FERTILE *CRYPTOCOCCUS NEOFORMANS* VAR. *NEOFORMANS* (*C. DENEOFORMANS*) ISOLATES FROM NATURAL ENVIRONMENT IN KOSOVO

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ABSTRACT

A total of 300 tree-associated samples were collected from green areas in three towns in Kosovo, and cultured to isolate *Cryptococcus* species. One soil sample from Prizren tested positive, yielding three isolates identified as *C. neoformans* var. *neoformans* (*C. deneoformans*), molecular type VNIV, two MATα and one MATa. Mating experiments revealed that they were fertile when crossed together, and MLST analysis showed unique sequence types not found in the global database. The study reports the presence of *C. neoformans* strains in the environment in Kosovo and highlights the importance of monitoring pathogen distribution and potential impacts of climate change.

LAY ABSTRACT

We collected tree samples from three main towns in Kosovo to isolate the human fungal pathogen *Cryptococcus*. The results showed that *C. neoformans* var. *neoformans* isolates, able to produce basidiospores, were present in the environment, confirming that population at risk can be potentially infected.

BRIEF REPORT

The genus *Cryptococcus* comprises about 30 species of environmental fungi, with *C. neoformans* and *C. gattii* species complexes being the most common cause of human infection.[1] Cryptococcosis is a life-threating infection in immunocompromised patients affected by HIV/AIDS, but also people with underlying diseases including diabetes mellitus, liver or kidney diseases, hematological malignancies, as well as chronic obstructive pulmonary diseases and viral

respiratory tract infections, identified lately as risk conditions.[2] In addition, cryptococcosis may also occur in immunocompetent hosts by accidental inoculation with contaminated material (primary cutaneous cryptococcosis).[3] Although virus and bacteria tend to be the pathogens that receive the most attention, in recent years this perception has changed rapidly and there is grown concern about the threats posed by environmental fungi to animal and humans also due to the drastic climate changes that are occurring.[4]

Kosovo is a country located in southeastern Europe, in the Balkan area, characterized by a prevalent hilly and mountainous terrain with valleys and plains. The climate is subcontinental with hot and dry summers and cold winters, especially in the mountainous areas. Due to these geographical and climate conditions this region was not highlighted as a risk area for *Cryptococcus* exposure by a recent niche modelling study.[5, 6] Despite the low suitable prediction and absence of reported cases of cryptococcosis in Kosovo, the presence of *Cryptococcus* in the environment cannot be excluded since no environmental surveys have been carried out in this region so far. Furthermore, clinical cases and environmental isolation from the neighbor countries, Serbia and Croatia, were documented in previous studies.[7-10] Therefore, the present study aims to investigate the prevalence and distribution of the two *Cryptococcus* pathogenic species complexes in the environment of Kosovo, and to form the basis for comparison with future studies allowing the monitoring of *Cryptococcus* and cryptococcosis epidemiology evolution in the country.

Tree-associated samples were collected from public gardens and green areas of three towns in Kosovo: Prishtina, the main town of the country, and Prizren and Gjakova, in the southwest, on the basis of a previous niche modelling prediction.[5] The types of samples were bark, swabs of trunk and fissures, and soil beneath the tree for a total of 300 samples from 30 trees (Supplementary material, Table S1) which were processed as previously described and cultured on *Cryptococcus*-selective medium Niger seed agar (NSA).[7, 11] All brown colonies grown on NSA were

first morphologically and biochemically identified as *Cryptococcus* spp. Thereafter, they were genotyped by specific multiplex PCRs to identify molecular and mating type.[12, 13] Finally, multi-locus sequence typing (MLST) using the ISHAM standard protocol was performed and profiles were compared with the main sequence types (ST) included in the global MLST database (https://mlst.mycologylab.org).[14] Fertility of the isolates were also tested by crossing the isolates with opposite mating types (MATα and MATa) and with reference strains (JEC20, VNIV-aD, and JEC21, VNIV-αD) on Murashige-Skoog agar (Merck KGaA, Darmstadt, Germany) for at least three weeks, at 25°C, in the dark.[15]

