichosporon mucoides	2	2.2
	Osijek	*Cryptococcus albidus*	1	1.1
KOSOVO	Prishtina	*Candida glabrata*	2	2.2
		Candida guilliermondii	1	1.1
		Candida magnoliae	2	2.2
		Candida pelliculosa	1	1.1
		Cryptococcus albidus	1	1.1
		Cryptococcus gattii	1	1.1
		Cryptococcus laurentii	1	1.1
		Pseudomycelium	1	1.1
		Rhodotorula mucilaginosa	2	2.2
	Mitrovica	*Candida colliculosa*	1	1.1
		Rhodotorula mucilaginosa	1	1.1
	Prizren	*Rhodotorula mucilaginosa*	1	1.1

Table 11 shows a different microorganisms isolated from different tree species and bird excreta from soil beneath the trees in Croatia. The higher number of microorganism was isolated from *O. europaea* (42/77; 54.5%) and *P. sylvestris*; (8/77; 10.4%). Also there are different microorganism isolated from bird excreta from soil beneath different trees (5/77; 6.5%).

Table 11. Fungal species isolated from different tree species and bird excreta beneath trees in Croatia.

Sample type	Name of the tree	Isolated microorganism	Nr of isolates	Total
Bird excreta	*Betula pendula**	*Cryptococcus albidus*	1	5
	*Ceratonia siliqua**	*Candida guilliermondii*	1	
		Cryptococcus laurentii	1	
	*Cercis siliquastrum**	*Candida colilulosa*	1	
		Candida guilliermondii	1	
Swabs from tree hollows	*Citrus reticulata*	*Cryptococcus albidus*	1	72
	Ficus carica	*Candida guilliermondii*	1	
		Cryptococcus laurentii	2	
	Juglans nigra	*Candida guilliermondii*	1	
		Candida tropicalis	1	
	Laurus nobilis	*Candida formata*	1	
		Cryptococcus albidus	1	
	Magnolia grandiflora	*Candida formata*	1	
	Morus	*Candida albicans*	1	
		Cryptococcus laurentii	2	
	Nerium oleander	*Candida guilliermondii*	1	
	Olea europaea	*Candida albicans*	1	
		Candida formata	1	
		Candida guilliermondii	2	

		Candida rigosa	1	
		Candida sake	1	
		Candida tropicalis	3	
		Candida utilis	1	
		Cryptococcus albidus	3	
		Cryptococcus gatti	2	
		Cryptococcus laurentii	15	
		Cryptococcus neoformans	3	
		Cryptococcus uniguttulatus	1	
		Rhodotorula mucilaginosa	4	
		Trichosporon mucoides	4	
	Paulownia elongate	*Candida parapsillosis*	1	
	Pinus sylvestris	*Candida colliulosa*	1	
		Candida crusei	1	
		Candida formata	2	
		Candida glabrata	1	
		Candida tropicalis	1	
		Cryptococcus neoformans	1	
		Cryptococcus terreus	1	
	Pittosporium	*Candida guilliermondii*	1	
	Prunus cesarus	*Candida tropicalis*	1	
	Prunus laurocerasus	*Candida colilulosa*	1	
		Candida guilliermondii	1	
	Quercus robur	*Candida sphaerica*	1	
		Candida tropicalis	1	
	Rosmarinus officinalis	*Cryptococcus uniguttulatus*	1	

	Sorbus domestica	*Candida tropicalis*	1	

*Name of the tree where swabs from bird excreta beneath them were collected.

Table 12 shows a different microorganism isolated from different tree species and from the swabs of the bird excreta beneath the trees collected in Kosovo.

Table 12. Fungal species isolated from different tree species and birds' excreta beneath the trees in Kosovo.

Sample type	Name of the tree	Isolated microorganism	Nr of isolates	Total
Bird excreta	*Pinus sylvestris**	*Cryptococcus laurentii*	1	7
	*Platanus occidentalis**	*Rhodotorula mucilaginosa*	2	
	*Popla tree**	*Candida magnolia*	2	
		Candida guilliermondii	1	
	*Querqus**	*Cryptococcus albidus*	1	
Swabs from tree hollows	*Platanus occidentalis*	*Psudomycelium*	1	8
	Willow tree	*Rhodotorula mucilaginosa*	1	
	Querqus	*Candida glabrata*	2	
		Cryptococcus gatti	1	
		Candida pellicullosa	1	
	Pinus sylvestris	*Rhodotorula mucilaginosa*	1	
		Candida colliculosa	1	

*Name of the tree where swabs from bird excreta beneath them were collected.

Table 13 shows the frequency of *Cryptococcus* spp, *Candida* spp, and other fungi isolated in samples collected from Croatia and Kosovo. The most common isolates in all samples were *Candida* spp, and other fungi (n = 55, 58.7%). Among cryptococci, other *Cryptococcus* spp, except *C. neoformans* and *C. gattii* species complex were the most isolated samples in both countries (n = 31, 34.8%).

There were 35 *Cryptococcus* species complex isolates in Croatia – *C. laurentii* (20/35, 57.1%), *C. albidus* (6/35; 17.1%), *C. neoformans* (4/35; 11.4%), *C. unigutulatus* (2/35; 5.7%), *C. gattii* (2/35; 5.7%) and *C. terreus* (1/35; 3.0 %). There were only three *Cryptococcus* species complex isolates in Kosovo – *C. albidus, C. gattii and C. laurentii*.

C. neoformans species complex was isolated only from samples collected in Croatia (4/77; 4.3%) and *C. gattii* species complex from samples in both countries. There were no significant differences between countries in the species of isolated microorganisms at 95% probability level, but at the 90% probability level the difference was significant. Fisher's exact test = 5.319; P = 0.10 (>0.05, NS).

Table 13. Frequency of *Cryptococcus* spp., *Candida* spp., and other fungi isolated in samples collected from Croatia and Kosovo.

		Cryptococcus neoformans species complex	*Cryptococcus gattii* species complex	Other *Cryptococcus* spp .	*Candida* spp. and other fungi	*Total*
Croatia	n	4	2	29	42	77
	%	4.3%	2.2%	31.6%	45.7%	
Kosovo	n	0	1	2	12	15
	%	0.0%	1.1%	2.2%	13.0%	
Total	n	4	3	31	54	92
	%	4.3%	3.3%	33.7%	58.7%	100%

Fisher's exact test = 5.319; P = 0.10 (>0.05, NS).

Figure 7 shows six different species of *Cryptococcus* isolated from various sample types in Croatia and Kosovo. In Croatia, the highest number of isolated species was *Cryptococcus laurentii*.

Figure 7. *Cryptococcus* species isolated in Croatia and Kosovo.

