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Interactions between the pathogenic fungus *Cryptococcus neoformans* and ants

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A R T I C L E I N F O

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A B S T R A C T

Despite the growing number of environmental surveys aimed to understand the ecology of the fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii*, little is known about their relationships with arthropods. In the present study we collected a large number of samples from trees and arthropods living on them to determine the occurrence of *Cryptococcus* in arthropods, to understand if they could represent a vehicle for dispersion in the environment, and finally to investigate how they might interact with the fungus. Samples were collected from seven different geographical areas of the world: northwestern Italy, southeastern Italy, Slovenia, Kosovo, Greece, Turkey, and Colombia. A total of 1396 trees were examined and 11,805 samples were collected, including 7492 arthropod samples. Arthropod positive samples, mostly from ants, were found only in northwestern and southeastern Italy, Greece, and Slovenia with an average rate of 0.2%. Thirty-three of positive trees hosted positive arthropods whereas in six of them arthropods resulted negative. In addition, for six trees, positive samples from arthropods were not associated with positive arboreal samples. In vitro experiments showed that ants can transfer cryptococcal yeasts from a contaminated substrate (soil or bark) to a sterile one and that the fungus can survive inside the digestive apparatus of ants. The present study showed that ants are potential vehicles for *C. neoformans* although the frequency of which they enter in contact with the fungus is low. Cryptococcal yeasts can survive within the bodies of ants, but it remains unclear whether the relationship they establish with their host is parasitic, commensal, or symbiotic.

1. Introduction

Human, animal, and environmental health are deeply interconnected, as acknowledged by the One Health approach. This holistic perspective aims to achieve optimal health outcomes recognizing the interconnections between people, animals, plants, and their shared environment (Mackenzie and Jeggo, 2019). Environmental, animal, and human health are being shaped by factors like climate change, global overpopulation, globalization, and biodiversity loss. Currently, an estimated two-thirds of newly developing infectious diseases are probably animal-related. To address these threats, a collaborative and multi-disciplinary approach, cutting across boundaries of animal, human, and environmental health, is needed to understand the ecology of each emerging zoonotic disease in order to undertake a risk assessment, and to develop plans for response and control. In recent decades, newly emerging fungal diseases affecting plants and animals have gained significant attention alongside viral and multidrug-resistant bacterial (Benedict et al., 2017).

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Cryptococcus neoformans and *Cryptococcus gattii* are encapsulated yeasts able to cause life-threatening invasive mycoses. According to recent estimates, cryptococcal infections result in 147,000 deaths annually worldwide (Denning, 2024) representing a serious concern to public health, as recently recognized by the World Health Organization that included these pathogens among the list of the most critical fungal pathogens (WHO, 2022). They are environmental fungi that have been isolated from various sources such as bird excreta, mammal feces, trees, plant debris, soil, and water (Cogliati, 2013). Despite the growing number of environmental surveys aimed at understanding the ecology of *Cryptococcus*, little is known about its interactions with arthropods. This class of animals is the most numerous on Earth and are highly adaptable to diverse environmental niches. Therefore, studying the interactions between *Cryptococcus* and arthropods could provide insights into how this fungal pathogen is able to disperse in the environment. To date, only a few studies reported the occasional isolation of *C. neoformans* or *C. gattii* from few arthropod samples. The first isolation is dated 1993 (Gezuele et al., 1993) and led to the identification of *C. gattii* yeasts from a wasp nest in Uruguay. *Cryptococcus gattii* of two different molecular types (VGI and VGII) were later isolated from fresh insect frass of Lepidoptera living in a trunk cavity of an *Eucalytpus* tree in Australia (Kidd et al., 2003). One year later another study reported the isolation of *C. neoformans* var. *grubii* from a honeybee hive in Turkey (Ergin et al., 2004). In Brazil, independent surveys isolated *C. neoformans* var. *grubii* VNI and VNII from *Odontomachus bauri* ants, and *C. gattii* VGII from *Atta sexdens* ants (Santos de Jesus et al., 2012; dos Santos Bentes et al., 2019). Evidence of association with social arthropods were also found when *C. gattii* VGII strains were isolated from termite nests and trails at Boa Vista, Brazil (Sales et al., 2017). Finally, an extensive survey carried out in Italy revealed that *C. neoformans* var. *grubii* (VNI) and *C. neoformans* var. *neoformans* (VNIV) were dispersed throughout the micro-ecosystem of an oak tree. This included both biotic and abiotic components, such as two ant species (*Camponotus piceus* and *Crematogaster scutellaris*), one bug species (*Pyrrhocoris apterus*), and one mite species (*Trombidium holosericeum*) (Cogliati et al., 2020).

In the present study we collected a large number of samples from trees and arthropods living on them to determine which is the occurrence of *Cryptococcus* in arthropods, to assess whether they could act as a vehicle for dispersion in the environment, and finally to investigate how they interact with the fungus.

2. Materials and methods

2.1. Sample collection and processing

Arboreal-associated samples and samples of arthropods living on the trees were collected in seven different geographical areas: northwestern Italy, southeastern Italy, Slovenia, Kosovo, Greece, Bursa province in Turkey, and Bucaramanga and Cundinamarca areas in Colombia (Fig. S1). The samples were collected during the main period of activity for arthropods, spring and summer season in the five European geographical areas and all year round in Colombia. Primarily, old trees presenting arthropods on the trunk were selected. Arboreal-associated samples were processed as previously reported (Cogliati et al., 2016) whereas arthropods were collected using different methods depending on the type of sample. Arthropods moving rapidly on the trunk, such as ants, were catched using a piece of adhesive tape and then transferred in a 2-ml sterile vial with tweezers, whereas arthropods with a bigger size such as bugs (Hemiptera) were caught using only tweezers. Finally, flying insect, such as bees, flies, and wasps, were attracted and caught using a bottle containing some water and sugar and then transferred in a 2-ml sterile vial. Once the arthropod samples were transported in the laboratory, vials were added with 1 ml of sterile distilled water and samples were vortexed for at least 1 min. Then arthropods contained in each vial were grinded using tweezers in order to keep the small volume and concentrate the presence of eventual yeast cells. Finally, they were vortexed for further 1 min. A 100-μl aliquot of the suspension was then distributed in two Petri dishes containing Niger seed agar medium (Pham et al., 2014) and incubated at 37 °C for at least 14 days.

2.2. Isolation and identification of C. neoformans and C. gattii

Petri dishes cultured with arthropod and arboreal samples were periodically observed for the presence of melanized yeast colonies which, in case, were collected and observed by light microscopy to check morphology of yeast cells. Colonies with spherical yeast cells and polar budding were further biochemically identified by testing assimilation of *myo*-inosytol, and urease activity.

Cryptococcus neoformans and *C. gattii* were distinguished culturing the yeasts on glycine-creatinine-bromothymol blue agar at 37 °C for five days.

2.3. Molecular typing

Genomic DNA extraction, and determination of molecular type and mating type by multiplex PCRs of all available *C. neformans* and *C. gattii* isolates, were performed as described elsewhere (Cogliati et al., 2000, 2015; Esposto et al., 2004; Feng et al., 2013). Strains H99 (VNI-αA), IUM 96–2828 (VNI-**a**A), JEC21 (VNIV-αD), JEC20 (VNIV-**a**D), WM163 (VGI-αB), IUM 96–2721 (VGI-**a**B) were used as reference strains.

