HHS Public Access

Author manuscript

Fungal Genet Biol. Author manuscript; available in PMC 2016 February 16.

Published in final edited form as:

Fungal Genet Biol. 2016 February ; 87: 22–29. doi:10.1016/j.fgb.2016.01.003.

Multilocus sequence typing analysis reveals that *Cryptococcus neoformans* var. *neoformans* is a recombinant population

Massimo Cogliati[a,*], **Alberto Zani**[a], **Volker Rickerts**[b], **Ilka McCormick**[b], **Marie Desnos-Ollivier**[c], **Aristea Velegraki**[d], **Patricia Escandon**[e], **Tomoe Ichikawa**[f], **Reiko Ikeda**[f], **Anne-Lise Bienvenue**[g,h], **Kathrin Tintelnot**[b], **Okan Tore**[j], **Sevim Akcaglar**[j], **Shawn Lockhart**[k], **Anna Maria Tortorano**[a], and **Ashok Varma**[i]

[a]Lab. Micologia Medica, Dip. Scienze Biomediche per la Salute, Università degli Studi di Milano, Milano, Italy

[b]Section Mycology, Robert-Koch Institute, Berlin, Germany

[c]Unité de Mycologie Moléculaire, Centre National de Référence Mycoses invasives et Antifongiques, Institut Pasteur, Paris, France

[d]Dept. of Microbiology, Medical School National and Kapodistrian University of Athens, Athens, Greece

[e]Grupo de Microbiología, Instituto Nacional de Salud, Bogotá, D.C., Colombia

[f]Dept. of Microbial Science and Host Defense, Meiji Pharmaceutical University, Tokyo, Japan

[g]Institut de Parasitologie et Mycologie Médicale, Hospices Civils de Lyon, France

[h]Malaria Research Unit, Université de Lyon, Lyon, France

[i]Molecular Microbiology Section, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA

[j]Dept. of Medical Microbiology, School of Medicine, Uludag University, Bursa, Turkey

[k]Antifungal and Fungal Reference Laboratories, Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, GA, USA

Abstract

Cryptococcus neoformans var. *neoformans* (serotype D) represents about 30% of the clinical isolates in Europe and is present less frequently in the other continents. It is the prevalent etiological agent in primary cutaneous cryptococcosis as well as in cryptococcal skin lesions of disseminated cryptococcosis. Very little is known about the genotypic diversity of this *Cryptococcus* subtype. The aim of this study was to investigate the genotypic diversity among a set of clinical and environmental *C. neoformans* var. *neoformans* isolates and to evaluate the

[*]Corresponding author at: Lab. Micologia Medica, Dip. Scienze Biomediche per la Salute, Università degli Studi di Milano, Via Pascal 36, 20133 Milano, Italy. massimo.cogliati@unimi.it (M. Cogliati).

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

Appendix A. Supplementary material: Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2016.01.003.

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. Maximum likelihood phylogenetic as well as network analysis strongly suggested the existence of a recombinant rather than a clonal population structure. Geographical origin and source of isolation were not correlated with a specific MLST genotype. The comparison with a set of outgroup *C. neoformans* var. *grubii* isolates provided clear evidence that the two varieties have different population structures.

Keywords

Cryptococcus; *C. neoformans* var. *neoformans*; *C. neoformans* var. *grubii*; MLST; Recombination

1. Introduction

Cryptococcus neoformans and *C. gattii* are two sibling yeast species responsible for cryptococcosis. This life-threatening disease is mainly associated with AIDS patients in the countries where the HIV infection burden is still high such as in sub-Saharan Africa and in South East Asia (Assogba et al., 2015; Park et al., 2009). In developed countries, however, the incidence of cryptococcosis in HIV-infected population is decreasing due to the introduction of high active antiretroviral therapy (HAART). In contrast, the disease is increasingly found in non-AIDS patients such as those with hematological neoplasms, recipients of organ transplantation, and victims of autoimmune diseases (Bratton et al., 2012; Henao-Martínez and Beckham, 2015; Sanchini et al., 2014).

C. neoformans is classified into two varieties, three serotypes and five molecular types. *C. neoformans* var. *grubii*, serotype A, is identified as the molecular types VNI, VNII and VNB, whereas *C. neoformans* var. *neoformans*, serotype D, belongs to molecular type VNIV. In addition, diploid or aneuploid intervarietal AD hybrids are identified as molecular type VNIII. *Cryptococcus gattii* has two serotypes, B and C, and four molecular types VGI, VGII, VGII, and VGIV (Heitman et al., 2010).

The recent *C. gattii* emergence from Vancouver Island (Canada) south to the Pacific Northwest of the United States, has contributed to the interest in *C. gattii* in different parts of the world and, at the same time, to highlight the differences from *C. neoformans* in both ecological distribution and clinical manifestations (Espinel-Ingroff and Kidd, 2015; Chen et al., 2014).

C. neoformans var. *grubii* is the prevalent agent of cryptococcosis and it is globally distributed. It is commonly isolated from pigeon and other bird excreta and soil as well as many species of trees (Cogliati, 2013). The main clinical manifestation and the cause of death is meningoencephalitis especially in immunocompromised patients (Kwon-Chung et al., 2014).

