



Original Article

***Cryptococcus neoformans* in the respiratory tract of squirrels, *Callosciurus finlaysonii* (Rodentia, Sciuridae)**

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Received 9 March 2015; Revised 16 April 2015; Accepted 14 May 2015

Abstract

Cryptococcosis is a fungal disease acquired from the environment, for which animals may serve as sentinels for human exposure. The occurrence of *Cryptococcus* spp. in the respiratory tract of 125 squirrels, *Callosciurus finlaysonii*, trapped in Southern Italy, was assessed. Upon examination of nasal swabs and lung tissue from each individual, a total of 13 (10.4%) animals scored positive for yeasts, 7 for *Cryptococcus neoformans* (*C.n.*) (5.6%) and 6 for other yeasts (4.8%). *C.n.* was isolated from the nostrils and lungs, with a high population size in nostrils. Two *C.n.* molecular types, VNI and VNIV, were identified, with *C.n.* var. *grubii* VNI the most prevalent. Phylogenetic analyses of ITS+ and *URA5* sequences revealed that *C.n.* isolates were genetically similar to isolates from a range of geographical areas and hosts. Results suggest that *C.n.* can colonize or infect the respiratory tract of *C. finlaysonii*. The high occurrence and level of colonization of nasal cavities might be an indicator of environmental exposure to high levels of airborne microorganism. The close phylogenetic relationship of *C.n.* strains from squirrels with those from human and other animal hosts suggests a potential role for these animals as "sentinels" for human exposure.

Key words: *Cryptococcus neoformans*, *URA5* RFLP, molecular type, squirrels, respiratory tract, Southern Italy.

Introduction

Cryptococcus spp. and *Candida* spp. are considered the most important agents of invasive fungal infections (IFIs) in immunocompromised or hospitalized patients with seri-

ous underlying diseases [1–4]. Despite some effective treatment options, such mycoses remain associated with high rates of morbidity and mortality [4]. Although *C. albicans* remains the most important cause of these opportunistic

mycoses worldwide, other species belonging to *Candida* non-*albicans* and *C. neoformans*/*C. gattii* species complex have been recognized as potential pathogens for humans [1, 3, 5, 6]. While *Candida* spp. infections are usually considered nosocomial, those of *Cryptococcus* spp. are acquired by the inhalation of infectious propagules present in the environment [4]. Cryptococcosis is a life-threatening fungal disease, mainly caused by *C. neoformans*/*C. gattii* species complex inhabiting different ecological niches [7]. Overall, *C. neoformans* (*C.n.*) is strongly associated with bird droppings, whereas *C. gattii* (*C.g.*) with angiosperm and gymnosperm trees [8]. The two species above include nine major molecular types (i.e., VNI, VNII, VNB, VNIII, and VNIV for *C.n.*, and VGI, VGII, VGIII, and VGIV for *C.g.*), which are unevenly distributed worldwide, under different climatic conditions [8, 9]. *C.n.* primarily affects individuals with impaired immunity, such as human immunodeficiency virus (HIV) positive patients, whereas *C.g.* mainly infects apparently immunocompetent hosts [10, 11]. Given the airborne nature of these microorganisms, the infection occurs mainly in the respiratory tract, without overt clinical presentations [12, 13]. Nonetheless, the infection occurs in a large range of domestic (i.e., dogs, cats) and wild species [13, 14], causing a plethora of clinical presentations from asymptomatic [12, 15–17] to either localised or disseminated forms of clinical condition [18–20].

The role played by asymptomatic animals as sentinel hosts for human exposure [14, 16] spurred the interest in isolating and characterizing these yeasts from squirrels that were found positive for *C.g.* on a single occasion [17].

The aim of the present study was to assess the occurrence of *Cryptococcus* spp. in the respiratory tract of squirrels (*Callosciurus finlaysonii*) trapped in southern Italy and to molecularly identify the isolates.

The potential association of *Cryptococcus* spp. strains from squirrels with those isolated from specific mammalian hosts or different ecological niches was also studied by phylogenetic analyses of the sequences of the internal transcribed spacer (ITS) regions (including ITS-1, 5.8S and ITS-2; ITS+) of ribosomal DNA and the orotidine monophosphate pyrophosphorylase (*URA5*) gene.

Materials and methods

Sampling procedures

One hundred and twenty-five squirrels were trapped in Maratea (Basilicata, Southern Italy) and humanely euthanized (i.e., inhalation of 60% CO₂) according to a project for the control of allochthonous wildlife populations and approved by the “Istituto Superiore per la Protezione e la Ricerca Ambientale” (12/08/2009 at n. 75 AG – 153998).