Multi-locus sequence typing (MLST) was performed for isolates from trees presenting both arboreal and arthropod positive samples to show their genetic relationship. Seven standard housekeeping loci, *CAP59, GDP1,* IGS1*, LAC1, PLB1, SOD1*, and *URA5*, were amplified and sequenced as described by the *Cryptococcus* MLST standard protocol (Meyer et al., 2009). Sequences were then compared with those present in the *Cryptococcus* MLST database (www.mycologylab.net) to assign the correct allele types (AT) and sequence types (ST).

2.4. Cryptococcus-ant in vitro experiments

2.4.1. Ants on contaminated soil

Two yeast suspensions in sterile distilled water (10^6 and 10^3 yeasts/ml) were prepared for one *C. neoformans* VNI strain (H99) and one VNIV strain (JEC21). Four ml of each suspension were distributed in a flask containing 10 g of autoclaved soil (before collected from a garden) and mixed. Soil was incubated for 72 h at 37 °C to allow the yeasts to adhere and adapt to the substrate, this also to reflect a more natural condition avoiding the presence of free yeast cells in water drops. After incubation it was transferred in a sterile 90-mm Petri dish. On the day of the experiment, live ants were collected from a colony of *Crematogaster scutellaris* inhabiting a tree near the laboratory. Four live ants were placed onto the contaminated soil in each Petri dish and allowed to move freely for 24 h at room temperature. Additionally, five ants were placed in a vial containing 1 ml of sterile distilled water, fragmented using tweezers, vortexed to mix, and 100 μl of the suspension was cultured on a Petri dish with Niger seed agar to confirm the absence of initial contamination with *Cryptococcus*. A control experiment was also set up using Petri dishes containing sterile soil. At the end of the interaction time, two out of the four ants were collected and transferred each in a 2-ml sterile vial containing 1 ml of 1% bleach for 3 min to sterilize the external surface of the ant. Washing with 1 ml of sterile distilled water and vortexing were performed twice, and then 100 μl of the washing water was cultured on a Petri dish containing Niger seed agar to confirm sterilization. Ants were dissected in three pieces, head, thorax, and abdomen, each of which was transferred in a new sterile vial. Ant parts were then grinded using tweezers, vortexed for 1 min, and finally 100 μl of the suspension were cultured on a Petri dish containing Niger seed agar. All plates were incubated for at least 7 day at 37 °C and observed periodically for the presence of melanized yeast colonies. For each plate the colony forming units (CFUs) of melanized yeasts were recorded.

The other two ants from every plate of the experiment were transferred each in a 5-ml container and fixed in a 4% formaldehyde solution diluted in phosphate buffer solution (PBS) for 48 h. Then the fixative solution was discarded and the ants were suspended in a solution PBS/absolute ethanol 1:1 and stocked at −20 °C for further processing (Fig. 1A).

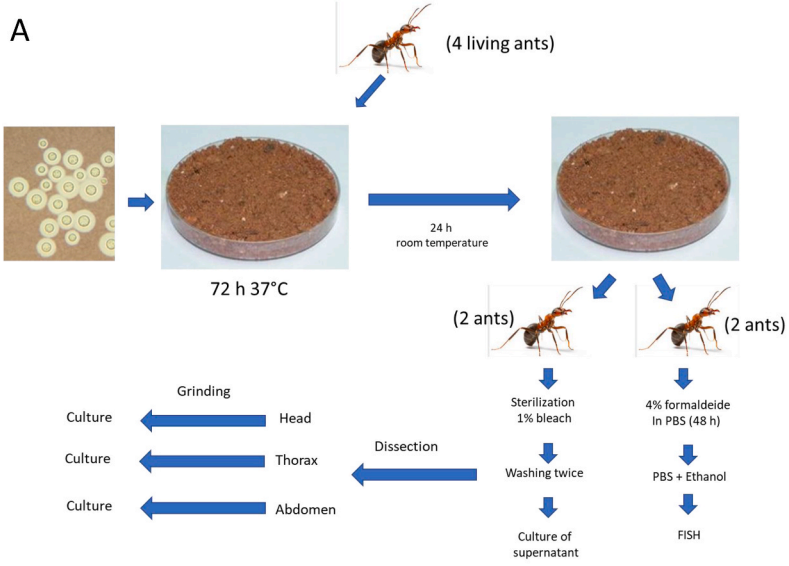
2.4.2. Soil contamination by ants

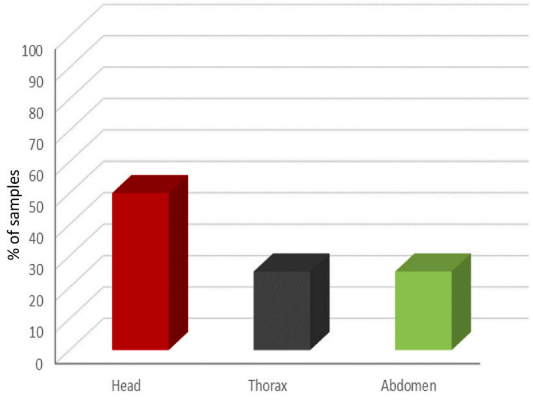
A yeast suspension in sterile distilled water (10^6 yeasts/ml) was prepared for one *C. neoformans* VNI strain and one VNIV strain. Four ml of each suspension were distributed in a flask containing 5 g of sterile soil and mixed. Soil was incubated for 72 h at 37 °C and then poured in a sterile 90-mm Petri dish divided in two halves by a paper strip. The other half of the plate was filled with 5 g of sterile soil. Ten live ants (collected in the environment as described above) were placed on the contaminated soil in each Petri dish, and then the dividing paper strip was removed and the ants were allowed to walk for 48 h at room temperature. A control experiment was also set up using Petri dishes containing only sterile soil. At the end of the interaction time two soil samples (1 g) from the contaminated half and four samples (1 g) from the sterile half were collected with a sterile spoon and transferred in sterile 10-ml tubes containing 3 ml of sterile distilled water. Tubes were vortexed for at least 1 min, suspension was let decant for 5 min and then 100 µl of the supernatant was cultured on a Petri dish containing Niger seed agar. All plates were incubated for at least 7 day at 37 °C and observed periodically for the presence of melanized yeast colonies. For each plate the CFUs of melanized yeasts were recorded (Fig. 2A).

In addition, two further samples (about 200 mg each) from contaminated soil and two from sterile soil were collected and processed for DNA extraction as described by the manufacturer of the soil extraction kit (DNeasy PowerSoil® Pro Kit, QIAGEN, Germany). DNA was then amplified using two primers targeting the 18S rDNA of *C. neoformans* as described elsewhere (Prariyachatigul et al., 1996).