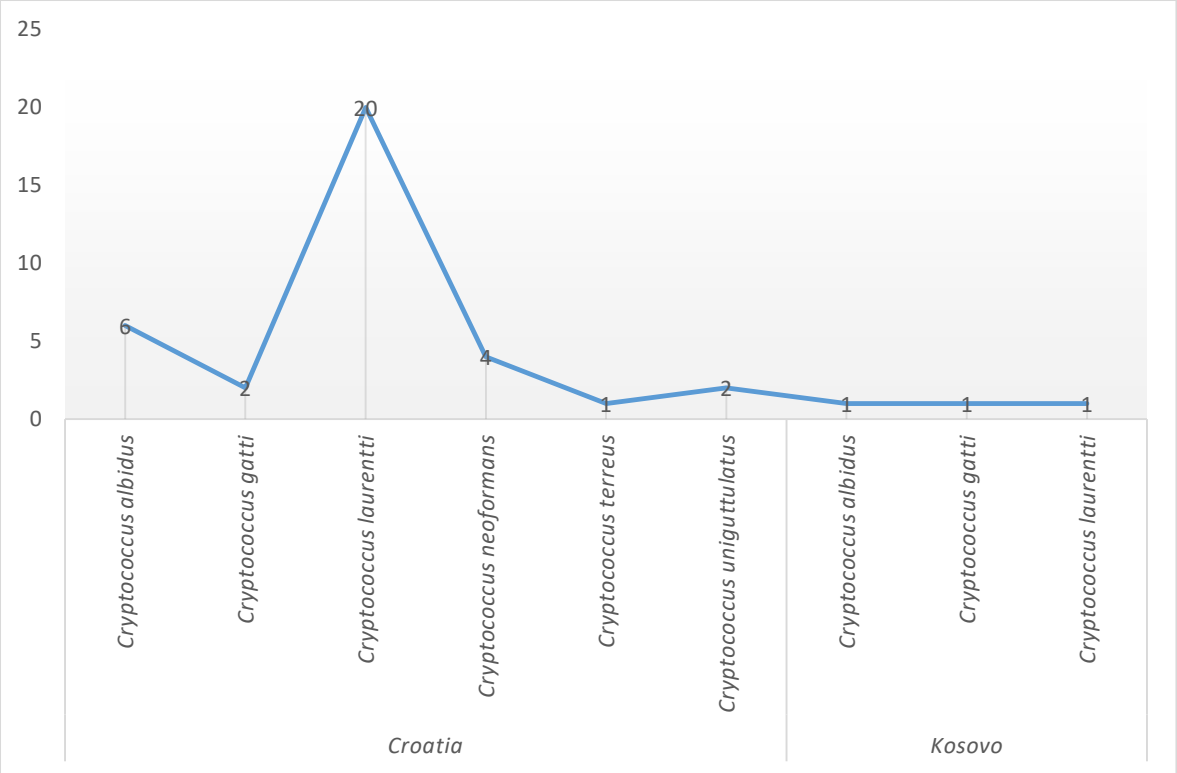
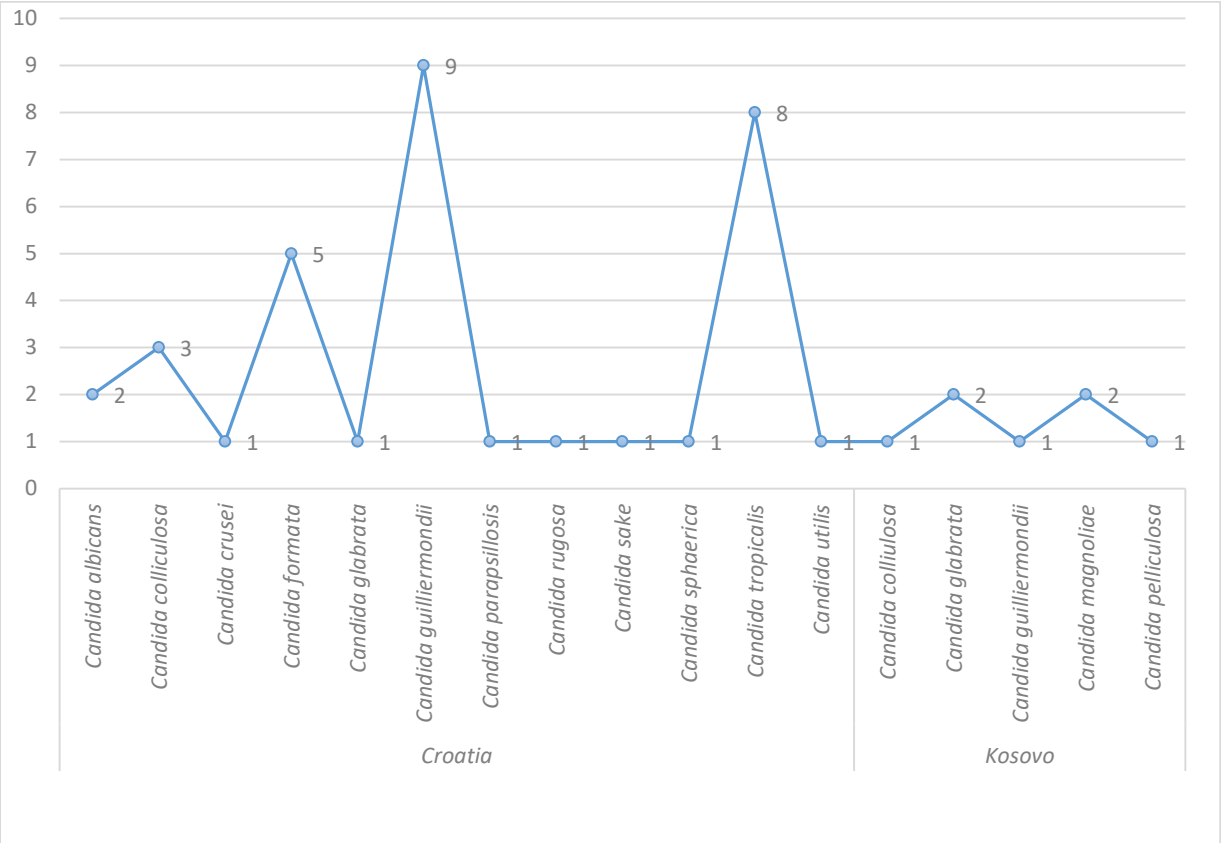


Figure 8 shows different species of *Candida* isolated from various sample types in Croatia and Kosovo. In Croatia, the highest number of isolated species was *Candida guilliermondii.* The number of different Candida species isolated from various sample types in Croatia and Kosovo is shown in Figure 5. In Croatia, the most frequent isolated species was *C. guilliermondii* (N=9) followed by *C. tropicalis* (N=8) *and C. formata* (N=5).

Figure 8. *Candida* species isolated in Croatia and Kosovo.



Characteristics of four *C. neoformans* species complex isolates from Croatia regarding the location, year and month of sample collection, sample type and tree species are shown in Table 14. Three isolates were found on island Krk in samples collected from *O. europea* and one isolate in city of Rijeka in sample collected from *P. sylvestris*.

Table 14. Description of four isolates of *Cryptococcus neoformans* species complex in Croatia according to location, month and year, sample type, tree species and growth temperature.

	N	**Country**	**City**	**Year**	**Month**	**Sample type**	**Tree species**
Cryptococcus neoformans species complex	3	CROATIA	KRK	2015	May	Swab sample from tree hollow	*Olea europea*
	1	CROATIA	RIJEKA	2016	June	Swab sample from tree hollow	*Pinus sylvestris*

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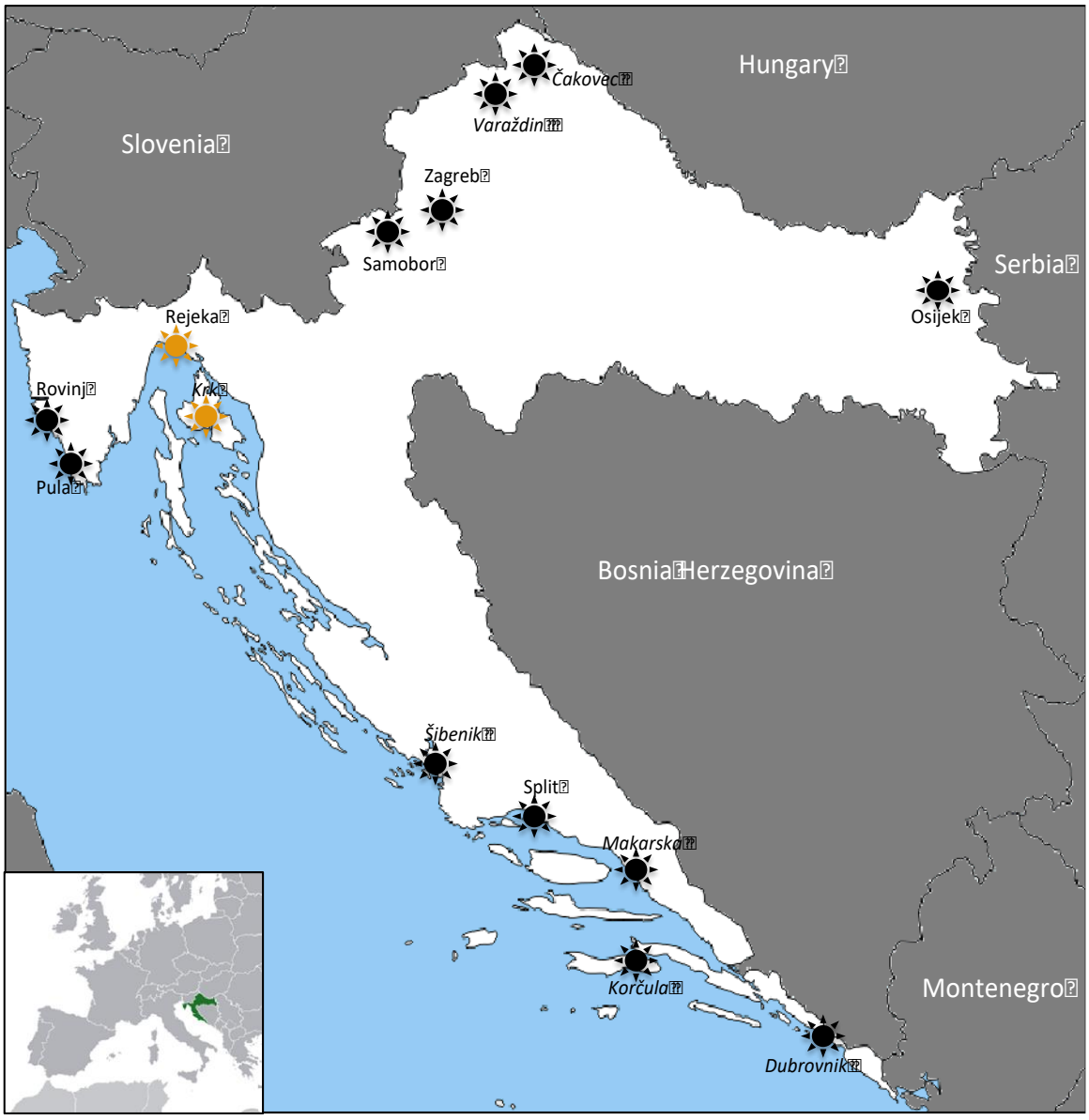
Geographic distribution of obtained samples and locations with samples positive for *C. neoformans* species complex are shown in Figure 9. All four isolates were recovered from cultures of tree hollow swabs collected in the Mediterranean area, while there were no isolates recovered from bird excreta or from samples collected in the continental area. The isolate from Rijeka was found in the hospital area and three isolates from the island of Krk were found along the promenade beside the sea.