The squirrels were preserved individually in plastic envelopes and stored at –20°C until sampling. Physical examination and necropsy of animals were carried out and anamnestic data (i.e., estimated age and gender) were collected along with information regarding the occurrence of pathological signs suggestive of respiratory infection. The occurrence of masses inside the nasal cavities, mucosal edema, and hyperaemia were considered suggestive of upper respiratory infection, whereas fibrinous pneumonia, pulmonary congestion, and edema were suggestive of lung disease.

The age (i.e., yearling and adult) was established by the examination of hair colour (i.e., at the border between the dark brown and the white coloration of the frons, the white spots on the dark brown frons, followed by the dark brown sideburns on the white genal-temporal area) [21]. Two nasal swabs, one for each nostril (n = 250), and lung tissue sample (n = 125) were aseptically collected from each animal. Sterile swabs were inserted into each nostril and rotated vigorously.

Isolation and identification

Samples were immediately cultured onto Staib medium [22] with 0.05% chloramphenicol and 0.1% biphenyl, incubated at 30°C and checked daily for 14 days. For each positive sample the colonies positive and negative for the production of a dark pigment on Staib medium were counted separately and expressed as colony-forming units per sample (CFU). The colonies were examined microscopically after Gram staining to avoid bacterial contamination, and up to four colonies were sub-cultured onto Sabouraud dextrose agar (SDA) slants for species identification. Yeasts were identified by microscopical features, urea hydrolysis and sugar assimilation using the API 20C AUX test (bioMérieux, Marcy-l'Etoile, France). *Cryptococcus* spp. was also identified by the presence of capsule upon Indian ink staining and *C. albicans* by the germ tube production test [23]. Canavanine Glycine Bromothimol Blue (CGB) medium was used to discriminate between *C.n.* and *C.g.*

DNA extraction and molecular identification

DNA was extracted from isolates grown on SDA for 72 h at 30°C using the ArchivePure™ DNA Yeast kit (5-Prime Inc., USA) according to manufacturers' instructions. In order to reduce capsule size, the *Cryptococcus* spp. isolates were subcultured into liquid yeast peptone glucose medium (YPG) (1% yeast extract, 1% peptone, 2% D-glucose) supplemented with 0.5M sodium chloride for 24 h at 25°C under agitation at 150 rpm. Cells, collected by

centrifugation, were washed with cold water, incubated at room temperature for 4 h in 2 ml of urea buffer (8M urea, 0.5M NaCl, 20mM Tris, 20mM EDTA, 2% SDS, pH 8) under agitation and centrifuged for 2 min at 4000 rpm [24]. The pellet was used for DNA extraction according to manufacturers' instructions.

Yeast isolates were molecularly identified by polymerase chain reaction (PCR) and sequencing of ITS+ for all species, and *URA5* alone for *Cryptococcus* spp. [25, 26].

Purified PCR products were sequenced using the TaqDyeDeoxy Terminator Cycle Sequencing Kit (v.2, Applied Biosystems) in an automated sequencer (ABI-PRISM 377). Sequences were aligned using the ClustalX program and compared, by Basic Local Alignment Search Tool (BLAST – <http://blast.ncbi.nlm.nih.gov/Blast.cgi>), with those available in the GenBank™ database.

Molecular genotyping

The *URA5* molecular types of *C. neoformans*/*C. gattii* species complex were identified by restriction fragment length polymorphism analysis (RFLP). The *URA5* gene was double digested with the restriction enzymes *Sau96I* (10U/μl) and *HhaI* (20U/μl) for 3 h at 37°C and separated by 3% agarose gel electrophoresis at 100V for 5 h. WM148 (serotype A, VNI), WM626 (serotype A, VNII), WM628 (serotype AD, VNIII) and WM629 (serotype D, VNIV) were used as reference strains [26]. RFLP patterns were assigned visually by comparing the isolate profile with those obtained from the reference strains.

Phylogenetic analysis

Phylogenetic analysis was performed using the ITS+ and *URA5* sequences of *Cryptococcus* spp. isolates from squirrels and available in the GenBank™ and Centraal bureau voor Schimmelcultures (CBS-KNAW) Fungal Biodiversity Center databases.