C. neoformans var. *neoformans* has not been extensively investigated and little is known about its ecology, distribution and clinical manifestations. The majority of clinical isolates were reported from Europe where it has a prevalence of 30% (Viviani et al., 2006), but it has also been found in North (Yan et al., 2002; Litvintseva et al., 2005) and South America (Pérez et al., 2008; Cortés et al., 2011; Meyer et al., 2003; Trilles et al., 2003) as well as in Asia (Sukroongreung et al., 1996; Feng et al., 2008; Capoor et al., 2008; Ikeda and Shinoda, 2000). Very few isolates have been recovered from the environment mainly from pigeon droppings. A recent large environmental survey carried out in Europe identified the association of this variety with different tree species (Cogliati et al., 2014). At present, little information about specific clinical manifestations is available although a skin tropism of this yeast has been shown. A study carried out on 108 *Cryptococcus* isolates recovered from patients with skin lesions clearly indicated that infection with serotype D isolates was one of the risk factors in cutaneous manifestations (Neuville et al., 2003).

The present study aims to investigate a large number of *C. neoformans* var. *neoformans* strains isolated from different geographical areas and from different sources in order to elucidate the genetic population structure of this variety.

2. Materials and methods

2.1. Isolates

Eighty-three *C. neoformans* var. *neoformans* isolates were investigated (Table 1). Twenty isolates were from Germany, 19 from Italy, 13 from Greece, 8 from Japan, 7 from France, 4 from Belgium, 4 from Denmark, 4 from Colombia, and 1 each from Australia, Thailand, Turkey and the USA. Twenty-six isolates were recovered from the environment (soil, trees, pigeon droppings and dust), 22 from cases of disseminated cryptococcosis, 17 from cases of documented primary cutaneous cryptococcosis, 4 from cases of probable primary cutaneous cryptococcosis, 9 from cases of secondary cutaneous cryptococcosis, and 5 from veterinary cases. All clinical cases were independent cases and no multiple isolates from the same patient were included in the study. In addition, data obtained from previous studies of 30 *C. neoformans* var. *grubii* isolates (11 VNB, 10 VNI, and 9 VNII) were included in the analysis as outgroup strains (Khayhan et al., 2013; Litvintseva et al., 2006; Sanchini et al., 2014; Cogliati et al., 2013; Meyer et al., 2009; Kaocharoen et al., 2013; Choi et al., 2010; Umeyama et al., 2013; Wiesner et al., 2012) (Table S1).

2.2. Clinical case definitions

The cases of primary cutaneous cryptococcosis presented a single cutaneous lesion often on the arms or the legs which are primarily due to a traumatic injury. No *Cryptococcus* antigens from serum were detected and no isolates from other body sites were recovered. The cases that presented isolated cutaneous lesions but were not supported by the other clinical evidences were defined as probable.

Secondary cutaneous cryptococcosis patients presented multiple skin lesions with no specific body sites, positive *Cryptococcus* antigens or positive cultures from other clinical samples (blood, CSF, urine).

Disseminated cryptococcosis cases presented positive cultures from multiple body sites, positive *Cryptococcus* antigens, but no skin lesions.

2.3. Molecular analysis

Molecular type and mating type were determined by multiplex PCRs as previously reported (Cogliati et al., 2000; Esposto et al., 2004). Multilocus sequence typing was performed according to the ISHAM consensus scheme (Meyer et al., 2009) and all sequences were deposited in the *Cryptococcus* MLST database (www.mycologylab.org).

The data of strains WM629, CBS7816, PD32, PD2270, PD1596, RKI 08-0429, RKI 04-0089, RKI 09-0388, RKI 09-0515, RKI 07-0173, RKI 05-0151, RKI 04-0061, RKI 09-0393, RKI 09-0545, RKI 09-0102, RKI 09-0103, RKI 11-0048, RKI 08-0591, RKI 08-0572, RKI 11-0047) were obtained from previous studies (Sanchini et al., 2014; Kaocharoen et al., 2013; Meyer et al., 2009; Danesi et al., 2014).

The concatenated sequences of the seven MLST genes (*CAP59*, *GPD1*, *IGS1*, *LAC1*, *PLB1*, *SOD1*, *URA5*) of the 83 *C. neoformans* var. *neoformans* and 30 *C. neoformans* var. *grubii* isolates were aligned by ClustalW algorithm (www.ebi.ac.uk) and the resulting file was converted in a Roehl data file by DnaSP software (Universitat de Barcelona, www.ub.edu/dnasp). Network analysis was performed using the median joining method included in the software Network v4.6 (Fluxus Technology Ltd., www.fluxus-engeneering.com).

Genetic population parameters and population comparisons were performed by DnaSP software whereas maximum likelihood phylogenetic analysis and average evolutionary divergence were calculated with the software Mega v6.0 (www.megasoftware.net).

The degree of recombination inside the population was also calculated using both the linkage disequilibrium test and the Watterson estimator (theta) method (DnaSP software). The linkage disequilibrium test is an extension of Fisher's exact probability test on contingency tables. The test consists in obtaining the probability of finding a table with the same marginal totals and which has a probability equal or less than the observed table. The null-hypothesis of non-random association between the two tested loci was confirmed if the probability was less than 0.05. The Watterson estimator (theta) method extrapolates two values that correspond to the expected theta value for a non-recombinant population and to the expected theta value for a free-recombinant population, and then it calculates the observed theta value in the investigated population.

3. Results

Molecular identification confirmed that all the *C. neoformans* var. *neoformans* isolates belonged to molecular type VNIV and that 63 were mating type α and 20 mating type **a** (Table 1).

The alignment of the 4092-bp sequences resulting by concatenating the seven MLST loci showed the presence of 425 polymorphic sites that identified 49 sequence types with a haplotype diversity (Hd) value of 0.965. *LAC1* and *URA5* were the most polymorphic loci

discriminating 16 and 15 haplotypes, respectively. In contrast, the *IGS1* locus was the least discriminatory locus with an Hd value of 0.241 (Table 2).