Figure 9. Locations marked in yellow indicate the towns where positive samples were recovered. The inset shows the geographic position of Croatia in Europe.



Three isolates of *C. gattii* species complex isolated in the two countries, in different year, from the same sample type but of different tree species are shown in Table 15.

Table 15. Description of four isolates of *Cryptococcus gattii* species complex in Croatia and Kosovo according to location, month and year, sample type, tree species and growth temperature.

	N	Country	City	Year	Month	Sample type	Tree species	37º Celsius
Cryptococcus gattii species complex	2	CROATIA	KRK	2015	May	Swab sample from tree hollow	Oak tree	2
	1	KOSOVO	PRISTINA	2016	June	Swab sample from tree hollow	*Olea europea*	1

Results obtained by the ATB FUNGUS 3 method showed that all isolates were as susceptible as wild-type strains, with MIC values very similar for all antifungals tested. Antifungal susceptibility according to the CLSI broth microdilution method, performed for one VNI and one VNIV isolate, confirmed the ATB FUNGUS 3 results (Table 16).

Table 16. Antifungal susceptibility of the four *C. neoformans* species complex isolates tested by ATB FUNGUS 3 and broth microdilution reported as minimal inhibitory concentration values (μg/mL).

Location	Molecular type	5-FC (ECV = 8 μg/mL)		AMB (ECV = 0.5 μg/mL)		FLZ (ECV = 8 μg/mL)		ITZ (ECV = 0.25 μg/mL)		VOZ (ECV = 0.25 μg/mL)	
		ATBF3	CLSI	ATBF3	CLSI	ATBF3	CLSI	ATBF3	CLSI	ATBF3	CLSI
Krk	VNIV	4	-	0.5	0.25	1	4	0.125	-	0.06	0.25
Krk	VNI	4	-	0.5	-	1	-	0.125	-	0.06	-
Krk	VNI	4	-	0.5	-	1	-	0.125	-	0.06	-
Rijeka	VNI	4	-	0.5	0.25	2	4	0.125	-	0.06	0.125

5-FC = 5-fluorocytosine; AMB = amphotericin B; FLZ = fluconazole; ITZ = itraconazole; VOZ = voriconazole; ECV = epidemiological cutoff value; ATBF3 = ATB FUNGUS 3 method; CLSI = CLSI standard broth microdilution method; - = not tested.

Characteristics of three *Cryptococcus gattii* species complex islates regarding the location, tree species, and antifungal susceptibility are shown in Table 17. MIC values obtained with ATB FUNGUS 3 were identical except the one strain isolated in Krk resulted with different antifungal susceptibility in fluconazole (4 µg/mL) in comparison with other two strains (1 µg/mL).

Table 17. Difference in antifungal susceptibility between three isolates of *Cryptococcus gattii* species complex isolated in Croatia and Kosovo.

Isolated	City	Location	Tree species	5- FC	AMB	FCA	ITRA	VRC
Cryptococcus gattii species complex	Krk	Park in the city	*Olea europea*	4	0.5	4	0.125	0.06
Cryptococcus gattii species complex	Krk	Island of Krk	*Olea europea*	4	0.5	1	0.125	0.06
Cryptococcus gattii species complex	Pristina	National park, Germia	*Quercus*	4	0.5	1	0.125	0.06

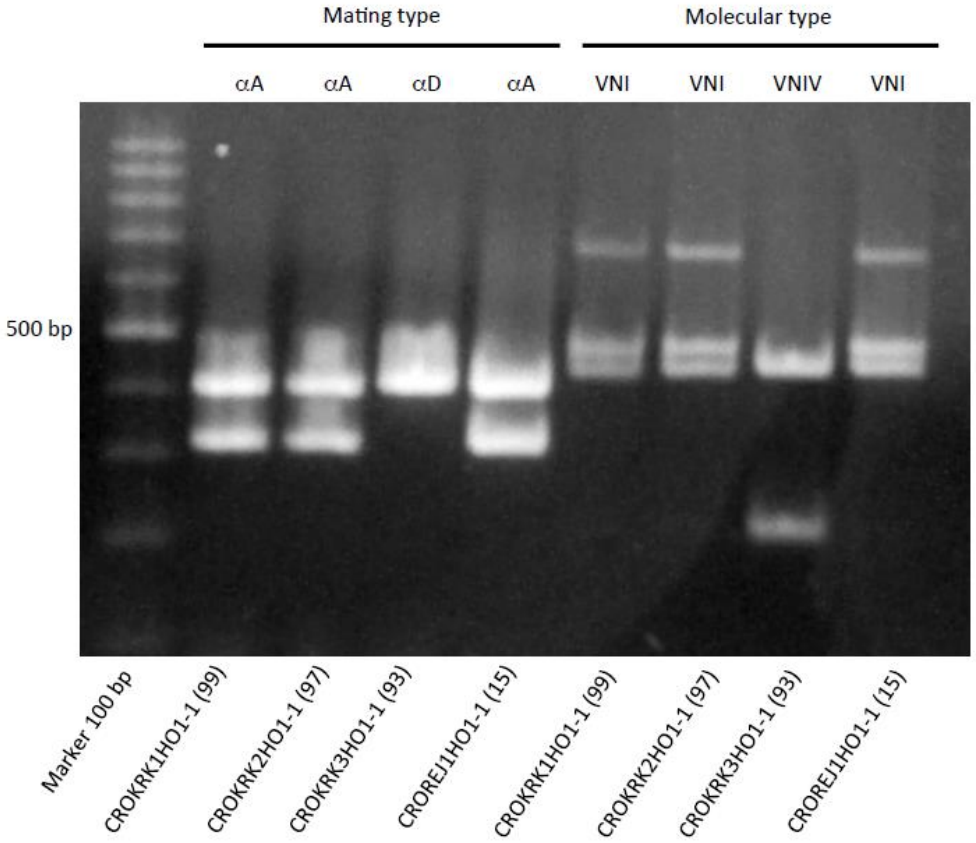
Table 18 shows mixed microorganisms isolated from the same sample type. *C. laurentii* and *C. guilliermondii* were isolated from *Ficus carica* (fig tree) in Šibenik, Croatia. The other two isolated microorganisms from the tree species in Split were *C. laurentii* and *C. famata.* In Kosovo, we isolated *C. albidus* and *R. mucilaginosa* from birds' excreta beneath the tree *Pinus sylvestris.*

Table 18. Samples with two different fungal species simultaneouslypresent

Isolated 1st microorganism	Isolated 2nd microorganism	Place	Country	Month, Year	Sample type	Tree species
Cryptococcus laurentii	*Candida guilliermondii*	Šibenik	Croatia	June, 2014	Tree hollow	*Ficus carica*
Cryptococcus laurentii	*Candida famata*	Split	Croatia	May, 2015	Tree hollow	*Morus*
Cryptococcus albidus	*Rhodotorula mucilaginosa*	Pristina	Kosovo	May, 2015	Birds' excreta beneath the trees	Birds' excreta beneath *Pinus Sylvestris*

Molecular analysis showed that four *C. neoformans* species complex isolates were classified into two different molecular types, VNI (n = 3) and VNIV (n = 1), all with mating type α (Figure 10). Three VNI isolates belnoged to serotype A and one VNIV isolate to serotype D. The three VNI isolates originated from two locations, Rijeka and Krk Island, whereas the VNIV isolate was found on Krk Island. Three isolates were found in tree hollows of *O. europea*, while one VNI isolate was also found in a tree hollow of a *P. sylvestris* (Table 18). The only isolate found in *P. sylvestris* tree hollows originated from the town of Rijeka and the isolates recovered from *O. europea* were found on Krk Island.