Phylogenetic trees, from ClustalX aligned sequences, were produced by the maximum-likelihood (ML) method (Kimura 2-parameter model) [27] and neighbor-joining (NJ) [28], based on the p-distance of each gene (i.e., ITS+ and *URA5*). Evolutionary distances were computed using the software package MEGA 6.0 (Center for Evolutionary Medicine and Informatics, Tempe, AZ). The reliability of internal branches was assessed using the bootstrap method with 1000 replicates. *Cryptococcus albidus* and *C. gattii* were chosen as the outgroups in the ITS+ and *URA5* sequence analyses, respectively.

Statistical analysis

The differences in mean CFU (mCFU) from different samples were statistically analysed using the Students t-test. A value of $P < 0.05$ was considered to be statistically significant.

Results

At the necropsy, no pathological lesions suggestive of upper respiratory disease were detected, whereas lesions suggestive of lung disease were observed in 86 animals. A total of 13 (10.4%) squirrels tested positive for yeasts, 7 (5.6%) for *Cryptococcus* spp., and 6 (4.8%) for other yeasts. *Cryptococcus* spp. were isolated both from the nostrils and lungs (4% and 1.6%, respectively), with a larger population size in the nostrils (i.e., 222 mCFU) than in lungs (3 mCFU) (Table 1). In particular, the mCFU of *Cryptococcus* spp. was larger than for other yeasts (i.e., 159 vs 2; $P < 0.05$) (Table 1).

Yeasts (i.e., 55 isolates) belonged to four species according to their phenotypical/biochemical data and molecular identification (Table 2). The PCR amplification from individual DNA samples, representing 42 isolates phenotypically identified as *Cryptococcus* spp., yielded amplicons of the expected size (500 bp for ITS+ and 790 bp for the *URA5* gene). Two sequence types for ITS+ (designed as A_i and B_i) and three for *URA5* genes (designed as A_u, B_u, C_u) were identified and deposited in the GenBank™ database (Table 2). One representative strain from each positive sample is stored at -80°C in the fungal collection of the mycology section at the Department of Veterinary Medicine, University of Bari, Italy (Table 2). The ITS+ and *URA5* sequences matched with those available in the Genbank™ database, showing a 100% nucleotide identity with *C.n.* isolated from humans (Table 2). ITS+ sequence types showed a nucleotide variation of 0.6%, whereas *URA5* a nucleotide variation ranging from 0.3% to 6.4%. The molecular types of *C.n.* were identified by RFLP as *C.n.* var. *grubii* VNI (26/42, 61.9%) and *C.n.* var. *neoformans* VNIV (16/42, 38.1%) (Table 2, Fig. 1).

C.n. var. *grubii* VNI was the most prevalent species isolated from nostrils (2.4%) and the only from lungs (1.6%). *C.n.* var. *neoformans* VNIV was isolated only from nostrils (1.6%) (Tables 1, 2). *C.n.* was isolated from both nostrils, except in one case.

Phylogenetic analyses of *C.n.* revealed concordance in grouping *C.n.* sequence types for the two loci, although the low variation in ITS+ herein detected did not yield strong bootstrap support for this gene. There was consistency in the topology of the trees inferred by the ML and NJ methods (for each locus, not shown). The squirrel isolates were

Table 1. Number and percentage (in brackets) of squirrels scoring positive for *Cryptococcus* spp. and other yeasts in the nasal cavity and lungs.

	Nasal cavity			Lungs			Total		
	Pos/Tot (125) (%)	Mean CFU (sd)	No. isolates (%)	Pos/Tot (125) (%)	Mean CFU (sd)	No. isolates (%)	Pos/Tot (125) (%)	Mean CFU (sd)	No. isolates (%)
<i>Cryptococcus</i> spp.	5 (4)	222 (221) ^a	36/49 (73.5)	2 (1.6)	3 (0)	6/6 (100)	7 (5.6)	159 (210.3) ^b	42/55 (76.4)
Other yeasts	6 (4.8)	2 (0.98) ^a	13/49 (26.5)	–	–	–	6 (4.8)	2 (0.98) ^b	13/55 (23.6)
Total	11 (8.8)	102 (181.3)	49/49	2 (1.6)	3 (0)	6/6	13 (10.4)	87 (169.7)	55/55 (100)

Note. Mean colony forming units (CFU)/sample with standard deviation (sd) and number of isolates tested are also reported. The statistically significant differences are indicated with the same superscript letters.

^{a,b} = Student's t-test, $P < 0.05$.

Table 2. Code number, ITS+ and *URA5* sequence types and GenBank™ accession numbers of the strains isolated from nostrils and lungs (*).