Maximum likelihood phylogenetic reconstruction showed that most of *C. neoformans* var. *neoformans* isolates were grouped in a unique cluster with an average evolutionary divergence of 0.008, twofold lower than that observed among *C. neoformans* var. *grubii* isolates. Four isolates (WM629, CBS7816, TRNCGB1HO1-1, IUM 01-0956) were situated between the two varieties with an ambiguous topology and one isolate (GRLMM26HO1-2) was unexpectedly more related to the VNI group (Fig. 1). A network analysis showed the same topology with a core region grouping the majority of *C. neoformans* var. *neoformans* isolates all linked by star-like branches. The topology of *C. neoformans* var. *grubii* population was very different with clear clusters and higher genetic distances. The two populations were linked by a long branch, along which were the five ambiguous isolates (Fig. 2A).

The investigated isolates were therefore divided in three groups, *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii* and putative intervarietal recombinants, and average evolutionary divergence between the populations was calculated. The results showed that *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* diverged 0.09 and both diverged from the putative recombinants around 0.05. The five putative recombinants were then checked to exclude mixed cultures and five single colonies from each of the original strains were collected and processed for molecular typing and ploidy determination by flow cytometry. The results confirmed that all strains from single colonies were VNIV **a**D or αD and that they were haploids (Fig. S1).

The linkage disequilibrium analysis of *C. neoformans* var. *neoformans* populations was also calculated for both the concatenated sequences and the single loci alignments. The results confirmed the absence of linkage disequilibrium in all the cases confirming that recombination in this population could not be excluded (Table 2). The Watterson estimator (theta) method results were in perfect agreement with that obtained in the linkage disequilibrium test strengthening for the hypothesis of a recombinant population (Table 2). In addition, the estimation of recombination events among the whole *C. neoformans* var. *neoformans* population reveals that at least 31 recombination events had occurred and that the most recombinant locus was *LAC1* (20 events) (Table 2).

Fig. 2B displays geographical origin of each isolate on the network tree. No correlation was observed between the country of isolation and the MLST profile. Only the eight Japanese isolates belonged all to the same genotype (ST168). Similarly, comparison between MLST profiles and source of isolation did not provide evidence of any specific correlation. ST135 and ST180 grouped together isolates from the environment with isolates from veterinary cases, from primary cutaneous cryptococcosis and from secondary cutaneous cryptococcosis whereas none of the environmental isolates shared the same ST with isolates from disseminated cryptococcosis (Fig. 2C).

4. Discussion

The present study investigated, for the first time by MLST, the genetic population structure of a large number of *C. neoformans* var. *neoformans* isolates from different geographical origin and from different sources. The results strongly support the hypothesis that this population is recombinant and are in agreement with the recent study carried out by other authors (Desnos-Ollivier et al., 2015) in a population of French clinical *C. neoformans* isolates. The high haplotype diversity as well as the low evolutionary divergence among *C. neoformans* var. *neoformans* population confirm that isolates are strictly correlated each to the other but they are characterized by high variability due to recombination. Statistical tests such as linkage disequilibrium analysis, Watterson estimator calculation, and recombining events evaluation also corroborate this hypothesis. Furthermore, the *C. neoformans* var. *neoformans* population investigated in the present study included 20 mating type a isolates recovered from both patients and the environment. This suggests that isolates of *C. neoformans* var. *neoformans* mating type **a** are more prevalent than those of *C. neoformans* var. *grubii* and therefore sexual reproduction may occur more frequently.

The high polymorphism observed for the *LAC1* locus suggests that the laccase enzyme plays a crucial role for both the host infection and the environmental survival of this yeast. The survival in a particular ecological niche, such as a tree, could depend on the capacity of this yeast to degrade a wide range of phenolic compounds present in lignin of the tree trunk. In addition, since laccase is a key enzyme for melanin production this variety could gain an advantage for growth on surfaces exposed to light, for example the bark of the tree.

Both maximum likelihood phylogenetic analysis and network analysis identified a group of five isolates with an ambiguous position on the tree. The average evolutionary divergence between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* groups was twice than that observed between the two groups and the ambiguous isolates suggesting that the five isolates could represent haploid strains generated by intervarietal recombination (Xu et al., 2000; Kavanaugh et al., 2006). Laboratory isolated haploid intervarietal recombinant clones have been reported by crossing between H99, a strain of *C. neoformans* var. *grubii* VNI, and JEC20, a strain of *C. neoformans* var. *neoformans* (Kwon-Chung and Varma, 2006). This is in contrast with the recent published proposal (Hagen et al., 2015), advocating that *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are separate species. Further investigations and a larger number of *C. neoformans* var. *neoformans* isolates are needed to clarify this important issue.

The comparison between MLST profiles and geographical origin showed that isolates recovered from different countries and from different continents were grouped in the same cluster confirming the role of recombination in shortening the genetic divergence. However, clonal expansion of some genotypes could occur in geographical areas where physical barriers exist that prevent recombination. This could be true in Japan where all the isolates investigated belonged to the same ST even though they were isolated from different patients, at different time points and from different regions of Japan.

A similar analysis to this one, which compared the source of isolates, showed that the environmental genotypes could be the source of infections for animal, primary cutaneous cryptococcosis and secondary cutaneous cryptococcosis. In contrast, this was not observed for the isolates from disseminated cryptococcosis cases, which shared an identical ST but only with those from primary and secondary cutaneous cryptococcosis cases. Further studies are required to confirm this discrepancy.

In conclusion, our study showed that *C. neoformans* var. *neoformans* population is not evolving primarily by clonal expansion, as observed for *C. neoformans* var. *grubii* (Khayhan et al., 2013), and that intravarietal recombination is largely occurring and intervarietal recombination could not be excluded.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Prof. F. Dromer and Prof. J. Kwon-Chung for providing some of the isolates and for her suggestions to improve this study, and Dr. T. Boekhout and Dr. B. Theelen for sequencing support.