Figure 10. Multiplex PCR results showing mating type, molecular type and serotype identification of the four Croatian *Cryptococcus neoformans* species complex isolates. H99 and JEC21 represents the reference strains, VNI-aA and VNIV-aD, respectively. The 100 bp DNA ladder (Promega, Madison, WI, USA) was used as molecular DNA ladder.



CROKRK = isolates from Krk Island; CRORIJ = isolate from Rijeka.

Comparison of tree species and molecular types is shown in Table 19.

Table 19 Comparison of tree species and molecular types in four *Cryptoccocus neoformans* isolates.

MOLECULAR TYPE	**TREE SPECIES**		**TOTAL**
	Olea europea	*Pinus sylvestris*	
VNI, ALPHA A	2	1	3
VNIV, ALPHA D	1	0	1
TOTAL	3	1	4

6. DISCUSSION

Cryptococcosis is a fungal disease affecting more than one million people per year worldwide. The main etiological agents of cryptococcosis are the two sibling species *C. neoformans* and *C. gattii* species complex that present numerous differences in geographical distribution, ecological niches, epidemiology, pathobiology, clinical presentation and molecular characters (85).

Molecular data reveal that *C. neoformans* and *C. gattii* species complexes are unexpectedly genetically diverse (20,102). Intraspecific genetic diversity has also been revealed as more genotyping methods have been applied for each serotype (103).

During a ECMM prospective survey of cryptococcosis in Europe (from July 1997 to December 1999) 655 cases were reported from 17 countries; 565 of the completed questionnaires were evaluable. Cryptococcosis was associated with HIV infection in 77% of cases (range 57.5–94%). Assessment of the laboratory data highlighted the lack of defined standard procedures for the diagnosis of cryptococcosis: the antigen test was not usually used for screening, the disease was mainly recognised when meningitis occurred (65% of patients) and, with the exception of a few cases, the extent of the infection was not investigated (104).

The disease is believed to be acquired from the environment by inhalation of fungal cells, initially leading to pulmonary infection. Cryptococcosis remains the second most common cause of AIDS-related mortality, only narrowly behind tuberculosis (20). Cases also occur in patients with other forms of immunosuppression and in seemingly immunocompetent individuals (105, 106, 107).

Most of the reported cases of human cryptococcosis in Croatia is registered as a cryptococcal meningitis, while in Kosovo cryptococcal meningitis is never diagnosed, but this may be due to inadequate investigation rather than absence of definite epidemiological data about the organism in Kosovo (57).

More attention should be considered for human cases of unexplained chronic meningitis that is not responding to conventional therapy as *C. neoformans* species complex could be main cause of such fetal meningitis (108).

A unique attempt for a prospective European survey was performed during a survey from 1997 to 1999 in which 655 cases from 17 countries were reported and 311 cryptococcal isolates were collected for molecular typing. Although the survey represented a milestone in the elucidation of the European epidemiology of cryptococcosis, the results underestimated the burden of the disease since many countries did not participate in the study. This study by Cogliati represents the first collaborative effort aimed to understand the environmental distribution of *C. neoformans* and *C. gattii* species complex on trees around the Mediterranean basin and in continental Europe. Data from the rest of the EU are either scarce or completely lacking, especially from Central and Eastern European countries where a higher incidence of cryptococcosis is expected due to a heavier burden of HIV infection compared to Western Europe (56).

In a review article in year 2013 from Cogliati *et al.,* on global molecular epidemiology of *C. neoformans* and *C. gattii* species complex, it is concluded that about 68,811 isolates of *C. neoformans/gattii* species complex are reported in the world. Most of these isolates are from Asia and Africa, followed by Central and South America, Europe, North America, and Oceania. In Europe, most isolates are reported from France, Spain, Italy, and United Kingdom representing 82% out of the total isolates (*n = 8,736).* Data are lacking from many countries of Africa, Asia, and especially Central and Eastern Europe (85).

Some countries in Europe published data only for clinical isolates. For instance, approximately a year ago, in April 2018, 46 sequentially obtained isolates from 19 patients in Ljubljana, Slovenia, resulted in 34.8% (n = 16/46; 34.8%) with *C. neoformans* species complex. This was a retrospective study of epidemiology of cryptococcosis in Ljubljana. Majority of the cases were caused by *C. deneoformans*, mating-type α was predominant. Despite antifungal therapy, a cryptococcal isolate could persist for years. Voriconazole, itraconazole and posaconazole were the most potent antifungal drugs (109).

So far, we have only had clinical isolates of *C.neoformans* species complex documented in Croatia, whereas in Kosovo there is a lack of information on clinical and epidemiological aspects of *C. neoformans* species complex. In total, there are two studies that provide facts about the clinical and epidemiological situation of *Cryptococcus* spp. in Croatia. One is a

retrospective study by Hagen and Missoni, which included 48 clinical *C. neoformans* species complex strains, obtained from 15 patients, 10 with HIV-negative and 5 with HIV-positive patients (57). The other is an extensive environmental survey by Cogliati *et al*., from 2016, conducted between 2012 and 2015. This study included nine European and three non-European countries. Croatia, was also one of the country included in the study, but there were no positive isolates found of Cryptococcus in Croatia (56).

The project web site SCrEEN was designed by Cogliati with the aim to supply an easy tool for data collection of clinical and environmental data. In addition, this on-line tool will help to expand the network and will contribute to obtain reliable and useful epidemiological data for public health management in Europe and in Mediterranean area (110).

SCrEEN Project is addressed to all clinicians and microbiologists who can contribute to add new epidemiological information about cryptococcosis and *Cryptococcus*. This website contains three different e-forms: one for clinical data collection for veterinary cases and one for environmental data collection. In each form is mandatory to fill the fields concerning the researcher contact details in order to verify the information received. For environmental data it is also mandatory to include the geographical coordinates of the location where the survey has been performed.

All information are stored in three different data bases accessible from this website. Databases are periodically updated. A list of network participants is available at section Network, and will be updated on the basis of the information collected.

The website include also a technical section including classical and molecular methods for identification and typing of *C. neoformans* and *C. gattii* species complex (110). We have also reported our data from our research on the web site.

Due to the fact that infections by *Cryptococcus* species are predominantly acquired from environmental exposures, comprehension of environmental populations of these pathogens is essential.

This study provides the first insight into the ecology of *Cryptococcus* species in Croatia and Kosovo, and thus into the epidemiological exposure of the inhabitants to these yeasts in the studied urban locations in both countries. The samples were collected from two countries, Croatia and Kosovo, between 2013 and 2016, from urban public places including squares, parks, gardens, hospital areas, and school playgrounds.

The sample types were different, although the highest number of samples was collected from tree hollows (87.7%) and lowest from swabs of birds' excreta beneath the tree (12.3%).

The deposition of pigeon excreta in public places can serve as a potential source of infectious pathogen of importance for public health, such as *C. neoformans* species complex. But in our research the amount of the excreta was limited and the probable reason was that frequent cleaning was observed in public areas. Study by Takahara *et al.,* collected samples from 11 squares, but there was no positivity of *C. neoformans* species complex, despite the presence of excreta in five of the suqares (111).

In addition, the geographic distribution of *C.neoformans* species complex is associated with the longitude and climate (40).

Interesting findings were observed regarding the seasonal prevalence of *Cryptococcus* colony recovery. The influence of weather on recovering *Cryptococcus* has been previously demonstrated, with the study's findings that *Cryptococcus* was more frequently isolated when rainfall decreased (112). Of the 1.439 wood samples investigated in India, 406 yielded members of *C. neoformans* species complex and 171 for *C, gattii.* Although both of pathogens were isolated through all the seasons, the overall prevalence of *C. neoformans* var *grubii* was significantly higher than that of *C. gattii* serotype B. Two pathogens shows seasonal variations in their prevalence found during the autumn followed by that in the summer. In contrast the lowest prevalence of *C. neoformans* var *grubii* was noted in the rainy season (112).

Granados analyzed for the relationship between occurrence of the serotypes of the *C. neoformans* species complex in tree species and climatic conditions registered during sampling in four cities in Columbia from 1992 to 2004. The results suggested that environmental climatic conditions, mainly humidity, temperature, evaporation and solar radiation can affect the occurrence of the different serotypes in a trees in a differential manner (113).

