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RESEARCH ARTICLE

Environmental distribution of *Cryptococcus neoformans* and *C. gattii* around the Mediterranean basin

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One sentence summary: The present survey established a wide network that, for the first time, collected abundant information concerning the environmental distribution and ecology of *Cryptococcus neoformans*/*C. gattii* species complex in the Mediterranean area.

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ABSTRACT

In order to elucidate the distribution of *Cryptococcus neoformans* and *C. gattii* in the Mediterranean basin, an extensive environmental survey was carried out during 2012–2015. A total of 302 sites located in 12 countries were sampled, 6436 samples from 3765 trees were collected and 5% of trees were found to be colonized by cryptococcal yeasts. *Cryptococcus neoformans* was isolated from 177 trees and *C. gattii* from 13. *Cryptococcus neoformans* colonized 27% of *Ceratonia*, 10% of *Olea*, *Platanus* and *Prunus* trees and a lower percentage of other tree genera. The 13 *C. gattii* isolates were collected from five *Eucalyptus*, four *Ceratonia*, two *Pinus* and two *Olea* trees. *Cryptococcus neoformans* was distributed all around the Mediterranean basin, whereas *C. gattii* was isolated in Greece, Southern Italy and Spain, in agreement with previous findings from both clinical and environmental sources. Among *C. neoformans* isolates, VNI was the prevalent molecular type but VNII, VNIV and VNIII hybrid strains were also isolated. With the exception of a single VGIV isolate, all *C. gattii* isolates were VGI. The results confirmed the presence of both *Cryptococcus* species in the Mediterranean environment, and showed that both carob and olive trees represent an important niche for these yeasts.

Keywords: *Cryptococcus*; *C. neoformans*; *C. gattii*; environment; Europe; epidemiology; molecular typing

INTRODUCTION

Cryptococcosis is a life-threatening fungal infection caused by the basidiomycetous yeasts in the *Cryptococcus neoformans* and *C. gattii* species complex. The infection is likely acquired from the environment by inhalation of spores or dehydrated yeast cells that are able to penetrate the pulmonary alveoli and then disseminate through the bloodstream causing soft tissue infections, pneumonia and most often meningoencephalitis (Kwon-Chung *et al.* 2014).

Cryptococcosis caused by *C. neoformans* is a major cause of mortality in patients with AIDS. An estimated one million cases of cryptococcal meningitis occur annually among people with HIV infection worldwide, resulting in nearly 625 000 deaths (Park

et al. 2009). Since the introduction of antiretroviral therapy, the cases of cryptococcosis and the number of deaths in people with advanced HIV infection have decreased substantially in developed countries. While cryptococcosis cases in HIV-infected patients have been decreasing, an increase in the number of cases has been reported in non-HIV patients due to the rising number of susceptible patients such as patients with hematological malignancies, organ transplant recipients and patients affected by autoimmune diseases, but also in patients without any other risk factor except that they were exposed to the pathogen (Bratton *et al.* 2012; Henao-Martínez and Beckham 2015).

In Europe, the epidemiology of cryptococcosis is difficult to establish for two reasons: there are only a few outdated reports on epidemiology of cryptococcosis from a limited number of countries and the lack of coordination to collect epidemiological data among scientists from EU countries. The epidemiological data thus far available on cryptococcosis are restricted to Croatia, France, Germany, Italy, Serbia, Spain, Portugal, the Netherlands and the United Kingdom (Baró *et al.* 1999; FIMUA Network 2002; Dromer *et al.* 2004; Mlinaric-Missoni *et al.* 2011; Hagen *et al.* 2012b; Patel *et al.* 2013; Arsic Arsenijevic *et al.* 2014; Bitar *et al.* 2014; Sanchini *et al.* 2014; Maduro *et al.* 2015). Data from the rest of the EU are either scarce or completely lacking, especially from Central and Eastern European countries where a higher incidence of cryptococcosis is expected due to a heavier burden of HIV infection compared to Western Europe (de Colombani *et al.* 2004).

A unique attempt for a prospective European survey was performed during a survey from 1997 to 1999 in which 655 cases from 17 countries were reported and 311 cryptococcal isolates were collected for molecular typing (Viviani *et al.* 2006). Although the survey represented a milestone in the elucidation of the European epidemiology of cryptococcosis, the results underestimated the burden of the disease since many countries did not participate in the study. At the national level, a recent study carried out in France reported 1850 cases of cryptococcosis from 2001 to 2010 and an incidence of 0.3 per 100 000 population/year with a fatality rate of 15% (Bitar *et al.* 2014), while 129 cases were recorded in a study carried out in Germany from 2004 to 2010 (Sanchini *et al.* 2014).

Because of its geographical location, Europe is also subjected to extensive immigration of people from both Asia and Africa where cryptococcosis represents the third highest cause of death among HIV-infected patients (Park *et al.* 2009; Assogba *et al.* 2015). This inevitably favors the spread of new genotypes in Europe through the introduction of the pathogen via vehicles, clothing and goods potentially contaminated as also been shown in the Vancouver outbreak caused by *C. gattii* (Kidd *et al.* 2007). Furthermore, the high flow of people to and from Europe for business and tourism allows the emergence of cryptococcosis cases acquired in endemic areas (Dromer, Ronin and Dupont 1992; Hagen *et al.* 2012a). The recent cryptococcosis outbreaks occurring on Vancouver Island (Canada), and the Pacific Northwest of North America showed how this fungal threat could spread rapidly in the environment once it has found a favorable niche (Byrnes and Marr 2011; Bartlett *et al.* 2012; Hagen *et al.* 2012a, 2013). The Centers for Disease Control and Prevention in the USA worked with local public health authorities to implement a plan to monitor the epidemiology of *C. gattii* in the states of Washington and Oregon where the reporting of this fungal disease is now mandatory. The coordination of such actions is a lengthy process in Europe and needs to be improved for early documentation of outbreaks as have been reported due to *C. gattii*.