Code number of representative strains	No. isolates	Sequence type - Accession No.		GenBank™ blast analysis 99-100% identity		Molecular identification	RFLP profile
		ITS+	<i>URA5</i>	ITS+	<i>URA5</i>		
CD1244, CD1245	8	A _i - KP835567	A _u - KP835572	KP068891, KP068877, KF958214, KF278463, EF081167	AJ555830, AF140184, AF032431, AY463674, AY463673	<i>C. neoformans</i> var. <i>neoformans</i>	VNIV
CD1246, CD1247	8	A _i - KP835567	B _u - KP835573	KP068891, KP068877, KF958214, KF278463, EF081167	KJ586335	<i>C. neoformans</i> var. <i>neoformans</i>	VNIV
CD1248, CD1249, CD1250, CD1251, CD1252, CD1253*, CD1254*	26	B _i - KP835568	C _u - KP835574	KP132181, KP132179, KJ777814, KP132177, GQ850220, KM085009	FN825632, FN825631, FN825630, FN825621, FN825608, FN825558	<i>C. neoformans</i> var. <i>grubii</i>	VNI
CD1255, CD1256, CD1257, CD1258	8	KP835569	nd	AB501149, AB501148, AB501150, AB501147, AB501145	nd	<i>Hanseniaspora thailandica</i>	nd
CD1259	2	KP835570	nd	GQ458025, EF643596, EF222225, EF196809	nd	<i>Debaryomyces hansenii</i>	nd
D1260	3	KP835571	nd	KP142532, KP132429, KP132428, KJ754141	nd	<i>Meyerozyma guilliermondii</i>	nd

Note. nd = not done.

found to be genetically homogeneous to those from human, animal and environment irrespective of their geographic origin (Fig. 2a, b). The phylogenetic analyses of ITS+ and *URA5* sequences revealed that the *C.n.* isolates clustered into two groups according to their RFLP molecular types.

A total of 13 yeast isolates were retrieved and molecularly and phenotypically identified as *Hanseniaspora thailandica* (8/13), *Debaryomyces hansenii* (anamorph: *Candida famata*, 2/13), and *Meyerozyma guilliermondii* (anamorph: *Candida guilliermondii*, 3/13). *H. thailandica* was isolated from four animals (3.2%), *D. hansenii* and *M. guilliermondii* from one animal (0.8%), respectively (Tables 1, 2). The strains were isolated only from one nostril. The phenotypical and biochemical identification was in agreement with the molecular delineation, except for *H. thailandica*, which was biochemically identified as *Kloeckera japonica*.

A higher prevalence of yeasts was registered in males (14.6%) and adults (13.7%) than in females (4.6%) and yearling (0%) animals (data not shown; $P > 0.05$). *C.n.* var. *grubii* VNI was isolated from lungs with lesions suggestive of lung infection.

Discussion

Among yeast species isolated from squirrels, *C.n.* was the most frequent species retrieved with a relative prevalence (4%) higher than those previously registered in feral (1.6%) and domestic cats (3.2%), as well as in dogs (1.1%) and wild birds (2.2%) [14, 15, 29]. The above finding might be the result of environmental exposure of this animal species to high airborne concentrations of the microorganism, particularly since squirrels spend most of their active time sniffing surfaces (i.e., decaying organic matter and soil),