In Italy, in 2012, Romeo *et al.* reported the occurrence of serotypes, genotypes and mating types of isolates of *C. neoformans/C. gattii* species complex recovered from environmental sources. An interesting evidence that emerges from this study is that during 1997-2011 the epidemiology of *C. neoformans* species complex in southern Italy remained almost unchanged (114).

In Mediterranean Europe, the number of *C. neoformans* species complex isolates in epidemiological survey is often lower (56).

In this study for the first time *C. neoformans* species complex was isolated from environmental samples in Croatia, while *C. neoformans* species complex was not found among environmental samples collected in Kosovo.

The differences in the prevalence between *C. neoformans* species complex across the seasons revealed that all our isolates belonged to season's spring-summer (May and June), contrary to the study by Randhawa *et al.*, which showed seasonal variations in their prevalence, with the highest prevalence found in autumn, followed by the summer.

Samples were collected monthly, year round, but the highest number of samples was recorded in June (54.6%), followed by May (14.8%) and November (11.1%).

Four *C. neoformans* species complex isolates were identified during this study in samples collected in Croatia (4/509, 0.8%). All four isolates were recovered from cultures of tree hollow swabs collected in the Mediterranean area, while there were no isolates recovered from bird excreta or from samples collected in the continental area. The isolate from Rijeka was found in the hospital area and three isolates from the island of Krk were found along the promenade beside the sea.

Croatia was included in extensive environmental survey carried out during 2012–2015 with eighteen environmental samples, but there was no positive isolate for *C. neoformans* or *C. gattii* species complex (56). In this study by Cogliati, *C. neoformans* species complex was isolated in different European countries, such as Cyprus, France, Greece, Italy, Libya, Portugal, Spain, and Turkey. The highest percentage of sampled trees was in Italy (43%), followed by other countries. The percentage of colonized trees relative to the two species was 4.6% for *C. neoformans* species complex, and 0.4% for *C. gattii* species complex. Although *C. gattii* species complex infections occurred mainly in tropical and subtropical climate zone over the past decade, *C. gattii* species complex infections in humans and animals in Europe have increased and have been reported as a rare cause of apparently autochthonous cryptococcal infections (115). This species was thought to be restricted to tropical and subtropical regions, but an outbreak due to *C. gattii* species complex infection, which occurred in Vancouver Island and North West Pacific Coast of America, has also expanded the geographical area of this pathogen to temperate regions (56).

Cogliati was the first who described the presence of the emerging pathogen *C. gattii* species complex in the Mediterranean environment. A high rate of positive samples were retrieved from carob tree (9.8%), and very low rate yielded from *Eucalyptus* trees. In that study, there were three types of trees from which this pathogen was obtained: carob tree - *Ceratonia siliqua*, Mediterranean stone pine - *Pinus halepensis*, and eucalyptus - *Eucalyptus camaldulensis* (56,116).

This is the first study that documented occurrence of *Cryptococcus gatti* species complex in two different states, Croatia and Kosovo.

In this study, we isolated *C. gattii* species complex from environmental samples in both countries, Croatia and Kosovo. Isolation rate of *C. gattii* species complex was 0.4% (n = 3/709). The frequency rate varied between the two countries.

Two isolates of *C. gattii* species complex were found in the city of Krk, in Croatia, and one in Prishtina, the capital city of Kosovo.

However, more recent epidemiological data indicate that this species can adapt to new climatic conditions, including those found in the Mediterranean area where this fungus is responsible for many human and animal infections (114).

Over the past decade, considerable data have resulted from approximately 400 studies revealing the occurrence of *Cryptococcus* species in approximately 50 tree species belonging to several families and genera. Although there is no evidence that *C. neoformans* species complex degrades wood by itself, it can survive with other fungi and bacteria on wood surfaces (117,118). Major evidence accumulated shows a close relationship between trees and yeasts of the *Cryptococcus* genus (56).

The epidemiological importance of the sampled trees would depend not only on the extent of their colonization but also on geographic distribution (119).

Our samples were collected from 622 tree hollows swabs (87.7%) in different tree species. In total, there were 53 different tree species. The species of the trees were specified every time the sample was obtained (101).The very first trees represented in higher number with collected samples belonged to *O. europea* (15.1%), followed by *P. sylvestris* (11.7%), and *C. sativa* (5.8%).

Because of their rarity, 12 different tree species were represented with only one collection of the sample. On the other site, samples from birds' excreta were collected in limited numbers. We tried to collect more samples from birds' excreta, but because of the streets being cleaned we could not find samples in higher numbers. Therefore, the number of the swabs collected from birds' excreta beneath the trees was (87/709; 12.3%).

A total of four trees colonized by *C. neoformans* species complex were found in Croatia. Three isolates of *C. neoformans* species complex were found in Krk, and one in Rijeka. Krk is the city where both *C. neoformans* and *C. gattii* species complex were isolated.

Both microorganisms were isolated from tree hollows. This information confirms that *C. neoformans* species complex co-habits in the same environmental niche as *C. gattii* species complex. It is worrying to note that *C. neoformans* species complex was recovered from a sample obtained from public places such as the park of the clinical hospital in Rijeka. This finding reveal the risk of exposure for immunosuppressed and even immunocompetent individuals in daily manner.

In the current study, positive samples of *C. neoformans* species complex belonged to two different trees, *P. sylvestris* and *O. europea*. These two trees are the trees with the highest representation in our research.

Different from the findings of Colon *et al.,* showing that carob trees are an important environmental niche for *C. gattii* in the Mediterranean area, our isolates of *C. gattii* species complex belonged to *Quercus robur* and *O. europea* (116).

Among our tree species, respectively tree hollows, *O. europea* was recorded as a host for *C. neoformans* and *C. gattii* species complex. These data show that *C. gattii* species complex is present in our environment, and it is associated with other trees than *Eucalyptus* tree. Our findings correlate with the findings of Cogliati *et al.* that both *C. neoformans* and *C. gattii* species complex are present in the Mediterranean environment in association with trees (85).

Furthermore, in a study by Cogliati from 2016, *Eucalyptus* tree and olive trees were the most colonized trees with *Cryptococcus* spp. In that study, *Cryptococcus neoformans* var *grubii* colonized 12 different tree genera, whereas *C. gattii* species complex was recovered only from trees typical for the Mediterranean environment like *Ceratonia, Olea, Eucalyptus,* and *Pinus pinea* (56).

A study by Ellabib *et al.* from 2016 described for the first time the distribution of species, mating types and molecular types of *C. neoformans/C. gattii* species complex from *Eucalyptus* trees and *Olive* trees in Libya. Out of 46 samples, 34 were isolated from pigeon excreta, 3 from *Eucalyptus* trees, and 9 from olive trees (102).

Another study shows the prevalence of *C. neoformans* species complex during the flowering season of *E. camaldulensis* at the Delta region in Egypt. Thirteen isolates of *C. neoformans* species complex were recovered from samples out of 200 *Eucalyptus* trees, including leaves, flowers and woody trunks (119).

Due to pathogenicity, all studies focus on two species, namely *C. neoformans* and *C. gattii* species complex inside genus *Cryptococcus.*

Other *Cryptococcus* non *neoformans* were also isolated in our study, which recently, infections caused by this pathogen have been increasingly recognized. The frequency of isolation was different in the two countries. In Croatia, there was 39.2% of isolates of other *Cryptococcus* species. Other species of *Cryptococcus* isolated in our study included: *Cryptococcus albidus, Cryptococcus laurentii, Cryptococcus terreus* and *Cryptococcus uniguttulatus.*

Cryptococcus laurentii was previously considered saprophyte and thought to be non-pathogenic to humans. However, in favourable circumstances like diminished immunity, it seems to be an important pathogen, also there is a case report of infection in immunocompetent child (120, 121). *Cryptococcus albidus* was reported in some case report. One of them is its presence in renal transplant recipient, who had been successfully treated with fluconazole therapy (122).Until today the last two *Cryptococcus* isolated in our study *C. terreus* and C. *uniguttulatus* are the two species that are not pathogenic to humans.

Šibenik, Krk, and Rijeka were the cities with the highest number of different pathogens isolated. In Kosovo, this number was much lower, but the highest number of pathogens was isolated is Prishtina.

Fifteen different species of Candida were isolated among 709 samples. These included *Candida albicans, C. colliculosa, C. krusei, C. formata. C. glabrata, C. guilliermondii, C. magnoliae, C. parapsilosis, C. pelliculosa, C. rugosa, C. sake, C. spherica, C. tropicalis, C. utilis*. Interestingly, *C. guilliermondii* was the most isolated yeast.