2.4.3. Contamination of bark by ants

A yeast suspension in sterile distilled water (10^6 yeasts/ml) was prepared for one *C. neoformans* VNI strain and one VNIV strain. Two ml of each of the two suspension were distributed in two 10-ml sterile tubes and then 0.5 g of autoclaved pieces of bark were added and incubated at room temperature for 24 h. Four 90-mm Petri dishes were prepared depositing about 0.5 g of sterile pieces of bark and 0.5 g of the inoculated bark in two distant points of each plate. Five live ants were collected in the environment (as described above) and placed in two plates, one inoculated with VNI and the other with VNIV, and were allowed to walk for four days on the whole plate at room temperature. The remaining two plates were used as control without ants. At the end of the interaction time the bark of each of the two sites was separately collected and transferred in a sterile 10-ml tube containing 3 ml of sterile distilled water. Tubes were vortexed for at least 1 min, suspension was let decant for 5 min and then 100 µl of the supernatant was cultured on a Petri dish containing Niger seed agar. A 100-µl quantity of the contaminated bark suspension was inoculated onto one culture plate, whereas the sterile



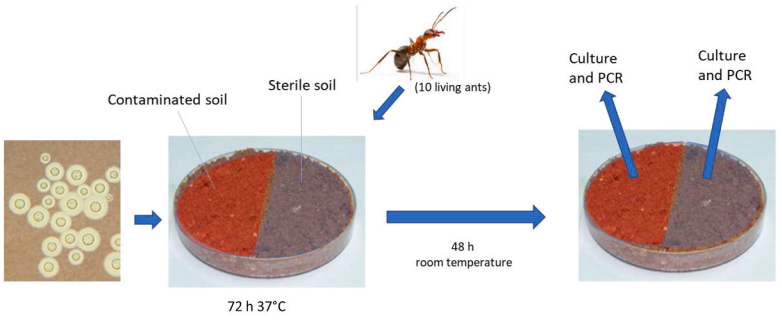


C

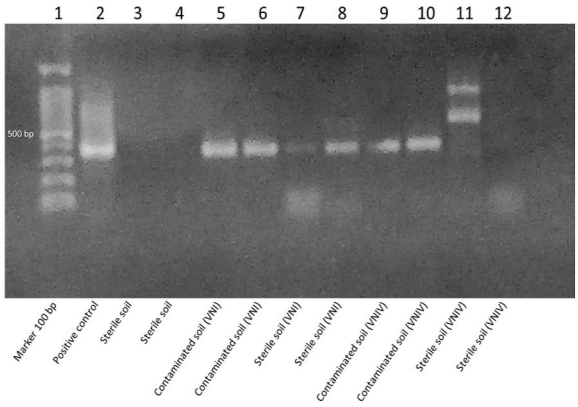
				CULTURE RESULTS (CFUs)							
Cryptococcus strain (molecular type)	Soil concentration (yeast cells/g)	No. of ants for FISH	No. of ants for culture	ANT1 External lavage	ANT1 Head	ANT1 Thorax	ANT1 Abdomen	ANT2 External lavage	ANT2 Head	ANT2 Thorax	ANT2 Abdomen
VNI	4×10^5	2	2	0	>100	2	23	0	0	0	0
VNI	4×10^2	2	2	0	0	0	0	0	1	0	0
VNIV	4×10^5	2	2	0	48	1	1	0	0	0	0
VNIV	4×10^2	2	2	0	4	0	0	0	0	0	0
Control	0	2	2	0	0	0	0	0	0	0	0

Fig. 1. A) Experimental scheme to prove that ants can be contaminated by soil inoculated with *Cryptococcus neoformans*. B) Histogram showing the percentage of positive cultures for each of the three body segments of ants. C) Colony forming units (CFUs) observed after culturing the different body segments of the two ants used in each plate.

A



B



C

		CONTAMINATED SOIL CULTURE			STERILE SOIL CULTURE			CONTAMINATED SOIL PCR		STERILE SOIL PCR	
Cryptococcus strain (molecular type)	Soil concentration (yeast cells/g)	No. of samples	No. of positive samples	CFUs	No. of samples	No. of positive samples	CFUs	No. of samples	No. of positive samples	No. of samples	No. of positive samples
VNI	$4x10^5$	2	2	>1000	4	4	>100	2	2	2	2
VNIV	$4x10^5$	2	2	>1000	4	4	3/5/11/12	2	2	2	0
Control	0	-	-	-	2	0	0	-	-	2	0

Fig. 2. A) Experimental scheme to prove that ants are able to transfer *Cryptococcus neoformans* from a contaminated soil to a sterile soil. B) Electrophoresis gel showing specific amplification of C. neoformans in contaminated and sterile soil. C) Colony forming units (CFUs) observed after culturing contaminated and sterile soil of the experiment.

bark suspension was cultured on five plates (100 µl each). Culture plates were incubated for at least 7 day at 37 °C and observed periodically for the presence of melanized yeast colonies. For each plate the CFUs of melanized yeasts were recorded (Fig. 3A).

2.5. Detection of C. neoformans yeasts by fluorescence in-situ hybridization (FISH) in the ant body

Ants fixed during the first experiment were prepared for FISH analysis as follows. Ants were decolorized with 6% hydrogen peroxide at room temperature for 2 h. The samples were then divided in head, thorax and abdomen, cut in the middle of each part and put in three different vials. Samples were washed with PBS supplemented with 0.2% of Tween 20, three times for 5 min each time, at room temperature. Then, the samples were incubated with hybridization buffer (20 mM Tris-HCl, 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 30% formamide) two times for 10 min at 42 °C. After that, the samples were hybridized with hybridization buffer containing 0.5 µM of each probe at 42 °C overnight. Two Cy3-labelled probes specific for *C. neoformans* targeting 28S rDNA were used: Cne448 (5′-CCAGCCCTTATCCACCGA-3′) and Cne205 (5′-GTCGCGTTACTTGGGAGT-3′). CY5-labelled probe EUK516 (5′-ACCAGACTTGCCCTCC-3′), universal for eukaryotic organisms, was also used as positive control (Martins et al., 2010). Some ant samples were also incubated only with hybridization buffer, at 42 °C overnight, as control. After one wash with PBST for 20 min at room temperature, samples were mounted on slides using a SlowFade Glass Soft-set Antifade Mountant with DAPI (Invitrogen, ThermoFisher Scientific, Monza, Italy). Slides were observed under a confocal microscope (Nikon A1-3D SIM; Nikon, Tokyo, Japan) and detection of *C. neoformans* was proved when Cy3 and Cy5-labelled probes resulted to be overlapped.