Out of the 300 samples only one soil sample, collected beneath a *Quercus cerris* tree in Prizren, resulted positive. Three brown colonies were isolated and identified as *C. neoformans* var. *neoformans* (*C. deneoformans*), molecular type VNIV. However, determination of mating type identified two isolates with MATα and one with MATa. The MATa isolate was able to produce filaments and basidiospores when crossed with both the MATα isolates as well as with reference strain JEC21. In contrast, both the MATα isolates were not fertile when crossed with the reference strain JEC20. Crossing between JEC20 and JEC21 strains, performed as control, produced abundant spores as expected (Figure 1 and Supplementary material, Table S2). MLST analysis identified a different ST for each of the three isolates: ST686, ST687, and ST688 (Supplementary material, Table S3). The three STs included eight new allele type (AT) sequences (GenBank accession numbers OR402890-OR402897) and did not match with any ST present in the MLST global database, therefore they were assigned as new ATs and STs by the database curator. Comparison with the main VNIV STs in the global MLST database showed that the MATα isolates were closely related and form a distinct cluster with respect to the other VNIV STs, whereas the MATa isolate was closely related to the large cluster including most of the VNIV STs (Figure 2).

The present results report for the first time the isolation of *C. neoformans* species complex strains from the environment in Kosovo where data about epidemiology of cryptococcosis and distribution of *Cryptococcus* are completely lacking. Based on niche modelling prediction performed in previous studies, at present, Kosovo seems to be an area with a low risk of human exposure to *C. neoformans*/*C. gattii* species complexes yeasts.[5, 6] The model identified a limited area in Kosovo for the possible presence of *C. neoformans* var. *neoformans* along the valley of Drini i Bardhè river where are located the towns of Prizren and Gjakova, therefore our survey was focused in that area (Supplementary material, Figure S1). Our results confirmed the model prediction showing a low frequency of isolation and the presence of yeasts belonging to *C. neoformans* var. *neoformans* in Prizren urban area. This scarce presence in the environment and the prevalence of *C. neoformans* var. *neoformans*, which is less virulent compared to *C. neoformans* var. *grubii*, could explain the absence of cryptococcosis cases reported from Kosovo.[16] However, recent studies reported how climate changes are influencing the distribution of yeasts and molds species, including *Cryptococcus*, in the environment.[4, 17] The increase of average temperatures, in particular during winters, could favor the expansion from the coastal areas towards internal areas, as Kosovo, of more virulent strains such as *C. neoformans* var. *grubii* and *C. gattii sensu stricto* that are sensitive to low temperatures.[18]

The MLST profile of the three VNIV isolates found during the present environmental survey were all unique and different from those included in the MLST global database confirming that VNIV lineage has a higher genotypical variability compared to VNI due to a more dynamic population which is characterized by a high rate of sexual recombination.[19, 20] However, studies reporting genotypes of VNIV isolates, in particular from the environment, are scarce and therefore their genotypical variability is largely underestimated. In addition, the three isolates from Kosovo were recovered from the same soil sample associated to a tree and were fertile when they were crossed

together. This was already shown for VNI isolates from the same tree but this is the first time that it is shown for VNIV, corroborating the hypothesis that sexual reproduction is an important evolution mechanism for *Cryptococcus* and that it can occur in the environment.[7]

In conclusion, this study showed that, although cryptococcosis cases are not reported from Kosovo the pathogen is present in the environment and it is able to reproduce both by asexual and sexual cycle. This means that the fungus could spread rapidly in case the environmental conditions become more favorable. Since climate changes are a global concern the above scenario is not so improbable and, therefore, monitoring of the environment represents an important tool to understand how distribution of pathogens is changing in the space and time and to predict future epidemiological scenarios.