There are also other studies that reported yeast isolation from trees. Maganti analyzed 11,100 samples obtained from tree hollows, shrubs, and avian droppings in Canada, and 88 positive yeast strains were isolated (123). Also, birds may act as carriers and transmitters in the environment of *C. neoformans* species complex and other potential yeas (57, 124).

Other yeasts isolated in the current study included: *Pseudomycelium, Rhodotorula mucilaginosa, Trichosporon mucoides.* They were isolated from various trees. Also, other pathogens from swabs of birds' excreta collected beneath the trees were isolated in both countries, such as *C. guillermondii, C. magnoliae, C. albidus, C.colilulos, R. mucilaginosa, C.laurentii and C.albidus.*. In total, there were 51 (7.0%) other microorganisms such as *Cryptococcus* isolated. More *Candida* species and less *Cryptococcus* species were isolated in Kosovo.

Pharmacologic management of cryptococcal infection usually consists of primary therapy with amphotericin B, with or without flucytosine, followed by maintenance therapy, or in some cases life-long suppressive therapy with fluconazole. During the 15-year study period (1994-2004) a total of 1,811 clinical episodes of *C. neoformans* species complex were submitted by 100 study centers in Africa, Europe, Latin America, the Pacific, and North America. In summary, Pfaller *et al.* confirmed that *in vitro* resistance to standard antifungal agents used in the treatment of cryptococcosis remained uncommon among isolates of *C. neoformans* species complex from five broad geographical regions and did not increase over the 15-year period (69).

It has been reported that the widespread use of fluconazole can lead to the emergence of less susceptible strains of *C. neoformans* species complex (125). Globally, there has been an increase in the percentage of *Cryptococcus* isolates found to have some degree of fluconazole resistance (126). It is unknown what causes the difference in the *in vitro* antifungal susceptibilities among isolates of different molecular types of the *C. neoformans/C. gattii* species complex. Sionov *et al.* suggested that fluconazole resistance may occur due to chromosome duplication during prolonged azole therapy, a process that could be favored in a certain molecular type (66).

In vitro antifungal susceptibility of *C. neoformans* species complex as determined with the techniques used, is not able to predict the early clinical outcome in patients with cryptococcosis. It is evident that concentration response relationships differ substantially and that patients with high pretreatment cerebrospinal fluid fungal burdens will commonly require either higher doses of amphotericin B, extended treatment courses, treatment with a combination of amphotericin B and other agents (fluconazole or flucytosine) or all three approaches for optimum treatment (127, 128).

Almeida *et al.* showed a high correlation between distinct genetic profiles identified by two molecular typing methods and the tendency to become resistant to antifungal drugs. They provided evidence that *C. neoformans* species complex may undergo phenotypic and genotypic changes during the early stages of infection in humans, prior to or during antifungal therapy (129).

Our study is the first to determine antifungal susceptibitliy of environmental isolates of *C. neoformans* species complex in Croatia and *C. gattii* species complex in both Croatia and Kosovo.

All the isolates of *C. neoformans* and *C. gattii* species complex were processed for antifungal susceptibility with ATB FUNGUS 3. The five investigated agents were flucytosine, amphotericin B, fluconazole, itraconazole, and voriconazole. The concentrations of these agents in the test ranged from 4 to 16 μg/mL for flucytosine, 0.5-16 μg/mL for amphotericin B, 1-128 μg/mL for fluconazole, 0.125-4 μg/mL for itraconazole, and 0.06-8 μg/mL for voriconazole. The strips were incubated at 35°C for 72 h and the MICs were read visually, according to the manufacturer's instructions (74).

Isolates of *C. neoformans* species complex were also tested with a standard broth microdilution method according to the CLSI for amphotericin B, fluconazole, and voriconazole (95). Since there are no clinical breakpoints defined by the CLSI for *C. neoformans* species complex, ECV were used as reference values to define a strain as wild type or non-wildtype (96, 97). Isolates were considered wild type when the minimal inhibitory concentration (MIC) value for amphotericin B was ≤0.5 μg/mL, for flucytosine ≤8 μg/mL, for fluconazole ≤8 μg/mL, for itraconazole ≤0.25 μg/mL, for voriconazole ≤0.25 μg/mL. Susceptibility testing according to CLSI was done in 2019, two years after other investigations were already finished.

For that reason, not all isolates of *C. neoformans* species complex (but two) were viable and available for testing. Unlike *C. neoformans* species complex, *C. gattii* species complex has not been included in CLSI guidelines for testing of yeasts and was tested only with ATB FUNGUS 3. The ECVs were used as reference values to define a strain as wild type or non-wildtype regardless of genotype: for amphotericin B was ≤1.00 μg/mL, for flucytosine ≤4 μg/mL, for fluconazole ≤8 μg/mL, for itraconazole ≤0.5 μg/mL, for voriconazole ≤0.25 μg/mL (96,97,130).

Environmental isolates of *C. neoformans* and *C. gattii* species complex were evaluated as to their *in vitro* responses to antifungals. Although the standardized CLSI methodology for susceptibility testing of *Candida* spp. was shown to be reliable and clinically useful, at least for superficial infections in HIV-infected patients, there are still technical problems for *C. neoformans* (128).

For *C. neoformans* species complex MIC values ty against fluconazole were not the same for all isolates, but are in concordance with a study by Thomson *et al.*, from 2009 (82). In our study three isolates had MIC value for fluconazole 1 μg/mL, and one isolate 2 μg/mL according to ATB FUNGUS 3. Two isolates tested according to CLSI standards had the same MIC 4 μg/mL in comparison to ATB FUNGUS 3 (1 μg/mL and 2 μg/mL, respectively). Although MIC values determined with CLSI standards were slightly higher, the interpretation was the same – all tested strains belonged to the wild-type category.

For *C. neoformans* species complex MIC values against itraconazole and voriconazole tested with ATB FUNGUS 3 only were the same for all four isolates of *C. neoformans* species complex. Susceptibility for itraconazole for all isolates of *C. neoformans* species complex was 0.125 μg/mL, and for voriconazole 0.006 μg/mL. For both antifungals all tested strains belonged to the wild-type category.

For *C. neoformans* species complex MIC values against amphotericin B was the same for all four isolates, 0.5 μg/mL according to ATB FUNGUS 3. Two strains tested according to CLSI standards had MIC value 0.25 and were susceptible to amphotericin B.

The tested antifungal compounds amphotericin B, 5-flucytosine, fluconazole, itraconazole, and voriconazole demonstrated high *in vitro* activity against environmental *C. neoformans* species complex isolates**.** This is in accordance with the antifungal susceptibility results for clinical isolates in a previous study in which all tested antifungals also showed high *in vitro* activity against *C. neoformans* species complex isolates (57). Some sudies demonstrated differences in antifungal susceptibility among molecular types, but the correlation between the molecular type and antifungal susceptibility is still an open issue (131,132).

A study in which isolates of *C. neoformans* species complex were isolated from birds' excreta in a public area in Malaysia using broth microdilution method and E test, it documented susceptibility of all environmental samples to amphotericin B, fluconazole, and itraconazole (78). Contrary to this finding, in a study by Dongmo *et al.* from 2016, all environmental isolates showed resistance to fluconazole, ketoconazole, and amphotericin. This is the only study with such a pattern of susceptibility (133).

There are also studies that compared susceptibility profile of clinical and environmental isolates of *C. neoformans* and *C. gattii* species complex. In one study, clinical isolates had lower susceptibility than the environmental isolates to amphotericin B and itraconazole, whereas environmental isolates had lower susceptibility than clinical isolates to fluconazole, voriconazole, and ketoconazole (134). In another study, by Gutsch, no significant difference was found between the antifungal susceptibility profiles of clinical and environmental isolates *C. neoformans* and *C. gattii* species complex ($p<0.06$) (81).

Comparison of antifungal susceptibility results obtained from clinical isolates of *C. neoformans* species complex in Croatia and our environmental sources revealed that both categories are susceptible to five antifungal agents. These facts were reinforced by the hypothesis that there is a strong relation between clinical and environmental isolates of *C. neoformans* species complex.

Antifungal susceptibility was performed also for *C. gattii* species complex. According to ATB FUNGUS 3 three isolates of *C. gattii* species complex showed the same susceptibility profile except one strain that showed higher MIC value for fluconazole in comparison to other two strains (4 μg/mL vs 1 μg/mL in other two isolates). All three isolates had MIC value of 4 μg/mL for 5-flucytozine, 0.5 μg/mL for amphotericin B, 0.125 μg/mL for itraconazole and 0.06 μg/mL for voriconazole. According to ECVs, *C. gatti* species complex strains belong to wild type for all tested antifungals. Although MICs for fluconazole were different among the strains, all were susceptible to fluconazole.