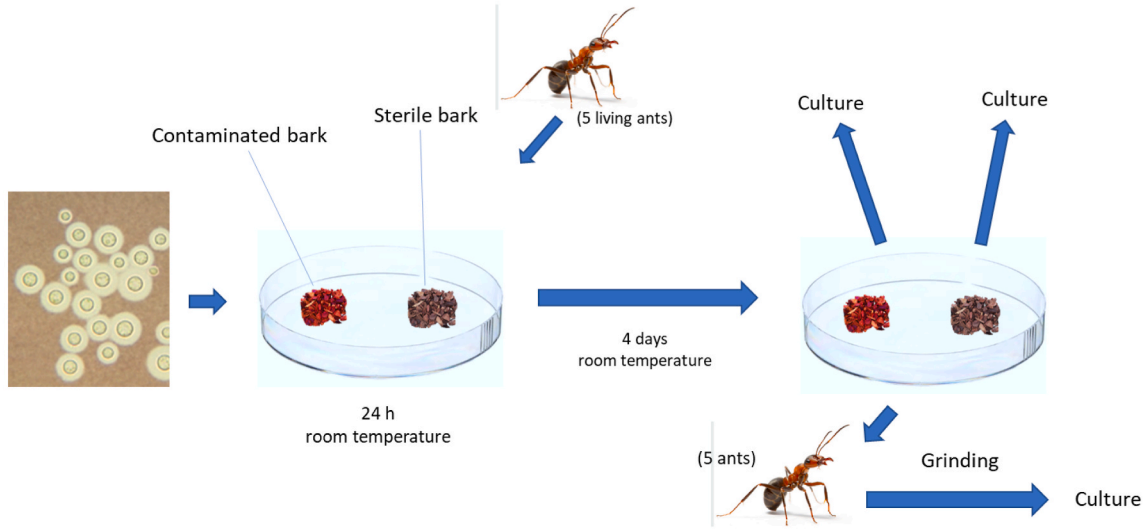
3. Results

3.1. Prevalence of positive samples among tree-associated samples and arthropods

A total of 11,805 samples were collected from 1396 trees, including 7492 samples from arthropods. The trees belonged to 39 different genera, being *Olea, Mangifera*, and *Platanus* the most prevalent (14%, 14%, and 12%, respectively) (Table S1). Forty-five trees (3.2%), belonging to 11 different genera, were colonized by *C. neoformans* or *C. gattii* yeasts, with 1.9% of positive samples from trunk fissure or hollow swabs, 1.3% from bark, 1.3% from soil beneath the tree, 5.9% from leaves, 1.8% from plant debris/decaying wood, and 0.2% from arthropods (Table 1). Sampled arthropods belonged to eight different Orders and ants represented 88% of the samples (Table S2). Among the 15 positive arthropod samples, ten were from ants of the genus *Formica*, three from *Cromatogaster scutellaris* ants, one from *Crematogaster auberti* ants, and one from *Apis mellifera*. *Formica* spp. positive samples were from seven olive trees (*Olea europaea*), one pine (*Pinus pinea*), and one *Eucalyptus camaldulensis*, all from southeastern Italy, whereas positive *C. scutellaris* ants were from a plane tree (*Platanus acerifolia*) and a horse chestnut tree (*Aesculus hippocastanum*) growing in northwestern Italy. The positive *C. auberti* ant was from a tamarisk tree (*Tamarix* spp.) growing in a Greek Aegean island (Schinoussa), and the sample from *Apis mellifera* was associated with a tree along the Adriatic coast of Slovenia (Table 1).

Thirty-three trees among the 1396 sampled trees (2.36%, 74% of positive trees) presented positive arboreal samples and negative arthropod samples, six trees (0.43%, 13% of positive trees) had both positive arboreal and arthropod samples, and six trees (0.43%, 13% of

A



B

			ANT CULTURE RESULTS (CFUs)				
Cryptococcus strain	Concentration (yeast cells/g)	No. of ants for culture	ANT1	ANT2	ANT3	ANT4	ANT5
VNI	$4x10^6$	5	>100	>100	40	25	>100
VNIV	$4x10^6$	5	>100	>100	>100	>100	>100
ANTS CONTROL	-	5	0	0	0	0	0

C

		BARK CULTURE RESULTS (CFUs)					
Cryptococcus strain	Concentration (yeast cells/g)	CONTAMINATED BARK CULTURE	STERILE BARK CULTURE 1	STERILE BARK CULTURE 2	STERILE BARK CULTURE 3	STERILE BARK CULTURE 4	STERILE BARK CULTURE 5
VNI	$4x10^6$	>1000	>1000	>1000	>1000	>1000	>1000
VNIV	$4x10^6$	>1000	>1000	>1000	>1000	>1000	>1000
Control VNI (w/o ants)	$4x10^6$	>1000	0	0	0	0	0
Control VNIV (w/o ants)	$4x10^6$	>1000	0	0	0	0	0

Fig. 3. A) Experimental scheme to prove that ants are able to transfer *Cryptococcus neoformans* from a contaminated bark to a sterile bark. B) Colony forming units (CFUs) observed after culturing contaminated and sterile bark of the experiment. C) CFUs observed after culturing each of the five ants used in the experiment.

positive trees) had positive arthropod samples but negative arboreal samples (Fig. 4).

3.2. Prevalence, molecular type, and mating type of C. neoformans and C. gattii isolates in positive samples

A total of 157 isolates were recovered from 71 samples of 45 trees, of which 25 isolates were from 15 samples of arthropods living on 12 trees,

Table 1
Type and number of samples collected during the survey and percentage of positive samples (p.ve).

Geographical area	No. of trees (p. ve, %)	Species of positive trees	No. of samples (p. ve, %)	Swabs of trunk and fissures (p. ve, %)	Bark (p.ve, %)	Soil (p.ve, %)	Leaves (p. ve, %)	Fruits/ seeds (p.ve, %)	Decaying wood (p.ve, %)	Arthropods (p. ve, %)	Positive arthropod species
Southeastern Italy	68 (23, 34%)	18 *Olea europaea* 2 *Pinus Pinea* 2 E*ucalyptus camaldulensis* 1 *Ilex aquifolium*	5289 (38, 0.7%)	322 (26, 8%)	295 (1, 0.3%)	322 (1, 0.3%)	0	0	0	4350 (10, 0.2%)	10 *Formica* spp.
Northwestern Italy	54 (6, 11%)	3 *Platanus acerifolia* 1 *Aesculus ippocastanum* 1 *Juglans nigra* 1 *Acer pseudolatanus*	2318 (16, 0.7%)	37 (2, 5%)	93 (6, 7%)	72 (5, 7%)	0	0	0	2116 (3, 0.14%)	3 *Crematogaster scutellaris*
Slovenia	905 (4, 0.4%)	3 *Pinus pinea* 1 *Olea europaea*	1226 (5, 0.4%)	1029 (4, 0.4%)	29	13	14	6	0	135 (1, 0.74%)	1 *Apis mellifera*
Turkey (Bursa)	78 (6, 7.7%)	*2 Pinus pinea* 4 *Olea europae*	1404 (8, 0.6%)	535 (6, 1.1%)	314 (2, 0.6%)	310	0	0	0	245	
Colombia (Cundinamarca & Bucaramanga)	248 (2, 0.8%)	*1 Mangifera indica* *1 Olea europaea*	1337 (12, 0.9%)	0	182	350 (5, 14%)	37 (3, 8.1%)	0	219 (4, 1.8%)	549	
Kosovo	30 (1, 3.3%)	1 *Quercus cerris*	212 (3, 1.4%)	58	29	29 (3, 10.3%)	0	0	0	96	
Greece (Aegean islands)	13 (3, 23%)	1 *Pinus* spp. 1 *Olea europaea* 1 *Tamarix* spp.	19 (4, 21%)	0	18 (3, 16.7%)	0	0	0	0	1 (1, 100%)	1 *Crematogaster auberti*
TOTAL	1396 (45, 3.2%)		11,805 (86, 0.7%)	1981 (38, 1.9%)	960 (12, 1.3%)	1096 (14, 1.3%)	51 (3, 5.9%)	6	219 (4, 1.8%)	7492 (15, 0.2%)	