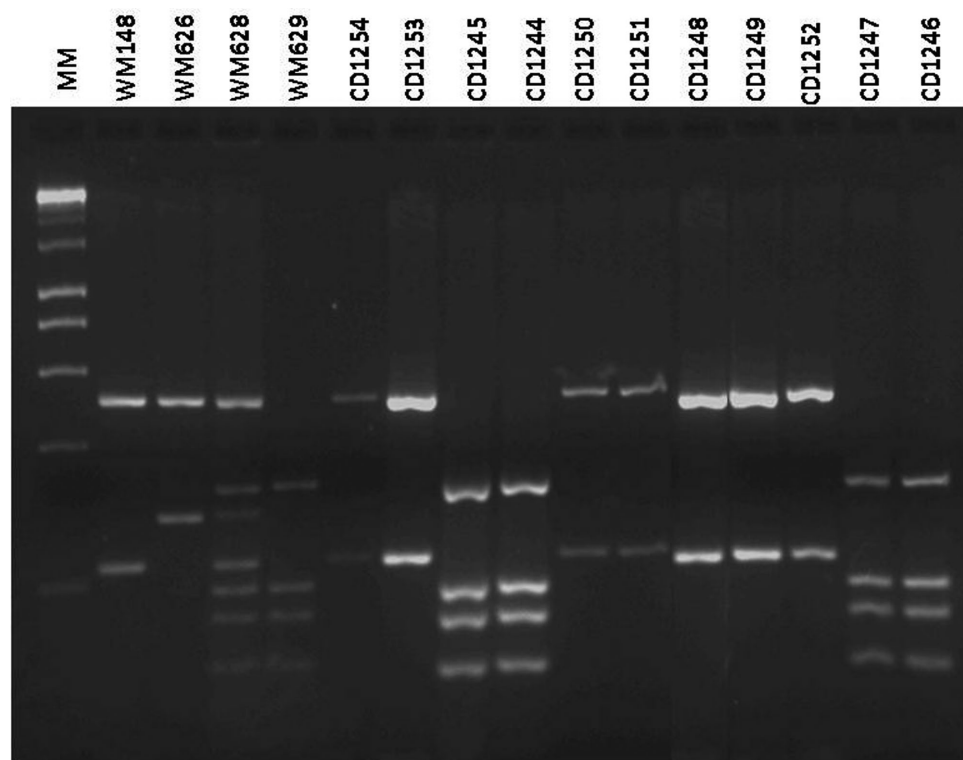


Figure 1. URA5 restriction fragment length polymorphism profiles, generated after double digestion with Sau96I and HhaI, of the *Cryptococcus neoformans* isolates. Left to right - MM: 100 bp Molecular Marker (Bioline), reference strains WM148 (VNI), WM626 (VNI), WM628 (VNI), WM629 (VNI) and representative strains indicated with laboratory code number (see Table 2).

which are a substrate for *Cryptococcus* spp. [30]. Indeed, a high prevalence of *C.g.* in nasal swabs of gray squirrels (*Sciurus carolinensis*) was previously registered in Vancouver Island, British Columbia, in association with soil, air, and vegetation [17, 31]. In addition, *C.g.* and *C.n.* were also isolated from *Ceratonia siliqua* [8, 32] a tree species present in the same area where squirrels were captured, thus confirming the role of environmental exposure in acquiring the infection. This finding is also supported by the high population size of *C.n.* in the nostrils, which suggests the presence of high airborne environmental contamination. The absence of lesions in the upper respiratory tracts on gross post-mortem examination suggests a nasal colonization. However, the isolation of *C.n.* (VNI) from the lungs and not from nostril of two squirrels with macroscopic lesions consistent with localized lung disease suggests the potential role of *C.n.* VNI as infectious agent. Moreover, it is difficult to draw any conclusion about the status of the microorganism within the lungs in the absence of histological examination of tissue and of cryptococcal antigen detection in the serum and only a nasal colonization might be envisaged.

The presence of molecular types VNI, followed by VNIV in sampled squirrels overlaps the distribution of

isolates from human cryptococcosis, with a higher prevalence of VNI in the south and VNIV in the north of Italy [9]. Indeed, molecular type VNI has been isolated from animals and the environment in southern Italy and the VNIV only in northern Italy [8, 33]. The retrieval of VNIV, for the first time, in southern Italy, suggests that climatic conditions are favourable for the survival of this molecular type and that squirrels are suitable carriers, since their geographic distribution depends on their climatic tolerance and/or the presence of different ecological niches [34, 35]. Therefore, the occurrence of the VNIV molecular type should be confirmed by environmental survey in this geographic area, as it might represent a source for human infection. Finally, the isolation of *C.n.* VNI only, from the lungs of two squirrels, confirms its virulence [35].

Based on phylogenetic analyses, *C.n.* isolated from squirrels were closely related to strains distributed worldwide irrespective of the geographic origin and host species and, in particular, to isolates from humans, in Italy [9]. Hence, this animal species might represent sentinel animals for human exposure.

The phylogenetic analyses of both genes (ITS+ and URA5) were consistent in clustering *C.n.* isolates from