For *C. gatttii* species complex susceptibility differs among regions of the world and genotypes. For example, VGII genotype exhibits higher ECVs for 5-flucytozine (ECV 95% 16μg/ml, ECV 99% 16μg/ml) and for fluconazole (ECV 95% 32μg/ml, ECV 99% 64μg/ml) compared to *C. neoformans* and others *C. gattii* species complex genotypes. Overall, according to literature data *C. gattii* species complex strains show higher MICs for fluconazole than *C. neoformans* species complex. In agreement, some studies have shown same results for other triazoles agents and propose slightly different ECVs values suggesting that genotype should be taking in consideration. It is important to note that clinical relevance of ECVs is not established and should be considered with caution.

In the current study, comparison of antifungal susceptibility profiles between two species showed that *C. gattii* species complex isolates were as susceptible as *C. neoformans* species complex to all tested antifungals.

The following techniques of microorganism typing have shown the best ability to differentiate between fungal serotypes and molecular types: PCR fingerprinting, PCR-RFLP, AFLP, and MLST. Thus, the accumulation of data generated by molecular methods may have a positive impact on the monitoring of resistant strains and on disease treatment. PCR-RFLP and AFLP have proven to be specific techniques for rapid detection of serotypes and molecular types of the *C. neoformans* species complex (135, 136).

In view of the important role played by mating type in the epidemiology an virulence of *C. neoformans* species complex, Esposto *et al*., designed a multiplex PCR method to distinguish the six mating type patterns (Aα, Dα, Aa, Dα, Aα /Da and Aa /Dα) of *C.neoformans* var *neoformans.*This method clearly distinguished the six different *C.neoformans* var *neoformans* matting type patterns by means of a single multiplex PCR.This was in advance compared to the previous molecular approach, in which the mating type was determined by ffour separate PCRs of the STE *20a* and STE20α alleles.

Thus, the multiplex PCR represents a rapid, simple and relatively economical tool for epidemiological and virulence studies, as it reduces the number of PCR sfrom four to one (98).

Different PCRs using specific primers for each of the mating type and serotype combination are performed to determine the mating type (a or α). Serotype of the *Cryptococcus* species isolates is performed by multiplex PCR.

Some studies have observed a correlation between the genotype and the antifungal susceptibility profile, showing that *C. neoformans* species complex AFLP1/VNI was found to be less susceptible to amphotericin B, fluconazole, itraconazole and flucytosine when compared to *C. deneoformans* AFLP2/VNIV and the interspecies hybrid AFLP3/VNIV (117,119, 136).

Molecular types of *C. neoformans* and *C. gattii* species complex are not equally distributed in the world, with VNIV being more often found in Europe, VGIII the most common molecular type of *C. gattii* recovered in the Americas, and VGI prevailing as the primary type of *C. gattii* in Oceania, Asia, and Europe (137).

Hagen *et al.* investigated retrospectively clinical isolates of *C. neoformans* species complex in Denmark and the Netherlands. The most predominant serotype in those two studies is serotype A, genotype VNI (88, 138).

It has been reported that 20% of cryptococcal infection are caused by multiple strain or molecular type, but generally only one isolate per infection is tested (96).

In a study by Herkert *et al.*, genotype AFLP1/VNI was the most frequent among the isolates studied and all *C. neoformans sensu stricto* isolates had low MICs of antifungal drugs. Microsatellite analysis revealed high diversity among the *C. neoformans sensu stricto* studied population with clinical, environmental and veterinary isolates being related to each other (139).

In 2012, Cogliati presented an atlas of the molecular types of *C. neoformans* and *C. gattii* species complex globally. This article shows the molecular type of these two species in different parts of the world. It is 'a cosmopolitan on the move', as Hagen named the book. Concerning the molecular determination, there is a lack of data from large parts of Africa, Asia, Eastern Europe, as well as from the United Kingdom, Ireland, Norway, and Finland. VNI is the prevalent molecular type worldwide, except in Australia and Papa New Guinea, where VGI prevails (85). According to recent studies, the VNI is the most common type in clinical and environmental isolates in different regions of Brazil, with a prevalence ranging from 64% to 95.7% (140).

In another study in year 2016, Cogliati *et al.,* investigated the genotypic diversity among a set of clinical and environmental *C. neoformans* var. *neoformans* isolates and to evaluate the relationship between genotypes, geographical origin and clinical manifestations. A total of 83 globally collected *C. neoformans* var. *neoformans* isolates from Italy, Germany, France, Belgium, Denmark, Greece, Turkey, Thailand, Japan, Colombia, and the USA, recovered from different sources (primary and secondary cutaneous cryptococcosis, disseminated cryptococcosis, the environment, and animals), were included in the study. All isolates were confirmed to belong to genotype VNIV by molecular typing and they were further investigated by MLST analysis (141).

Amplified fragment length polymorphism fingerprinting showed that most Danish cryptococcal isolates (n = 61; 56.5%) representing *C. neoformans* species complex (56 isolates; 51.9%) belonged to the major genotype AFLP1/VNI, and a small proportion (n = 5; 4.6%) belonged to the genotype AFLP1B/VNII (87).

The high percentage of VNIII and VNIV hybrid strains reported by the ECMM epidemiological survey of cryptococcosis suggests that in Italy and other European countries serotype A and D populations are not genetically isolated but are able to recombine by sexual reproduction. This is in contrast to the hypothesis that serotype A and D populations are diverging towards a clonal evolution and suggests that they are members of a unique variety, *C. neoformans* var *neoformans* (142). Boekhout *et al.*, proposed the presence of hybridization in *C.neoformans* species complex and *C.bacilisporus.* They favour the scenario that the fungus uses both (para) sexual and asexual reproduction strategies. Consequently, genetic material can be transferred between isolates of different genetic background, which may result in strains with an altered virulence and/or resistance to antifungal agents (46).

In another study, by Ferreira *et al.* from 2012, a total of 49 environmental samples were collected from several locations in North Portugal, and resulted with 28 *C. neoformans* species complex isolates. The distribution of the *C. neoformans* species complex genotypes among the environmental isolates was as follows: 32.1% for VNI, 14.3% for VNIII, and 53.6% for VNIV. *C. gattii* species complex was not found among the environmental isolates (87).

Some reports suggested that *C. neoformans* var *grubii* serotype A and mating types alpha isolates are more virulent than serotype D and mating types isolates (128).

In our study, molecular data obtained from all environmental isolates of *C. neoformans* species complex recovered here, revealed that they were of different serotypes and different mating types.

Based on molecular typing, all *C. neoformans* species complex isolates were classified into two different molecular types, VNI and VNIV. Serotype A and serotype D were both found in environmental samples. Both serotypes were found in Krk, isolated from the same tree *Olea europea.* Most isolates from the Cogliati data belonged to VNI molecular type (59%), although VNIII and VNIV molecular types were also reported in most of the countries representing 18.5% and 18.3%, respectively. According to those data, *C. neoformans* specie complex molecular type distribution in Europe has not been properly defined yet.

The molecular analysis of environmental isolates in Croatia demonstrated the presence of molecular types VNI and VNIV, which were already identified in clinical samples during previous studies. Molecular type VNIII has only been detected in clinical samples so far (56, 57).The impact of different molecular types on patient outcome in terms of clinical manifestations or attributable mortality rates is still unknown. Controversial results have been generated regarding the correlation between molecular types, virulence, and mortality rate (143, 144).

Determination of the mating type of four isolates showed that our results are in concordance with the observation made by Cogliati, showing that mating type αA was the prevalent mating type among *C. neoformans* var *grubii*, whereas αD mating type was recovered in Italy (71 isolates), Spain (1 isolate), and Turkey (6 isolates) (85).

Mating type alpha (MAT α) was predominant in all isolates of *C. neoformans* species complex (n = 4, 100%).

Comparing our results with results of clinical isolates of *C. neoformans* species complex from Croatia, we can conclude that there is a relationship between the clinical and environmental isolates of *C. neoformans* species complex. Serotype A (genotype AFLP1) and serotype D (AFLP 2) were both found in clinical and environmental isolates of *C. neoformans* species complex in Croatia.

Serotype AD (genotype AFLP 3) was also found in clinical isolates. We did not isolate any hybrids between VNI and VNIV, as Missoni reported for clinical isolates (57).

The current study suggests that there are different serotypes and different mating types in Croatia; serotype VNI alpha A was found in 75% of the isolates, and VNIV alpha D in 25% of the isolates.