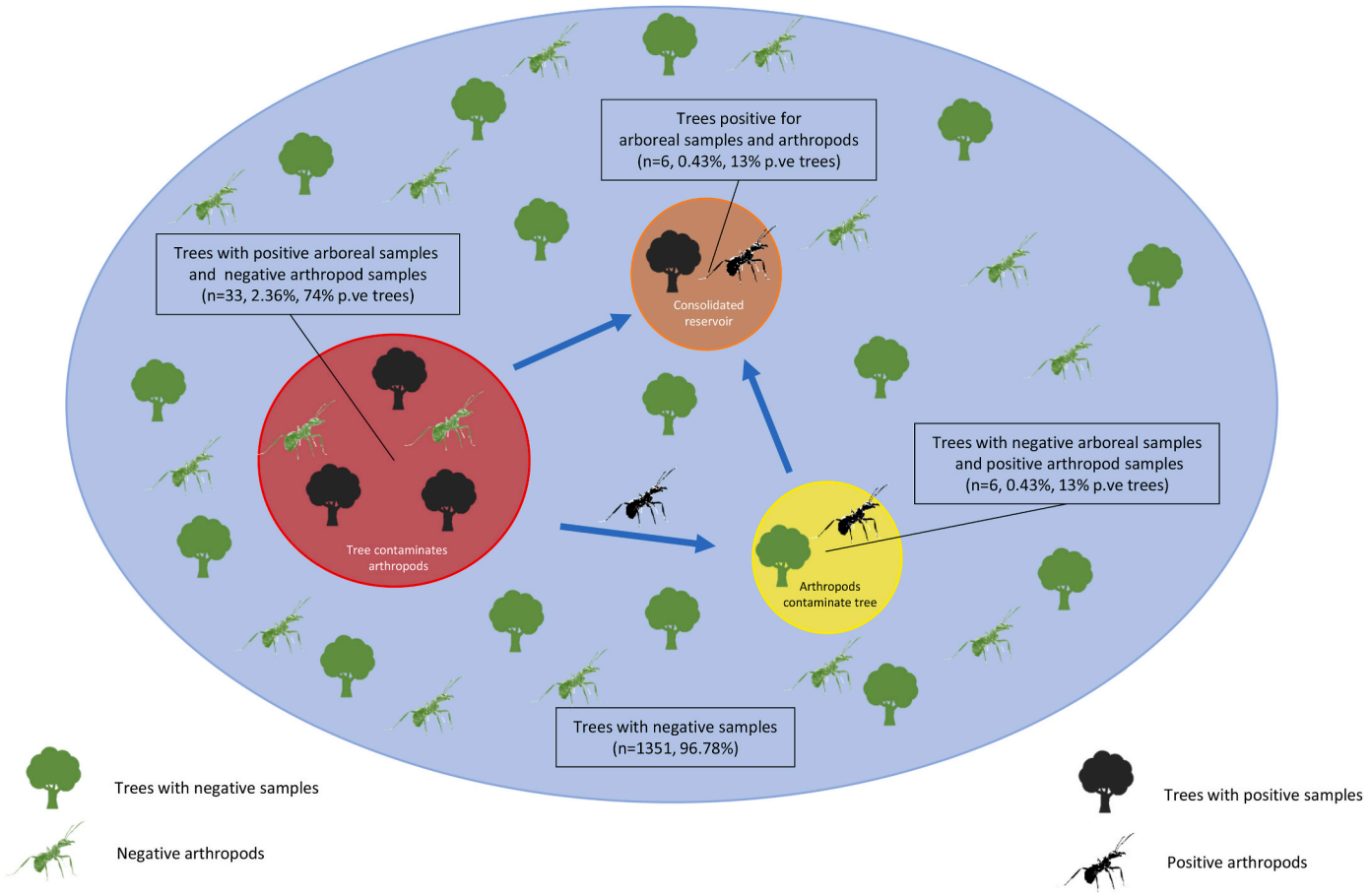


Fig. 4. Schematic representation of the possible interactions between *Cryptococcus neoformans*, trees, and arthropods. Arthropods can be occasionally contaminated by *C. neoformans* yeast when they attend colonized trees (red area). Yeasts can be carried by arthropods on the external cuticule or can be ingested and survive in the digestive apparatus. Colonized arthropods can transport the yeasts on a different tree probably contaminating it through their excrements (yellow area). Arthropods and other animal communities living on the tree contribute to extend the colonized area to the whole tree which becomes a consolidated reservoir of the fungus (orange area). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Molecular typing of the 157 *Cryptococcus* isolates collected during the present study.

Geographical area	Arboreal isolates (n = 132)	*Cryptococcus* species, molecular-mating type	Arthropod isolates (n = 25)	*Cryptococcus* species, molecular type, mating type
Southeastern Italy	5	*C. neoformans* var. *grubii*, VNI-αA		
	3	*C. neoformans* var. *grubii*, VNI-αA	2	*C. neoformans* var. *grubii*, VNI-αA
	17[a]	*C. neoformans* species complex	8[a]	*C. neoformans* species complex
	2	*C. gattii*, VGI-αB		
Northwestern Italy	35	*C. neoformans* var. *grubii*, VNI-αA	12	*C. neoformans* var. *grubii*, VNI-αA
	17	*C. neoformans* var. *neoformans*, VNIV-αD		
Slovenia	4	*C. neoformans* var. *grubii*, VNI-αA	1[a]	*C. neoformans* species complex
Turkey, Bursa	6	*C. neoformans* var. *grubii*, VNI-αA	0	
Colombia, Cundinamarca & Bucaramanga	13[a]	*C. neoformans* species complex	0	
	2[a]	*C. gattii* species complex		
Kosovo	2	*C. neoformans* var. *neoformans*, VNIV-αD	0	
	1	*C. neoformans* var. *neoformans*, VNIV-αD		
Greece, Aegean islands	25	*C. neoformans* var. *grubii*, VNI-αA	2	*C. neoformans* var. *grubii*, VNI-αA

[a] Not available for molecular typing.

and 132 isolates from 56 arboreal samples of 39 trees. Only 16 isolates from five arthropod samples, and 100 isolates from 20 trees were available for molecular typing. The 16 isolates from arthropods were all *C. neoformans* var. *grubii* VNI-αA, whereas the arboreal isolates were 75% (75/100) *C. neoformans* var. *grubii* VNI-αA, 3% (3/100) VNI-αA, 22% (19/100) *C. neoformans* var. *neoformans* VNIV-αD, 1% (1/100) VNIV-αD, and 2% (2/100) *C. gattii* VGI-αB. The VNI-αA isolates were found in the same olive tree (*O. europaea*) growing in southeastern Italy, the VNIV-αD isolate was recovered from a tree (*Quercus cerris*) in Kosovo together with other VNIV-αD isolates, which were also isolated from other two trees (*Platanus acerifolia*) in northwestern Italy. The two *C. gattii* isolates colonized two different trees in southeastern Italy: one

Table 3
Multi-locus sequence typing profile of *Cryptococcus neoformans* isolated from ants, the related arboreal isolates, and the two *C. gattii* isolates.

STRAIN	Geograhical origin	Source	Tree code	Species of the host tree	Molecular type	Sequence type
ITMPS1ANT1-1	Northwestern Italy	*Crematogaster scutellaris*	MPS1	*Aesculus hippocastanum*	VNI	ST23
ITMPS1ANT2-1	Northwestern Italy	*Crematogaster scutellaris*	MPS1	*Aesculus hippocastanum*	VNI	ST23
ITMPS1BK1-1	Northwestern Italy	Bark	MPS1	*Aesculus hippocastanum*	VNI	ST23
ITMPS1BK2-1	Northwestern Italy	Bark	MPS1	*Aesculus hippocastanum*	VNI	ST23
ITMPV15BK1-1	Northwestern Italy	Bark	MPV15 (close to MPV16)	*Platanus acerifolia*	VNIV	ST160
ITMPV16BK1-1	Northwestern Italy	Bark	MPV16	*Platanus acerifolia*	VNIV	ST160
ITMPV16ANT1-1	Northwestern Italy	*Crematogaster scutellaris*	MPV16	*Platanus acerifolia*	VNI	ST533
ITVEG96ANT1-1	Southeastern Italy	*Formica* spp.	VEG96	*Olea europaea*	VNI	ST23
ITMON48ANT1-1	Southeastern Italy	*Formica* spp.	MON48	*Olea europaea*	VNI	ST63
ITCOP39HO1-1	Southeastern Italy	Trunk fissure	COP39	*Pinus pinea*	VGI	ST156
ITOST4HO1-1	Southeastern Italy	Trunk fissure	OST4	*Olea europaea*	VGI	ST156
GRSCH1ANT1-1	Greece	*Crematogaster auberti*	SCH1	*Tamarix* spp.	VNI	ST264
GRSCH1ANT1-2	Greece	*Crematogaster auberti*	SCH1	*Tamarix* spp.	VNI	ST264
GRSCH1BK1-1	Greece	Bark	SCH1	*Tamarix* spp.	VNI	ST63

Isolates from the same tree are indicated with the same tree code.

pine (*P. pinea*) and one olive tree (*O. europaea*) (Table 1, Table 2).