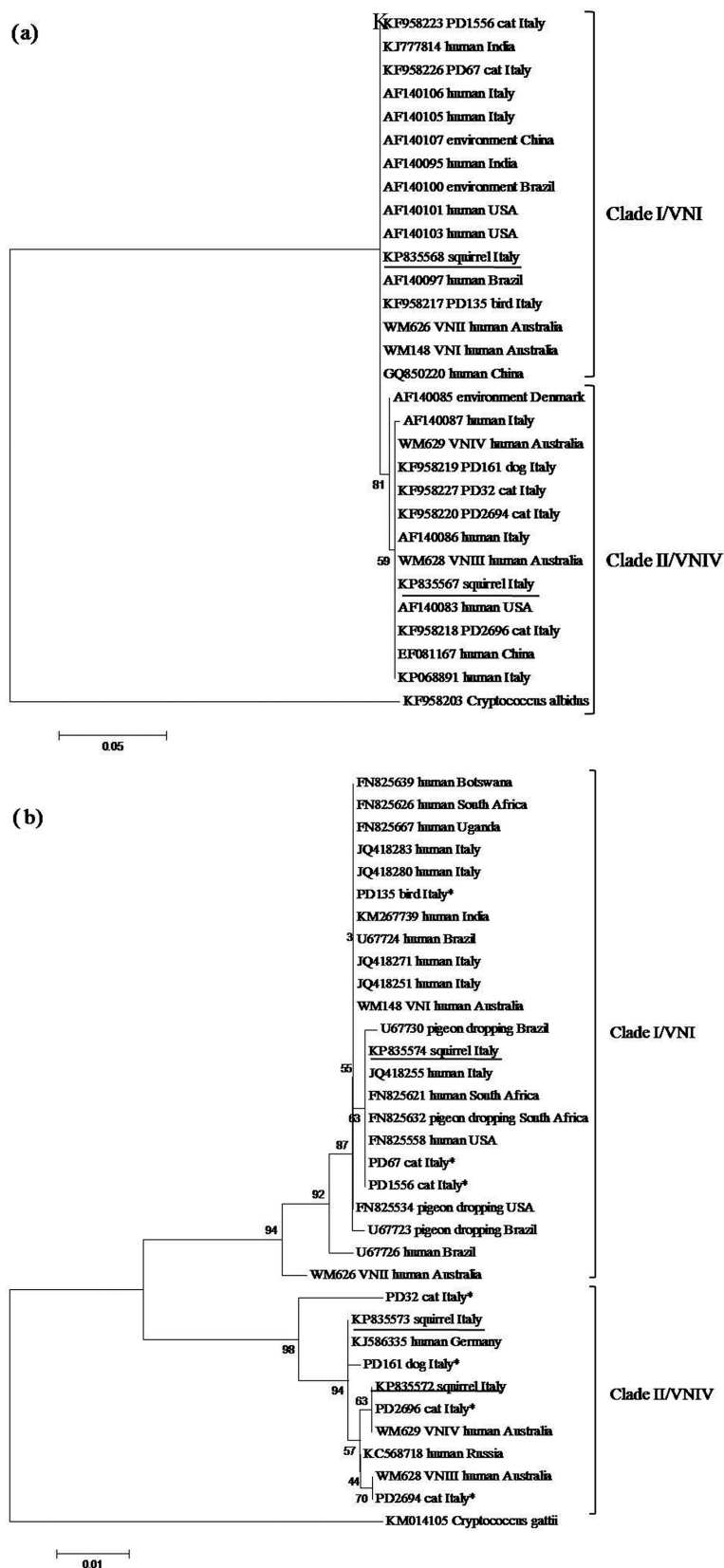


Figure 2. Dendrograms of ITS+ (a) and *URA5* (b) sequences for *Cryptococcus neoformans* compared with those available in the GenBank™ and CBS (*) databases. GenBank™ accession number and/or laboratory code, hosts and geographical origin of each strain are reported. Trees were inferred by the Maximum Likelihood method and sequences herein generated are underlined.

squirrels in two groups, in accordance with the RFLP genotyping.

The low prevalence and population size of *D. hansenii* and *M. guilliermondii* might be due to the medium employed or to the fact that they may be components of the nasal mucosa of squirrels, as previously reported in other animal species [36, 37]. In addition *D. hansenii* and *M. guilliermondii* are routinely isolated from insects, soil, plants, the atmosphere, seawater, the exudates of various trees, and processed foods, indicating that the microbiota in hosts is affected by their diet and behaviour [38].

The high prevalence of *H. thailandica*, previously isolated, exclusively from natural substrates (i.e., from insect frass, flower, lichen) in Thailand, indicates that this yeast belongs to the microbiota of the upper respiratory tract of this animal species which, in turn, is native to the Indochinese area [39].

The role of predisposing factors (age and sex) in the occurrence of infection in squirrels, needs to be confirmed, considering that adult males could be more exposed than females and yearlings due to their continual sniffing of surfaces [30].