References

Assogba K, et al. Neuromeningeal cryptococcosis in sub-Saharan Africa: killer disease with sparse data. J Neurosci Rural Pract. 2015; 6:221–224. [PubMed: 25883484]

Bratton EW, et al. Comparison and temporal trends of three groups with cryptococcosis: HIV-infected, solid organ transplant, and HIV-negative/non-transplant. PLoS ONE. 2012; 7:e43582. [PubMed: 22937064]

Capoor MR, et al. Current scenario of cryptococcosis and antifungal susceptibility pattern in India: a cause for reappraisal. Mycoses. 2008; 51:258–265. [PubMed: 18399907]

Chen SC, Meyer W, Sorrell TC. *Cryptococcus gattii* infections. Clin Microbiol Rev. 2014; 27:980–1024. [PubMed: 25278580]

Choi YH, et al. Prevalence of the VNIc genotype of *Cryptococcus neoformans* in non-HIV-associated cryptococcosis in the Republic of Korea. FEMS Yeast Res. 2010; 10:769–778. [PubMed: 20561059]

Cogliati M, et al. Molecular epidemiology of Italian clinical *Cryptococcus neoformans* var. *grubii* isolates. Med Mycol. 2013; 51:499–506. [PubMed: 23286351]

Cogliati M, et al. Genotyping *Cryptococcus neoformans* var. *neoformans* with specific primers designed from PCR-fingerprinting bands sequenced using a modified PCR-based strategy. Med Mycol. 2000; 38:97–103. [PubMed: 10817225]

Cogliati M. Global molecular epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii*: an Atlas of the molecular types. Scientifica. 2013; 2013:675213. [PubMed: 24278784]

Cogliati M, et al. Environmental survey of *Cryptococcus neoformans* and *C. gattii* in European and Mediterranean area. Mycoses. 2014; 57(Suppl. 1):30.

Cortés JA, et al. Fungal bloodstream infections in tertiary care hospitals in Colombia. Rev Iberoam Micol. 2011; 28:74–78. [PubMed: 21241818]

Danesi P, et al. Multilocus sequence typing (MLST) and M13 PCR fingerprinting revealed heterogeneity amongst *Cryptococcus* species obtained from Italian veterinary isolates. FEMS Yeast Res. 2014; 14:897–909. [PubMed: 24981157]

Desnos-Ollivier M, et al. Cryptococcosis serotypes impact outcome and provide evidence of *Cryptococcus neoformans* speciation. MBio. 2015; 6:e00311. [PubMed: 26060271]

Espinel-Ingroff A, Kidd SE. Current trends in the prevalence of *Cryptococcus gattii* in the United States and Canada. Infect Drug Resist. 2015; 11:89–97. [PubMed: 25999744]

Esposto MC, et al. Determination of *Cryptococcus neoformans* var. *neoformans* mating type by multiplex PCR. Clin Microbiol Infect. 2004; 10:1092–1094. [PubMed: 15606637]

Feng X, et al. Genotype and mating type analysis of *Cryptococcus neoformans* and *Cryptococcus gattii* isolates from China that mainly originated from non-HIV-infected patients. FEMS Yeast Res. 2008; 8:930–938. [PubMed: 18671745]

Hagen F, et al. Recognition of seven species in the *Cryptococcus gattii/Cryptococcus neoformans* species complex. Fungal Genet Biol. 2015; 78:16–48. [PubMed: 25721988]

Heitman, J., et al. Cryptococcus: From Human Pathogen to Model Yeast. ASM Press; Washington, DC, USA: 2010.

Henao-Martínez AF, Beckham JD. Cryptococcosis in solid organ transplant recipients. Curr Opin Infect Dis. 2015; 28:300–307. [PubMed: 26098495]

Ikeda R, Shinoda T. Mycological and serological diagnosis of cryptococcosis. Nihon Ishinkin Gakkai Zasshi. 2000; 41:241–244. [PubMed: 11064322]

Kaocharoen S, et al. Molecular epidemiology reveals genetic diversity amongst isolates of the *Cryptococcus neoformans/C. gattii* species complex in Thailand. PLoS Negl Trop Dis. 2013; 7:e2297. [PubMed: 23861989]

Kavanaugh LA, Fraser JA, Dietrich FS. Recent evolution of the human pathogen *Cryptococcus neoformans* by intervarietal transfer of a 14-gene fragment. Mol Biol Evol. 2006; 23:1879–1890. [PubMed: 16870684]

Khayhan K, et al. Geographically structured populations of *Cryptococcus neoformans* variety *grubii* in Asia correlate with HIV status and show a clonal population structure. PLoS ONE. 2013; 8:e72222. [PubMed: 24019866]

Kwon-Chung KJ, et al. *Cryptococcus neoformans* and *Cryptococcus gattii*, the etiologic agents of cryptococcosis. Cold Spring Harb Perspect Med. 2014; 4:a019760. [PubMed: 24985132]

Kwon-Chung KJ, Varma A. Do major species concepts support one, two or more species within *Cryptococcus neoformans*? FEMS Yeast Res. 2006; 6:574–587. [PubMed: 16696653]

Litvintseva AP, et al. Multilocus sequence typing reveals three genetic subpopulations of *Cryptococcus neoformans* var. *grubii* (serotype A), including a unique population in Botswana. Genetics. 2006; 172:2223–2238. [PubMed: 16322524]

Litvintseva AP, et al. Comparative analysis of environmental and clinical populations of *Cryptococcus neoformans*. J Clin Microbiol. 2005; 43:556–564. [PubMed: 15695645]

Meyer W, et al. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerg Infect Dis. 2003; 9:189–195. [PubMed: 12603989]