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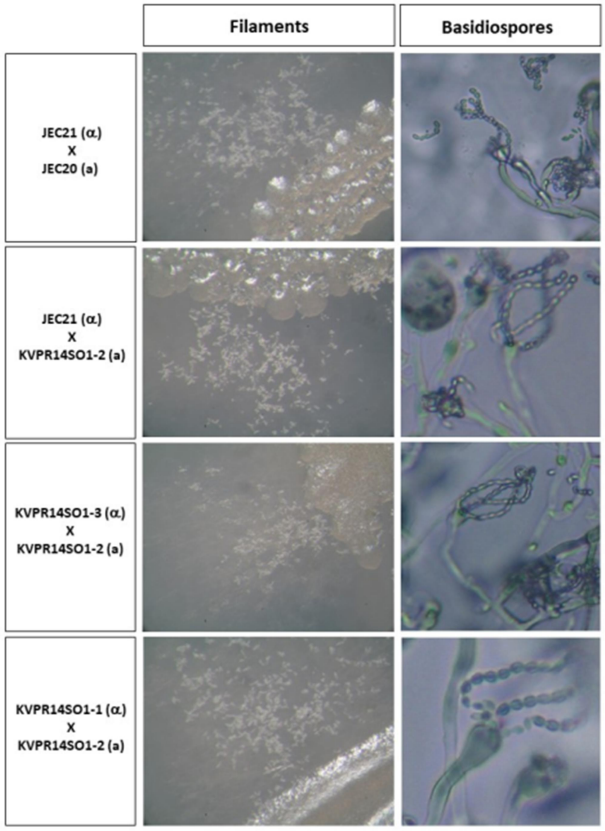

Figure 1. Mating experiments carried out on Murashige and Skoog agar medium at 25°C in the dark after three weeks of incubation. Isolates from Kosovo (KVPR14SO1-1, KVPR14SO1-2, KVPR14SO1-3) with opposite mating type were crossed together and with reference strains (JEC20 and JEC21). Left panel: edge of the mixture between opposite mating types from where hyphae arise (50X magnification with a stereomicroscope, Zeiss, Essingen, Germany). Right panel: microscopy showing basidia and chains of basidiospores (400X magnification with a light microscope Axioskope 40, Zeiss).

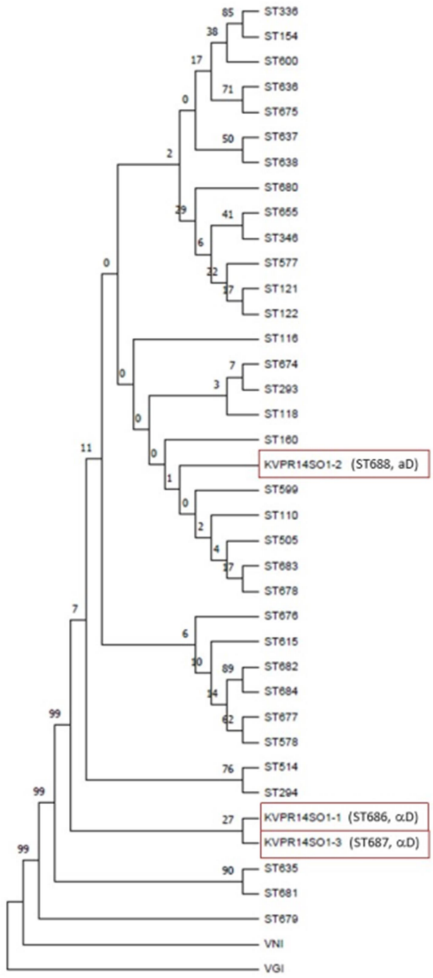

Figure 2. Maximum likelihood phylogenetic tree inferred using the concatenated sequences of seven MLST loci (*CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1*, *URA5*). The sequences of isolates from Kosovo (red box) were compared with the main VNIV sequence types present in the *Cryptococcus* global MLST database. The Tamura-Nei substitution model and the bootstrap statistical analysis with 1000 replicates were applied. Bootstrap values are reported beside each node. VNI and VGI sequences were used as outgroups.