3.3. MLST profiles

A total of 12 *C. neoformans* and two *C. gattii* isolates were available for MLST analysis. Seven isolates were from ants and seven from arboreal samples. For three trees (two from Italy and one from Greece) both ant and arboreal isolates from the same tree were selected (Table 3). The two *C. gattii* VGI isolates belonged to ST156, the ten VNI isolates belonged to four genotypes ST23, ST63, ST264, and ST533, whereas the two VNIV arboreal isolates belonged to the same genotype, ST160. For one tree both ant and arboreal isolates belonged to the same ST, on the contrary, for the other two trees, the isolates from ants belonged to a genotype different from that isolated in the bark of the tree. The VNIV genotype ST160 was identified from the bark of two neighbor plane trees. Two VNI isolates recovered from ants in two different sites of southeastern Italy belonged to ST23 and ST63 and were identical to those collected in northwestern Italy and Greece during the present study (Table 3).

3.4. Ants can be contaminated by soil

Cultures of ants exposed to soil contaminated with *C. neoformans* showed that 50% of ants (4 out of 8) were positive. In each experiment, two and three out of the four ants were contaminated with VNI and VNIV strains, respectively. None of the ants exposed to sterile control soil were positive.

Cryptococcus neoformans VNI and VNIV were isolated in ants exposed to both high and low concentrations, whereas no CFUs were observed in plates cultured with washing water after sterilization of ants with bleach. Ant heads were the samples with the higher percentage of positives (50%, 4/8), whereas two thorax and two abdomen samples were positive (25%, 2/8) (Fig. 1B and C).

3.5. Soil can be contaminated by ants

After 48 h of interaction between soil and ants from the half plate containing the sterile soil four samples were collected and cultured for each of the two experiments carried out using VNI and VNIV strains, respectively. In both experiments all samples were positive although CFUs of VNI were more numerous than VNIV: >100 CFUs vs. 3–12 CFUs. PCRs confirmed the presence of the fungus only for VNI experiment due to the low yeast concentration observed in the soil samples collected from the VNIV experiment. Experiment in the plate containing only sterile soil led to negative samples (Fig. 2B and C).

3.6. Ants can be contaminated by bark and can contaminate sterile bark

Both the cultures of the contaminated and sterile bark pieces from the two experiments with ant interaction resulted positive for *C. neoformans*. In addition, also cultures of single ants were positive, with an average concentration >500 yeasts/ant observed for both plates inoculated with VNI and VNIV isolates. In contrast, in the other two control experiments without ants only the inoculated pieces of bark were positive whereas from the sterile ones did not grow any yeast colony (Fig. 3B and C).

3.7. Detection of Cryptococcus yeast cells inside ant body after contamination

Observation of the microscopy samples stained with FISH probes specific for *C. neoformans* showed the presence of the yeast in all three main body segments of the ants: head, thorax, and abdomen. Since the ant cuticle is known to have a thickness between 1 and 100 μm, all images were obtained by detecting the presence of *C. neoformans* immediately below the cuticle. Fluorescent masses of fungi were detected in the anterior part of the head. In particular, *C. neoformans* was observed nearby the mouthpart, suggesting its presence at the pharyngeal mucosa. In particular, the fungus was detected using a 60× magnification in the anterior part of the head from 50.62 to 83.44 μm under the cuticle (Fig. 5). The presence of *C. neoformans* was also confirmed in both thorax and abdomen of the ants (Figs. 6–7). As for the thorax, *C. neoformans* was detected at 270 μm under the cuticle. In general, we observed that the fungus is more abundant in the abdomen than in the other segments of the body since aggregates of fungi are evident in several points of the abdomen. No specific signal was observed in the control group of non-contaminated ants.

4. Discussion

This study represents the first attempt to explore the relationship between the pathogenic fungus *C. neoformans* and arthropods. During the environmental survey 3.2% of the sampled trees were positive for *C. neoformans* or *C. gattii* with the highest prevalence observed in *Olea*, *Pinus* and *Eucalyptus* in agreement with a previous survey carried out in Europe reporting a frequency of about 5% (Cogliati et al., 2016). A small percentage of arthropods (0.2%) was contaminated by *C. neoformans,* suggesting that they interact only occasionally, and likely when arthropods inhabit or frequent a tree colonized by the fungus. Most of the positive arthropods were ants except one bee. This reflects the sampling method that focused on collecting arthropods present on trees. In our survey, ants represented the most numerous arthropods living on trees and, therefore, the probability to find *Cryptococcus* yeasts in these insects was higher than in other arthropods. Furthermore, sampling method