Meyer W, et al. Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. Med Mycol. 2009; 47:561–570. [PubMed: 19462334]

Neuville S, et al. Primary cutaneous cryptococcosis: a distinct clinical entity. Clin Infect Dis. 2003; 36:337–347. [PubMed: 12539076]

Park BJ, et al. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS. 2009; 23:525–530. [PubMed: 19182676]

Pérez C, et al. *Cryptococcus neoformans*, *Cryptococcus gattii*: serotypes in Venezuela. Mycopathologia. 2008; 166:149–153. [PubMed: 18512129]

Sanchini A, et al. Molecular typing of clinical *Cryptococcus neoformans* isolates collected in Germany from 2004 to 2010. Med Microbiol Immunol. 2014; 203:333–340. [PubMed: 24838744]

Sukroongreung S, et al. Serotypes of *Cryptococcus neoformans* isolated from patients prior to and during the AIDS era in Thailand. Mycopathologia. 1996; 135:75–78. [PubMed: 9063001]

Trilles L, et al. Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. Med Mycol. 2003; 41:383–390. [PubMed: 14653514]

Umeyama T, et al. Determination of epidemiology of clinically isolated *Cryptococcus neoformans* strains in Japan by multilocus sequence typing. Jpn J Infect Dis. 2013; 66:51–55. [PubMed: 23429086]

Viviani MA, et al. Molecular analysis of 311 *Cryptococcus neoformans* isolates from a 30-month ECMM survey of cryptococcosis in Europe. FEMS Yeast Res. 2006; 6:614–619. [PubMed: 16696657]

Wiesner DL, et al. Cryptococcal genotype influences immunologic response and human clinical outcome after meningitis. MBio. 2012; 3:e00196–12. [PubMed: 23015735]

Xu J, Vilgalys R, Mitchell TG. Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*. Mol Ecol. 2000; 9:1471–1481. [PubMed: 11050543]

Yan Z, Li X, Xu J. Geographic distribution of mating type alleles of *Cryptococcus neoformans* in four areas of the United States. J Clin Microbiol. 2002; 40:965–972. [PubMed: 11880424]

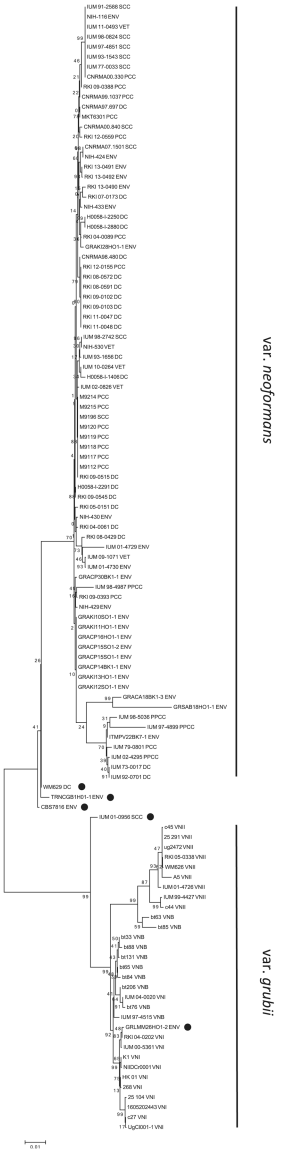

Fig. 1.

Maximun likelihood phylogenetic reconstruction including 83 *C. neoformans* var. *neoformans* (VNIV) and 30 *C. neoformans* var. *grubii* (VNI, VNII, VNB) isolates. Black dots indicate VNIV isolates with an ambiguous position. Numbers near the nodes represent the bootstrap values obtained for 1000 replications.