In conclusion, data herein presented provides the first evidence regarding the occurrence of *C.n.* in the nasal cavities or lungs of *C. finlaysoni* and highlights the role of this animal species as an indicator of the presence of the yeast in the environment. In addition, the close phylogenetic relationship of *C.n.* strains from squirrels with those from other animal species suggests the role of these animals as “sentinels” for humans. Future studies are needed in order to assess the role of *C. neoformans* as colonizer or infectious agent by fungal culture, histopathology of tissue and by antigen detection in the serum of these or other wild animal species.

Acknowledgments

We kindly thank Dr Bronwyn Campbell for revising the English text.

Declaration of interest

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

References

1. Montagna MT, Lovero G, Borghi E et al. Candidemia in intensive care unit: a nationwide prospective observational survey (GISIA-3 study) and review of the European literature from 2000 through 2013. *Eur Rev Med Pharmacol Sci* 2014; **18** (5): 661–674.
2. Johnston DL, Lewis V, Yanofsky R et al. Invasive fungal infections in paediatric acute myeloid leukaemia. *Mycoses* 2013; **56** (4): 482–487.
3. Morace G, Borghi E, Iatta R et al. Antifungal susceptibility of invasive yeast isolates in Italy: the GISIA3 study in critically ill patients. *BMC Infect Dis* 2011; **11**: 130.
4. Nucci M, Queiroz-Telles F, Tobón AM, Restrepo A, Colombo AL. Epidemiology of opportunistic fungal infections in Latin America. *Clin Infect Dis* 2010; **51** (5): 561–570.
5. Arendrup MC, Boekhout T, Akova M et al. European Society of Clinical Microbiology and Infectious Diseases Fungal Infection Study Group; European Confederation of Medical Mycology. ESCMID/ECMM joint clinical guideline for the diagnosis and management of rare invasive yeast infections. *Clin Microbiol Infect* 2014; **Suppl 3**: 76–98.
6. Tortorano AM, Prigitano A, Lazzarini C et al. A 1-year prospective survey of candidemia in Italy and changing epidemiology over one decade. *Infection* 2013; **41** (3): 655–662.
7. Cogliati M. Global Molecular Epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii*: An Atlas of the Molecular Types. *Scientifica (Cairo)* 2013; **2013**: 675213.
8. Romeo O, Scordino F, Chillemi V et al. *Cryptococcus neoformans*/*Cryptococcus gattii* species complex in southern Italy: an overview on the environmental diffusion of serotypes, genotypes and mating-types. *Mycopathologia* 2012; **174** (4): 283–291.
9. Cogliati M, Zamfirova RR, Tortorano AM et al. Fimua Cryptococcosis Network. Molecular epidemiology of Italian clinical *Cryptococcus neoformans* var. *grubii* isolates. *Med Mycol* 2013; **51** (5): 499–506.
10. Kourbeti IS, Mylonakis E. Fungal central nervous system infections: prevalence and diagnosis. *Expert Rev Anti Infect Ther* 2014; **12** (2): 265–273.
11. Iatta R, Hagen F, Fico C et al. *Cryptococcus gattii* infection in an immunocompetent patient from Southern Italy. *Mycopathologia* 2012; **174** (1): 87–92.
12. Connolly JH, Krockenberger MB, Malik R et al. Asymptomatic carriage of *Cryptococcus neoformans* in the nasal cavity of the koala (*Phascolarctos cinereus*). *Med Mycol* 1999; **37** (5): 331–338.
13. Malik R, Wigney DI, Muir DB et al. Asymptomatic carriage of *Cryptococcus neoformans* in the nasal cavity of dogs and cats. *J Med Vet Mycol* 1997; **35** (1): 27–31.
14. Cafarchia C, Romito D, Iatta R et al. Role of birds of prey as carriers and spreaders of *Cryptococcus neoformans* and other zoonotic yeasts. *Med Mycol* 2006; **44** (6): 485–492.
15. Danesi P, Furnari C, Granato A et al. Molecular identity and prevalence of *Cryptococcus* spp. nasal carriage in asymptomatic feral cats in Italy. *Med Mycol* 2014; **52** (7): 667–673.
16. Morera N, Hagen F, Juan-Sallés C et al. Ferrets as sentinels of the presence of pathogenic *Cryptococcus* species in the Mediterranean environment. *Mycopathologia* 2014; **178** (1–2): 145–151.
17. Duncan C, Schwantje H, Stephen C et al. *Cryptococcus gattii* in wildlife of Vancouver Island, British Columbia, Canada. *Journal Wild Dis* 2006; **42** (1): 175–178.
18. Mischnik A, Stockklauser J, Hohneder N et al. First case of disseminated cryptococcosis in a Gorilla gorilla. *Mycoses* 2014; **57** (11): 664–671.