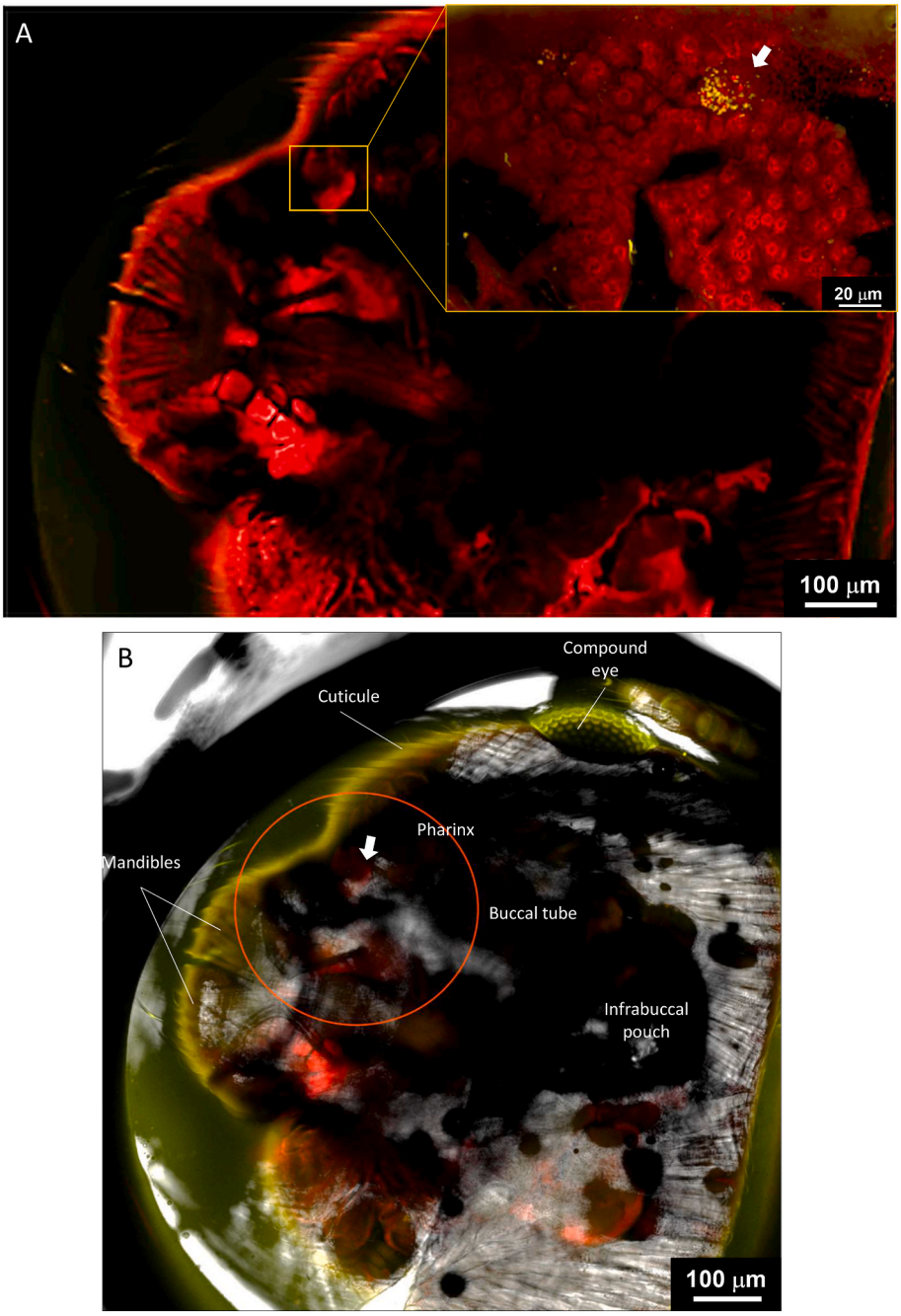


Fig. 5. A) Fluorescence microscopy image of an ant head section, 10× magnification. Red signal indicates area labelled with pan-eukaryotic EUK516 probe, and green signal indicates areas labelled with Cne448 and Cne205 probes specific for *C. neoformans*. The inset shows the 60× magnification of an area where *C. neoformans* yeasts were detected (arrow). B) Light and fluorescence microscopy overlapping image showing the different ant anatomic parts and the location of *C. neoformans* yeasts (arrow). Red circle indicates the area observed by microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

might also have affected the type of flying insects collected that were mostly bees and lepidoptera attracted by sugar solution. The bias could also depend on the specific geographical areas investigated in the present study. On the other hand, four out of seven previous studies reporting *Cryptococcus* in arthropods isolated the fungus from ants and seems to confirm our findings, although these studies were limited to restricted areas and based on few samples (Gezuele et al., 1993; Kidd et al., 2003; Ergin et al., 2004; Santos de Jesus et al., 2012; Sales et al., 2017; dos Santos Bentes et al., 2019; Cogliati et al., 2020). It could be also hypothesized that social insects have a high probability to enter in contact with *Cryptococcus* since they are often numerous and interact actively with the member of the same colony and with the environment.

Cryptococcus neoformans VNI was the most frequently isolated molecular type from both arboreal and arthropod samples as previously reported by other studies worldwide (Cogliati, 2013). Additionally, *C. neoformans* VNIV and *C. gattii* VGI were identified in arboreal samples from northwestern and southeastern Italy, respectively, regions where these molecular types had been previously reported (Cogliati et al., 2016; Montagna et al., 2018). All VNI isolates shared sequence types found in the MLST global database, including globally distributed ST23 and ST63. Meanwhile, the two VGI isolates corresponded to ST156, which is endemic to the Mediterranean region (Hagen et al., 2012;

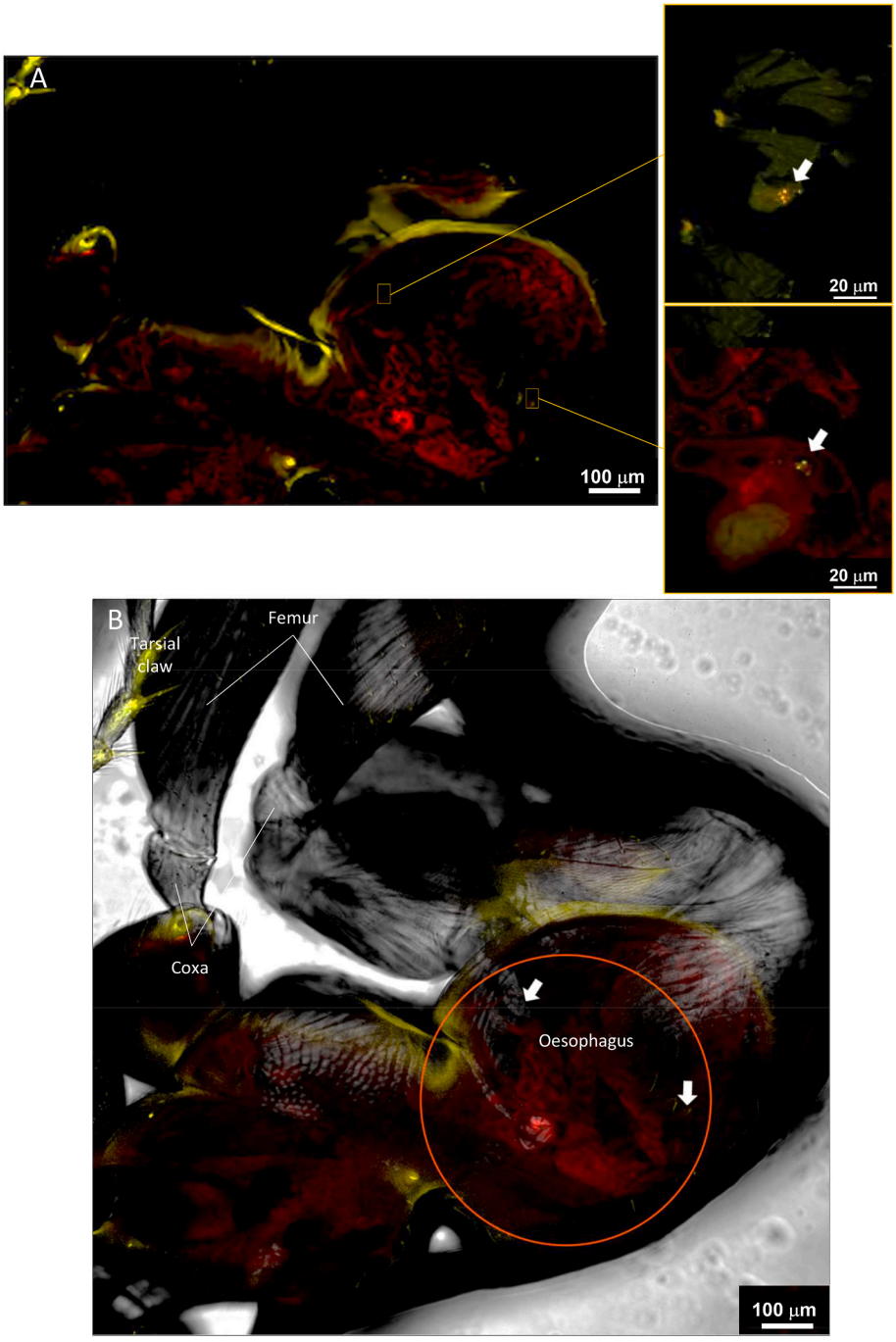


Fig. 6. A) Fluorescence microscopy image of an ant thorax section, 10× magnification. Red signal indicates area labelled with pan-eukaryotic EUK516 probe, and green signal indicates areas labelled with Cne448 and Cne205 probes specific for *C. neoformans*. The insets show the 60× magnification of two areas where *C. neoformans* yeasts were detected (arrows). B) Light and fluorescence microscopy overlapping image showing the different ant anatomic parts and the location of *C. neoformans* yeasts (arrows). Red circle indicates the area observed by microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Cogliati et al., 2019). Notably, arboreal and arthropod isolates from one tree shared the same ST, suggesting the tree acts as a stable reservoir for *C. neoformans*. Ants from this tree likely belong to a long-established colony exposed repeatedly to fungal contamination from bark, trunk fissures, and surrounding soil. In contrast, isolates from the other two trees displayed mismatched genotypes between ants and arboreal samples, implying exogenous contamination. This finding suggests that ants may act as vectors, as evidenced by trees where ant samples tested positive while arboreal samples were negative. A similar result was observed in a previous study reporting a tree heavily colonized by two genotypes of *C. neoformans* in all its biotic and abiotic components including arthropods living on it, but from which a completely different genotype was isolated from a flying ant male likely originating from another colony (Cogliati et al., 2020).