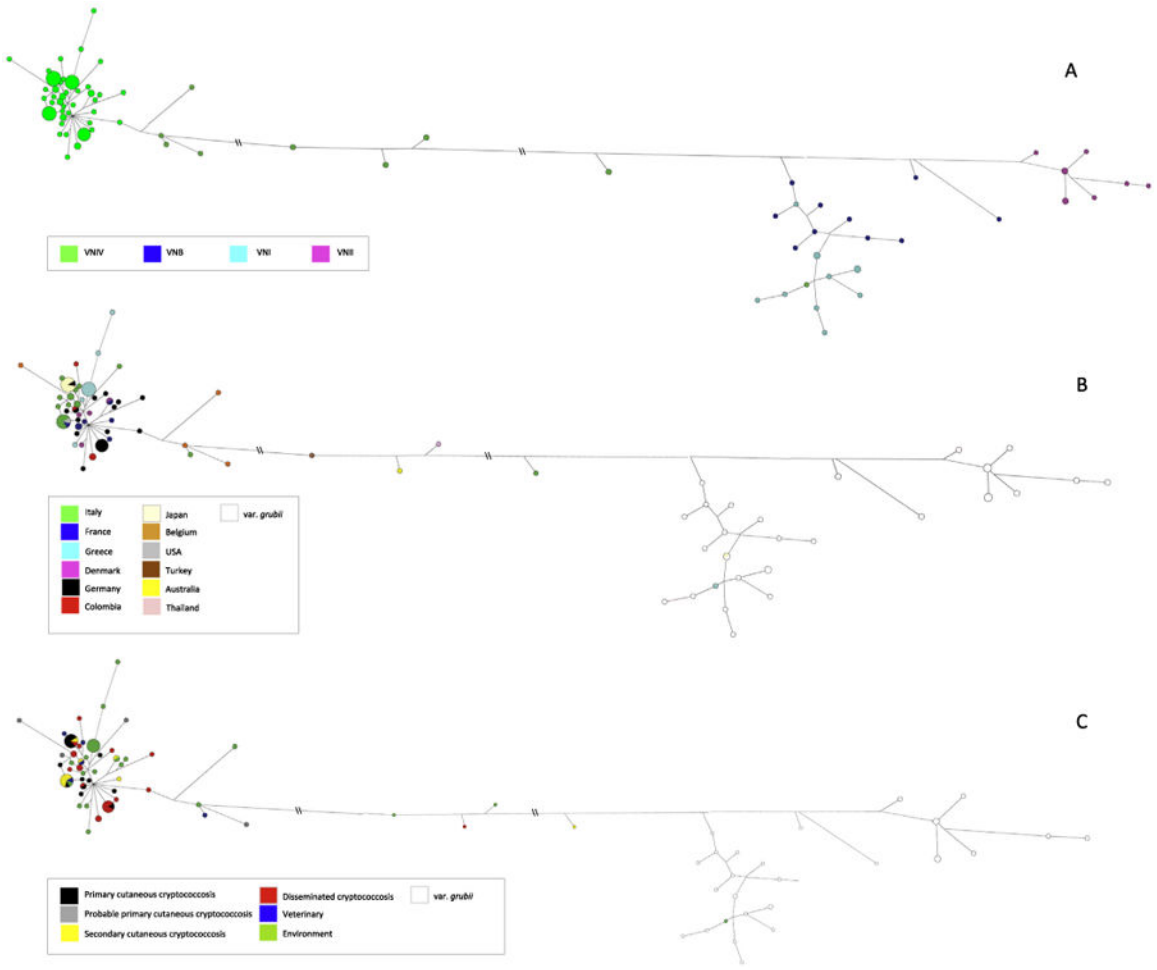

Fig. 2.

Network analysis performed by median joining algorithm. Panel A shows the different genotypes, panel B shows the different geographical origin, and panel C the different source of isolation. Double slash means that the branch has been shortened to fit the image.

Table 1

Clinical and molecular information of the 83 *C. neoformans* var. *neoformans* isolates investigated in the present study.

Strain code	Category	Origin	Source	Date	Underlying disease	Molecular type	Mating type	Sequence type	*CAP59*	*GPD1*	*IGS1*	*LAC1*	*PLB1*	*SOD1*	*URA5*	Reference
WM629	DC	Australia	Blood	1987	AIDS	VNIV	αD	**117**	16	21	30	19	13	1	19	*Meyer*
IUM 01-4729	ENV	Belgium	Pigeon droppings	2001	–	VNIV	αD	**510**	16	21	24	18	13	20	32	*Cogliati*
IUM 01-4730	ENV	Belgium	Dust	2001	–	VNIV	αD	**514**	16	40	24	20	13	20	32	*Cogliati*
IUM 97-4899	PPCC	Belgium	Skin	1997	No risk factors	VNIV	αD	**507**	16	3	26	39	14	20	34	*Cogliati*
IUM 98-5036	PPCC	Belgium	Skin	1998	Diabete	VNIV	αD	**506**	16	3	24	20	13	20	32	*Cogliati*
H0058-I-1406	DC	Colombia	CSF	2002	AIDS	VNIV	αD	**335**	27	28	30	19	14	17	41	*Escandon*
H0058-I-2250	DC	Colombia	CSF	2004	AIDS	VNIV	αD	**336**	16	22	32	14	14	18	17	*Escandon*
H0058-I-2291	DC	Colombia	CSF	2004	AIDS	VNIV	αD	**160**	16	21	30	19	13	17	19	*Escandon*
H0058-I-2880	DC	Colombia	CSF	2007	AIDS	VNIV	αD	**336**	16	22	32	14	14	18	17	*Escandon*
NIH-424	ENV	Denmark	Pigeon nest	1970	–	VNIV	αD	**180**	20	21	26	21	19	17	21	*Kwon-Chung*
NIH-429	ENV	Denmark	Pigeon nest	1970	–	VNIV	αD	**512**	16	21	31	19	13	19	16	*Kwon-Chung*
NIH-430	ENV	Denmark	Pigeon nest	1970	–	VNIV	**a**D	**509**	16	20	24	16	14	20	16	*Kwon-Chung*
NIH-433	ENV	Denmark	Pigeon nest	1970	–	VNIV	**a**D	**515**	17	21	28	13	14	17	16	*Kwon-Chung*
CNRMA00.330	PCC	France	Skin	2000	No risk factor	VNIV	αD	**135**	27	22	43	24	13	17	20	*Dromer*
CNRMA00.840	SCC	France	Skin	2000	Hematological malignancy	VNIV	αD	**125**	16	21	45	21	13	21	22	*Dromer*
CNRMA07.1501	SCC	France	CSF	2007	AIDS	VNIV	αD	**180**	20	21	26	21	19	17	21	*Dromer*
CNRMA97.697	DC	France	CSF	1997	AIDS	VNIV	αD	**121**	16	21	32	19	13	17	20	*Dromer*
CNRMA98.480	DC	France	CSF	1998	AIDS	VNIV	αD	**511**	16	21	29	16	14	17	24	*Dromer*
CNRMA99.1037	PCC	France	Skin	1999	No risk factor	VNIV	αD	**122**	16	21	32	24	13	17	20	*Dromer*
MKT6301	PCC	France	Skin	2011	No risk factor	VNIV	αD	**121**	16	21	32	19	13	17	20	*Bienvenu*
RKI 04-0061	DC	Germany	–	2004	Liver disorder	VNIV	αD	**110**	15	21	24	21	13	20	22	*Rickerts*
RKI 04-0089	PCC	Germany	Skin, hand	2004	Chronical asthma, corticosteroids	VNIV	αD	**486**	16	22	32	19	13	17	22	*Rickerts*
RKI 05-0151	DC	Germany	Blood	2005	Diabetes	VNIV	αD	**531**	16	29	24	20	13	20	18	*Rickerts*
RKI 07-0173	DC	Germany	–	2007	Liver disorder	VNIV	αD	**530**	31	21	83	13	37	59	53	*Rickerts*
RKI 08-0429	DC	Germany	CSF	2008	AIDS	VNIV	αD	**487**	16	21	32	24	13	17	32	*Rickerts*
RKI 08-0572	DC	Germany	–	2008	No risk factors	VNIV	αD	**116**	16	21	29	13	14	17	24	*Rickerts*
RKI 08-0591	DC	Germany	–	2008	No risk factors	VNIV	αD	**116**	16	21	29	13	14	17	24	*Rickerts*
RKI 09-0102	DC	Germany	–	2009	Sarcoidosis	VNIV	αD	**116**	16	21	29	13	14	17	24	*Rickerts*