The European molecular typing showed that most isolates belonged to VNI (59%) molecular type, although VNII and VNIV molecular types were also reported in most of the countries, representing 18.5% and 18.3%, respectively (85).

Isolates recovered from environmental and clinical sources have often shown genotypic similarities (85). Compared to clinical isolates from Croatia, there is a similarity concerning serotype A and D, but we did not isolate any AD serotype.

The presence of pigeons and *Eucalyptus* trees in the vicinity of some particular places such as rest homes and hospitals should be considered as a risk factor for the immunocompromised population (145).

This is the first report proving the presence of *C. neoformans* species complex in the environment of Croatia. The results of the study show the potential risk of exposure for inhabitants, especially on the Croatian coast, to certain molecular types, particularly VNI and VNIV, which can be expected in clinical cases of cryptococcosis. Although a limitation of this study is the small number of detected environmental isolates, their antifungal susceptibility pattern suggests that no strains with low susceptibility to the most common antifungals should be expected in patients with cryptococcosis and no guideline modifications are needed at the moment. Further investigation regarding the correlation of molecular types with clinical outcome and antifungal susceptibility are warranted.

7. CONCLUSION

This is the first environmental study that has covered two countries, Croatia and Kosovo, investigating the epidemiological distribution, antifungal susceptibility and molecular characterization of *C. neoformans* species complex. According to hypothesis and aims, the conclusions based on the achieved results of this study are the following:

● Three out of four (3/4; 75%) of C. *neoformans* complex isolates in Croatia belonged to serotype A and all (4/4, 100%) had mating type α confirming the hypothesis that these are the predominant types in Croatia.

● The tested antifungal compounds amphotericin B, 5-flucytosine, fluconazole, itraconazole, and voriconazole demonstrated high *in vitro* activity against environmental *C. neoformans* species complex isolates confirming the hypothesis that environmental *C. neoformans* species complex isolates are susceptible to antifungals. Environmental *C. gattii* species complex isolates belonged to wild type for all tested antifungals as well according to ECVs.

● *C. neoformans* species complex was founded in environmental samples collected in public places in two cities, Krk and Rijeka, on Croatian coast, but we could not isolate *C. neoformans* species complex from the samples collected in Kosovo.

● Antifungal susceptibility testing obtained by the ATB FUNGUS 3 method showed that all *C. neoformans* species complex isolates from Croatia were as susceptible as wild-type strains, with MIC values very similar for all antifungals tested. Antifungal susceptibility according to the CLSI broth microdilution method, performed for two isolates available, confirmed the ATB FUNGUS 3 results. No *in vitro* resistance to amphotericin B, 5-flucytosine, fluconazole, itraconazole, and voriconazole was observed with either of two methods applied.

● The four *C. neoformans* species complex isolates from Croatia were classified into two different molecular types, VNI (n = 3) and VNIV (n = 1), the finding expected according to previous studies. VNI is the most prevalent molecular type in the Mediterranean basin, and molecular type VNIV, although less common than VNI, was already detected in the environment of Croatia's neighbouring countries.

● The four *C. neoformans* species complex isolates from Croatia were found in swabs taken from tree hollows and none were found from samples taken from the bird excreta in the soil beneath the trees. Three isolates were found in *O. europa* and one in *P. sylvestris.* Our findings suggest that trees present an important environmental niche and stable reservoir for *C. neoformans* species complex.

● All four isolates of *C. neoformans* species complex were recovered from cultures of tree hollow swabs collected in the Mediterranean area, while there were no isolates recovered from the continental part of Croatia.

● As *C. neoformans* species complex isolates were founded in environmental samples form Croatia only, the comparison of antifungal susceptibility, serotypes, molecular and mating types was not possible with isolates from Kosovo. However, this is the first study showing the presence of *C. gattii* species complex in the samples obtained from tree hollows in both countries, Croatia and Kosovo. Three isolates of *C. gattii* species complex were isolated from two tree species, *Quercus* and *O. europea.*

8. SAŽETAK

Cryptococcus neoformans species complex je oportunistički kvasac koji uzrokuje po život opasne infekcije kod imunokompromitianih bolesnika. Cilj ovog istraživanja je bio istražiti epidemiološku distribuciju, osjetljivost na antifungike i molekularne karakteristike *C. neoformans* species complex u Hrvatskoj i Kosovu.

U razdoblju od 2013. do 2016. godine, uzeto je 709 uzoraka iz duplji drveća i sasušenih ptičjih sekreta s tla ispod drveća na različitim zemljopisnim lokacijama.

U uzrocima uzetim iz duplji drveća izolirana su četiri izolata *C. neoformans* species complex u dva grada, Krku i Rijeci, na jadranskoj obali, dok u Kosovu nije utvrđen niti jedan izolat. Testirani antifungici pokazali su dobru *in vitro* djelotvornost na izolate *C. neoformans species* complex iz okolišnih uzoraka. Tri izolata *C. gattii* species complex su izolirana u Hrvatskoj i Kosovu te su također pripadali divljem tipu na temelju epidemioloških graničnih vrijednosti. Tri od četiri (3/4; 75%) izolata *C. neoformans* complex u Hrvatskoj pripadala su serotipu A a svi (4/4, 100%) su pokazali tip α. Za četiri izolata *C. neoformans* species complex u Hrvatskoj utvrđeno je da pripadaju molekularnim tipovima VNI (n = 3) and VNIV (n = 1).

Ovo je prvo istraživanje koje je pokazalo prisutnost *C. neoformans* species complex u okolišnim uzorcima u Hrvatskoj i u kojem je određena njihova osjetljivost na antifungike. Rezultati istraživanja su pokazali potencijalni rizik za izloženost stanovnika, osobito na jadranskoj obali, molekularnim tipovima VNI i VNIV koji se mogu očekivati u kliničkim slučajevima kriptokokoze. Iako je ograničenje ove studije mali broj otkrivenih izolata u okolini, rezultati osjetljivosti na antifungike pokazuju da kod bolesnika s kriptokokozom ne treba očekivati izolate sa samnjenom osjetljivosšću na najvažnije antifungike.

Ključne riječi: *Cryptococcus neoformans, Cryptococcus gattii*, environment, molecular typing

9. SUMMARY

Cryptococcus neoformans species complex is an opportunistic yeast that causes life-threatening infections in immunocompromised hosts. The aim of the study was to investigate the epidemiological distribution, antifungal susceptibility and molecular characterization of *C. neoformans* species complex Croatia and Kosovo.

During 2013-2016, 709 samples were collected from trunk hollows and birds' excreta in the soil beneath the trees in different geographical locations.

Four *C. neoformans* species complex isolates was found in environmental samples of tree hollows collected in two cities, Krk and Rijeka, on Croatian coast, but not in Kosovo. The tested antifungal compounds demonstrated high *in vitro* activity against environmental *C. neoformans species* complex isolates. Three *C. gattii* species complex were isolated in both, Croatia and Kosovo and belonged to wild type for all tested antifungals as well according to ECVs. Three out of four (3/4; 75%) of *C. neoformans* species complex isolates in Croatia belonged to serotype A and all (4/4, 100%) had mating type α. The four *C. neoformans* species complex isolates from Croatia were classified into two different molecular types, VNI (n = 3) and VNIV (n = 1).

This is the first report proving the presence of *C. neoformans* species complex in the environment of Croatia and determining their susceptibility to antifungals. The results of the study show the potential risk of exposure for inhabitants, especially on the Croatian coast, to molecular types VNI and VNIV, which can be expected in clinical cases of cryptococcosis. Although a limitation of this study is the small number of detected environmental isolates antifungal susceptibility pattern of *C. neoformans* species complex suggests that no strains with low susceptibility to the most common antifungals should be expected in patients with cryptococcosis.

Keywords: *Cryptococcus neoformans, Cryptococcus gattii*, environment, molecular typing

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11. CURRICULUM VITAE

Donjeta Hajdari was born in 1977 in Prishtina, Kosovo. She completed her first years of medical studies at Karl Franzens University in Graz, Austria, but graduated from the Medical Faculty, University of Prishtina in 2004. She completed her residency and postgraduate studies in microbiology at the National Institute of Public Health in Prishtina, Kosovo, in 2013, where she was appointed a specialist in microbiology and the Head of the Laboratory for Molecular Diagnostics and Serology in 2017. She also worked as Health Inspector for the Ministry of Health in Prishtina. She has participated in many local and national activities, where she has been invited to give her contribution to various topics. She participated in international meetings in Italy and Bulgaria, and training in the field of microbiology in Germany and Finland.