When C. *neoformans* yeasts, experimentally inoculated in soil, were tested for their ability to contaminate ants, the results showed that the yeasts were ingested by ants as proved by their presence along all the digestive apparatus of ants. The yeasts were not only detected by FISH but their viability was also confirmed by cultures. This suggests that ants are potentially able to vehiculate yeasts and disperse them in the

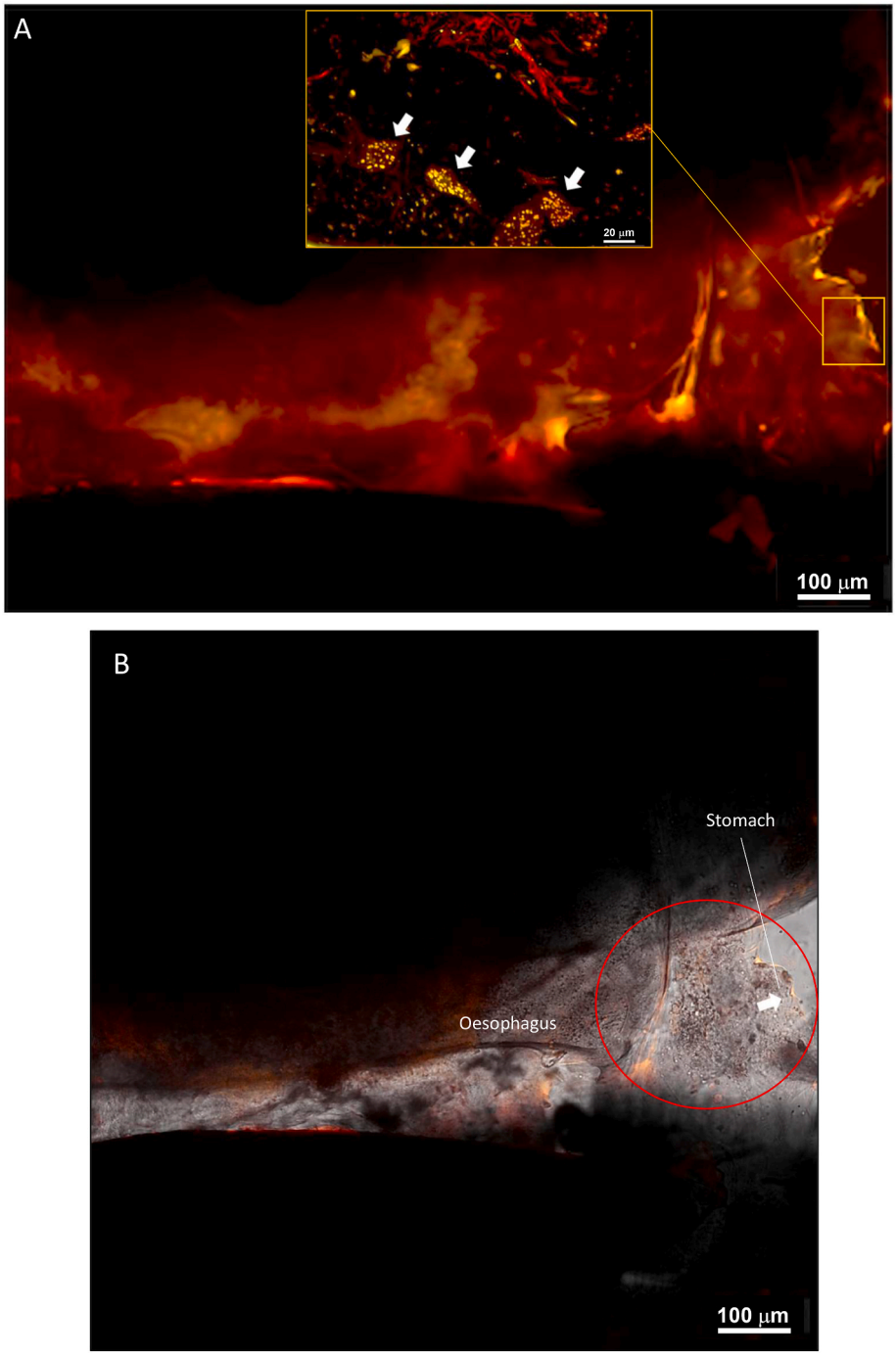


Fig. 7. A) Fluorescence microscopy image of an ant abdomen section, 10× magnification. Red signal indicates area labelled with pan-eukaryotic EUK516 probe, and green signal indicates areas labelled with Cne448 and Cne205 probes specific for *C. neoformans*. The inset shows the 60× magnification of an area where *C. neoformans* yeasts were detected (arrows). B) Light and fluorescence microscopy overlapping image showing the different ant anatomic parts and the location of *C. neoformans* yeasts (arrows). Red circle indicates the area observed by microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

environment likely through excrements. A hypothesis that seems to be supported by the findings of *Cryptococcus* yeasts in insect frass deposited on the bark of a tree in Australia (Kidd et al., 2003). In a second experiment the ability of ants to vehiculate *Cryptococcus* yeasts from a contaminated soil to a sterile soil was tested. Cultures of sterile soil showed that viable yeasts were present and that ants acted as vehicles during contamination. Since soil was shown to be a suitable substrate for both asexual and sexual reproduction of *C. neoformans*, its contamination by ants could play an important role for the spread of the pathogen in the environment (Cogliati, 2018). Similarly, our results show that ants are able to transfer *Cryptococcus* yeasts from contaminated bark to sterile bark corroborating the hypothesis that flying arthropods during their flights from a tree to another might contribute to the contamination of trees even for long distances. Bees, wasps, termites, and flying ant males were already shown to be contaminated by *Cryptococcus* yeasts in previous studies (Gezuele et al., 1993; Ergin et al., 2004; Cogliati et al., 2020). In particular, monitoring cryptococcal contamination of bees and their hives could be employed as an index for the presence of the

pathogen in the restricted area around hives where the pollen is collected (Wirta et al., 2022). Finally, the in vitro experiments here carried out showed no differences in the ability of *C. neoformans* VNI or VNIV molecular types to contaminate ants as previously shown by the investigation of a tree colonized by these two molecular types which were equally present in arthropods samples (Cogliati et al., 2020).

5. Conclusions

The present study showed that ants are potential vehicles for *C. neoformans* although the frequency of which they enter in contact with the fungus is low. Cryptococcal yeasts can survive within the bodies of ants, but it remains unclear whether the relationship they establish with their host is parasitic, commensal, or symbiotic. This requires further detailed microscopy analyses by electron or scan microscopy. From One Health point of view this study add new insights about the ways of dispersion of *C. neoformans* suggesting that the anthropic impact on Earth ecosystems could in future modify the equilibrium of arthropod communities as well as that of related microbial communities including fungal human pathogens.

CRediT authorship contribution statement

Massimo Cogliati: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sevim Akçağlar:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Okan Tore:** Writing – review & editing, Data curation. **Tadeja Matos:** Writing – review & editing, Data curation. **Rok Tomazin:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Irena Zdovc:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Donjeta Pllana-Hajdari:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Patricia Escandon:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Sara Epis:** Writing – review & editing, Data curation. **Giulia Maria Cattaneo:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Francesca Serio:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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