Strain code	Category	Origin	Source	Date	Underlying disease	Molecular type	Mating type	Sequence type	*CAP59*	*GPD1*	*IGS1*	*LAC1*	*PLB1*	*SOD1*	*URA5*	Reference
RKI 09-0103	DC	Germany	–	2009	Sarcoidosis	VNIV	αD	**116**	16	21	29	13	14	17	24	*Rickerts*
RKI 09-0388	PCC	Germany	Skin	2009	No risk factor	VNIV	αD	**519**	26	22	32	24	13	17	20	*Rickerts*
RKI 09-0393	PCC	Germany	Skin	2009	No risk factor	VNIV	αD	**505**	15	21	31	15	13	19	18	*Rickerts*
RKI 09-0515	DC	Germany	BAL	2009	Solid organ Tx	VNIV	αD	**168**	22	21	30	22	14	17	18	*Rickerts*
RKI 09-0545	DC	Germany	CSF	2009	AIDS	VNIV	αD	**160**	16	21	30	19	13	17	19	*Rickerts*
RKI 11-0047	DC	Germany	CSF	2010	AIDS	VNIV	αD	**116**	16	21	29	13	14	17	24	*Rickerts*
RKI 11-0048	DC	Germany	CSF	2010	AIDS	VNIV	αD	**116**	16	21	29	13	14	17	24	*Rickerts*
RKI 12-0155	PCC	Germany	Skin	2012	No risk factor	VNIV	αD	**116**	16	21	29	13	14	17	24	*Rickerts*
RKI 12-0559	PCC	Germany	Skin	2012	No risk factor	VNIV	αD	**513**	16	21	43	21	13	20	18	*Rickerts*
RKI 13-0490	ENV	Germany	Pigeon droppings	2013	–	VNIV	**a**D	**522**	27	21	53	19	14	19	16	*Rickerts*
RKI 13-0491	ENV	Germany	Pigeon droppings	2013	–	VNIV	**a**D	**523**	29	21	83	21	14	58	53	*Rickerts*
RKI 13-0492	ENV	Germany	Pigeon droppings	2013	–	VNIV	**a**D	**524**	29	21	83	40	14	58	53	*Rickerts*
GRACA18BK1-3	ENV	Greece	Eucalyptus tree	2013	–	VNIV	**a**D	**502**	26	38	30	22	13	19	18	*Velegraki*
GRACP14BK1-1	ENV	Greece	Pine tree	2013	–	VNIV	**a**D	**499**	26	21	30	22	13	19	18	*Velegraki*
GRACP15SO1-1	ENV	Greece	Pine tree	2013	–	VNIV	**a**D	**499**	26	21	30	22	13	19	18	*Velegraki*
GRACP15SO1-2	ENV	Greece	Pine tree	2013	–	VNIV	**a**D	**499**	26	21	30	22	13	19	18	*Velegraki*
GRACP16HO1-1	ENV	Greece	Pine tree	2013	–	VNIV	**a**D	**499**	26	21	30	22	13	19	18	*Velegraki*
GRACP30BK1-1	ENV	Greece	Plane tree	2013	–	VNIV	**a**D	**498**	26	20	30	17	13	19	18	*Velegraki*
GRAKI10SO1-1	ENV	Greece	Olive tree	2013	–	VNIV	**a**D	**499**	26	21	30	22	13	19	18	*Velegraki*
GRAKI11HO1-1	ENV	Greece	Olive tree	2013	–	VNIV	**a**D	**499**	26	21	30	22	13	19	18	*Velegraki*
GRAKI12SO1-1	ENV	Greece	Plane tree	2013	–	VNIV	**a**D	**503**	26	39	30	22	13	19	18	*Velegraki*
GRAKI13HO1-1	ENV	Greece	Plane tree	2013	–	VNIV	**a**D	**503**	26	39	30	22	13	19	18	*Velegraki*
GRAKI28HO1-1	ENV	Greece	Olive tree	2013	–	VNIV	**a**D	**500**	26	22	44	17	14	23	23	*Velegraki*
GRLMM26HO1-2	ENV	Greece	Olive tree	2013	–	VNIV	**a**D	**489**	1	1	1	1	1	27	1	*Velegraki*
GRSAB18HO1-1	ENV	Greece	Pine tree	2013	–	VNIV	**a**D	**501**	26	37	30	38	13	19	18	*Velegraki*
ITMPV22BK7-1	ENV	Italy	Oak tree	2014	–	VNIV	αD	**496**	16	3	30	19	13	17	19	*Cogliati*
IUM 01-0956	SCC	Italy	Skin, head	2001	AIDS	VNIV	αD	**521**	27	13	12	6	9	8	13	*Cogliati*
IUM 02-0826	VET	Italy	Cat	2002	–	VNIV	**a**D	**517**	22	21	30	24	21	17	20	*Cogliati*
IUM 02-4295	PPCC	Italy	Skin	2002	No risk factors	VNIV	**a**D	**516**	22	3	31	24	14	17	20	*Cogliati*
IUM 73-0017	DC	Italy	CSF	1973	No risk factors	VNIV	αD	**508**	16	3	31	24	14	17	16	*Cogliati*
IUM 77-0033	SCC	Italy	Skin, right ear	1977	No risk factors	VNIV	αD	**135**	27	22	43	24	13	17	20	*Cogliati*