19. Sykes JE, Sturges BK, Cannon MS et al. Clinical signs, imaging features, neuropathology, and outcome in cats and dogs with central nervous system cryptococcosis from California. *J Vet Intern Med* 2010; **24** (6): 1427–1438.
20. Malik R, Alderton B, Finlaison D et al. Cryptococcosis in ferrets: a diverse spectrum of clinical disease. *Aust Vet J* 2002; **80** (12): 749–755.
21. Mazzoglio PJ, Blengio L, Bertolino S. Individual recognition in *Callosciurus finlaysonii* (Horsfield, 1824) (Rodentia, Sciuridae) introduced into Italy. *Mammalia* 2007; **71**: 122–125.
22. Staib F. New concepts in the occurrence and identification of *Cryptococcus neoformans*. *Mycopathol Mycol Appl* 1963; **19**: 143–145.
23. Cafarchia C, Camarda A, Romito D et al. Occurrence of yeasts in cloacae of migratory birds. *Mycopathologia* 2006; **161** (4): 229–234.
24. Bolano A, Stinchi S, Preziosi R et al. Rapid methods to extract DNA and RNA from *Cryptococcus neoformans*. *FEMS Yeast Res* 2001; **1** (3): 221–224.
25. Katsu M, Kidd S, Ando A et al. The internal transcribed spacers and 5.8S rRNA gene show extensive diversity among isolates of the *Cryptococcus neoformans* species complex. *FEMS Yeast Res* 2004; **4** (4–5): 377–388.
26. Meyer W, Castañeda A, Jackson S et al. IberoAmerican Cryptococcal Study Group. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerg Infect Dis* 2003; **9** (2): 189–195.
27. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980; **16** (2): 111–120.
28. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987; **4** (4): 406–425.
29. Duncan C, Stephen C, Lester S. Follow-up study of dogs and cats with asymptomatic *Cryptococcus gattii* infection or nasal colonization. *Med Mycol* 2005; **43** (7): 663–666.
30. Bertolino S, Lurz P. *Callosciurus* squirrels: worldwide introductions, ecological impacts and recommendations to prevent the establishment of new invasive populations. *Mammal Rev* 2012; **43**: 22–33.
31. Datta K, Bartlett KH, Baer R et al. *Cryptococcus gattii* Working Group of the Pacific Northwest. Spread of *Cryptococcus gattii* into Pacific Northwest region of the United States. *Emerg Infect Dis* 2009; **15** (8): 1185–1191.
32. Colom MF, Hagen F, Gonzalez A et al. *Ceratonia siliqua* (Carob) trees as natural habitat and source of infection by *Cryptococcus gattii* in the Mediterranean environment. *Med Mycol* 2012; **50** (1): 67–73.
33. Danesi P, Firacative C, Cogliati M et al. Multilocus sequence typing (MLST) and M13 PCR fingerprinting revealed heterogeneity amongst *Cryptococcus* species obtained from Italian veterinary isolates. *FEMS Yeast Res* 2014; **14** (6): 897–909.
34. Lo Passo C, Pernice I, Gallo M et al. Genetic relatedness and diversity of *Cryptococcus neoformans* strains in the Maltese Islands. *J Clin Microbiol* 1997; **35** (3): 751–755.
35. Dromer F, Mathoulin S, Dupont B et al. Individual and environmental factors associated with infection due to *Cryptococcus neoformans* serotype D. French Cryptococcosis Study Group. *Clin Infect Dis* 1996; **23** (1): 91–96.
36. Cordeiro Rde A, Bittencourt PV, Brilhante RS et al. Species of *Candida* as a component of the nasal microbiota of healthy horses. *Med Mycol* 2013; **51** (7): 731–736.
37. Desnos-Ollivier M, Ragon M, Robert V et al. *Debaryomyces hansenii* (*Candida famata*), a rare human fungal pathogen often misidentified as *Pichia guilliermondii* (*Candida guilliermondii*). *J Clin Microbiol* 2008; **46** (10): 3237–3242.
38. Jin JS, Touyama M, Yamada S et al. Alteration of a human intestinal microbiota under extreme life environment in the Antarctica. *Biol Pharm Bull* 2014; **37** (12): 1899–1906.
39. Jindamorakot S, Ninomiya S, Limtong S et al. Three new species of bipolar budding yeasts of the genus *Hanseniaspora* and its anamorph *Kloeckera* isolated in Thailand. *FEMS Yeast Res* 2009; **9** (8): 1327–1337.