Strain code	Category	Origin	Source	Date	Underlying disease	Molecular type	Mating type	Sequence type	*CAP59*	*GPD1*	*IGS1*	*LAC1*	*PLB1*	*SOD1*	*URA5*	Reference
IUM 79-0801	PCC	Italy	Skin, left leg	1979	Common variable immunodeficiency	VNIV	αD	**520**	27	3	43	24	13	17	20	*Cogliati*
IUM 91-2588	SCC	Italy	Skin, hand	1991	AIDS	VNIV	αD	**135**	27	22	43	24	13	17	20	*Cogliati*
IUM 92-0701	DC	Italy	CSF	1992	AIDS	VNIV	αD	**508**	16	3	31	24	14	17	16	*Cogliati*
IUM 93-1543	SCC	Italy	Skin	1993	AIDS	VNIV	αD	**135**	27	22	43	24	13	17	20	*Cogliati*
IUM 93-1656	DC	Italy	CSF	1993	AIDS	VNIV	αD	**279**	22	22	31	22	14	17	34	*Cogliati*
IUM 97-4851	SCC	Italy	Skin	1997	AIDS	VNIV	αD	**135**	27	22	43	24	13	17	20	*Cogliati*
IUM 98-0824	SCC	Italy	Skin, right hand and arm	1998	Solid tumor prostate	VNIV	αD	**135**	27	22	43	24	13	17	20	*Cogliati*
IUM 98-2742	SCC	Italy	Skin, left hand	1998	AIDS	VNIV	αD	**112**	16	22	31	24	14	17	16	*Cogliati*
IUM 98-4987	PPCC	Italy	Skin	1998	Solid tumor breast	VNIV	αD	**518**	26	21	31	15	21	19	15	*Cogliati*
NIH-530	VET	Italy	Cow	1972	–	VNIV	αD	**112**	16	22	31	24	14	17	16	*Kwon-Chung*
PD1596	VET	Italy	Cat	2010	–	VNIV	αD	**252**	22	22	30	19	14	23	41	*Danesi*
PD2270	VET	Italy	Cat	2011	–	VNIV	αD	**135**	27	22	43	24	13	17	20	*Danesi*
PD32	VET	Italy	Cat	2009	–	VNIV	αD	**294**	16	21	24	20	13	22	32	*Danesi*
M9112	PCC	Japan	Skin	1985	No risk factors	VNIV	αD	**168**	22	21	30	22	14	17	18	*Ikeda*
M9117	PCC	Japan	Skin	1988	Severe cellular immunity deficiency	VNIV	αD	**168**	22	21	30	22	14	17	18	*Ikeda*
M9118	PCC	Japan	Skin	1988	No risk factors	VNIV	αD	**168**	22	21	30	22	14	17	18	*Ikeda*
M9119	PCC	Japan	Skin	1988	No risk factors	VNIV	αD	**168**	22	21	30	22	14	17	18	*Ikeda*
M9120	PCC	Japan	Skin	1983	No risk factors	VNIV	αD	**168**	22	21	30	22	14	17	18	*Ikeda*
M9196	PCC	Japan	CSF	1989	Malignant lymphoma	VNIV	αD	**168**	22	21	30	22	14	17	18	*Ikeda*
M9214	PCC	Japan	Skin	1989	No risk factors	VNIV	αD	**168**	22	21	30	22	14	17	18	*Ikeda*
M9215	PCC	Japan	Skin	1993	Systemic lupus erythematosus	VNIV	αD	**168**	22	21	30	22	14	17	18	*Ikeda*
CBS7816	ENV	Thailand	Cuckoo droppings	1997	–	VNIV	αD	**126**	17	21	28	19	14	1	20	*Kaochaoen*
TRNCGB1H01-1	ENV	Turkey	Pine tree	2013	–	VNIV	αD	**497**	16	21	24	20	13	1	32	*Tore O*
NIH-116	ENV	USA, Virginia	Pigeon nest	1960	–	VNIV	αD	**135**	27	22	43	24	13	17	20	*Kwon-Chung*

PCC = primary cutaneous cyrptococcosis; PPCC = probable primary cutaneous cryptococcosis; SCC = secondary cutaneous cryptococccosis; DC = disseminated cryptococcosis; ENV = environment; VET = veterinary; CSF = cerebrospinal fluid; BAL = Bronchoalveolar lavage.

Table 2

Genetic population parameters defining the population structure of the 83 var. *neoformans* isolates investigated.

Locus	Sequence length (bp)	Polymorphic sites	Haplotypes	Haplotype diversity (Hd)	Recombining events	Linkage disequilibrium	Theta (no recombination; free recombination)
CAP59	560	41	9	0.761	4	No	–
GPD1	558	58	5	0.241	1	No	–
IGS1	781	57	11	0.772	1	No	–
LAC1	481	149	15	0.866	20	No	–
PLB1	534	40	7	0.584	0	No	–
SOD1	537	73	11	0.628	2	No	–
URA5	639	55	16	0.865	3	No	–
All loci	4092	425	49	0.965	31	No	87.1 (484.0; 17.4)

Parameters were calculated by DnaSP v.5 software.