Few studies to assess the occurrence of the *C. neoformans*/*C. gattii* species complex in the environment have been performed in Europe. *Cryptococcus neoformans* was mainly reported to be associated with bird excreta (Colom Valiente *et al.* 1997; Garcia-Hermoso *et al.* 1997; Pernice *et al.* 1998; Boekhout *et al.* 2001; Montagna *et al.* 2003; Lagrou *et al.* 2005; Cafarchia *et al.* 2006), and only few isolates were recovered from arboreal sources (Criseo *et al.* 1995; Criseo and Gallo 1997; Lo Passo *et al.* 1997; Campisi *et al.* 2003; Bauwens *et al.* 2004, Chowdhary *et al.* 2012, Colom *et al.* 2012). *Cryptococcus gattii* was recovered for the first time from the European environment in Southern Italy (Montagna *et al.* 1997; Romeo, Scordino and Criseo 2011), and then also in the Netherlands (Chowdhary *et al.* 2012) and Spain (Colom *et al.* 2012). However, these surveys were limited to a restricted territory and carried out at different periods, as such the results are geographically and temporally fragmented.

This study represents the first collaborative effort aimed to understand the environmental distribution of *C. neoformans* and *C. gattii* on trees around the Mediterranean basin and in continental Europe. In addition, isolates and associated metadata collected during the survey represent an important source for the comparison and correlation of European and global clinical data.

MATERIALS AND METHODS

Network and study design

The ISHAM Working Group for Genotyping of *C. neoformans* and *C. gattii* (http://www.isham.org/WorkingGroups/Genotyping_neoformans_gattii) established a network to survey the distribution of *C. neoformans* and *C. gattii* in the environment focusing on sampling around the Mediterranean basin. Thirty-two centers from nine European countries and three non-European countries (Israel, Libya and Turkey) participated in the study. Each participating center was required to collect samples from trees and soil especially in urban area where the finding of the pathogens could represent a menace for humans, and to record a defined set of metadata, including sampling site and date, type of sample, tree species, daily mean temperature, number of collected samples and number of positive samples. Sample collection and cultivation were performed in each participating center according to predefined methods described below. Both the metadata and the isolates were sent to the coordinating center of the study at the Medical Mycology Laboratory, Università degli Studi di Milano (Italy). All isolates were coded and stocked, and then processed for molecular analyses. Three additional non-European centers, one from Australia and two from the USA, joined the study and participated in the molecular strain typing effort.

Environmental samples

Samples were collected in the geographical areas where the different participating centers were located mainly from the widest public gardens in urban areas but also from some rural areas. In each site, a minimum of 10 and a maximum of 100 samples were collected depending of the extension of the sampled area and the density of the trees using a non-random sampling methodology.

The sources of samples were hollows and fissures of trees, flowers, leaves, bark, fruits, decaying wood, soil underneath trees and bird excreta on or near the tree. All the sampled trees were identified as far as possible to the species level.

Hollows and fissures on the tree trunk

Samples were collected by rubbing the inner of the hollows or fissures of the trees with a sterile cotton-tipped swab moistened in a solution of sterile distilled water supplemented with chloramphenicol (10 mg L^{-1}). The swab was placed into a tube with 3 mL of the solution and the tube was shaken for 5 min without removing the swab. The swab was removed and the suspension was left to sediment at least for 10 min. A total of 100 μl of the supernatant and 100 μl of the diluted supernatant (1:10 in sterile distilled water) were inoculated onto two different Niger seed agar plates (Kwon-Chung and Bennett 1992). The plates were incubated at 37°C for at least 10 days. All brown colored colonies grown on the plates were isolated for further species identification.

Flowers and leaves

About 10–20 g of the sample were collected and sealed in zip-lock bags. A portion of the sample (5 g) was transferred in a sterile mortar and fragmented with a pestle. The fragments were suspended in 50 mL sterile distilled water and vortexed for about 2 min at maximum speed. Sediment was allowed to settle for 15–20 min. A total of 2 mL of the supernatant was mixed with 8 mL of sterile distilled water containing chloramphenicol (10 mg L^{-1}). A total of 100 μl of the supernatant and 100 μl of the diluted supernatant were inoculated in two different Niger seed agar plates. The plates were incubated at 37°C for at least 10 days. All brown colonies grown on the plates were collected for identification.

Bark and decaying wood

Samples were obtained by scraping the surface of the wood with a scalpel. The obtained shavings were sealed in a zip-lock bag. Following vigorous grinding with a mortar, 1 g of the sample was suspended in 50 mL of sterile distilled water containing chloramphenicol at 10 mg L^{-1}, shaken for 2 min and allowed to settle for 30 min. A total of 100 μl of the supernatant and 100 μl of the diluted supernatant (1:10 in sterile distilled water) were inoculated onto two different Niger seed agar plates. The plates were incubated at 37°C for at least 10 days. All brown colonies grown on the plates were collected for identification.

Soil

Approximately 10–20 g of soil was collected and sealed in a zip-lock bag. Part of the soil (5 g) was suspended in 50 mL sterile distilled water and mixed by vortexing for about 2 min at maximum speed. Sediment was allowed to settle for at least 15 min. A total of 2 mL of supernatant was mixed with 8 mL of sterile distilled water containing chloramphenicol (10 mg L^{-1}). A total of 100 μl of the supernatant and 100 μl of the diluted supernatant were inoculated on two different Niger seed agar plates. The plates were incubated at 37°C for at least 10 days. All brown colonies grown on the plates were collected for identification.

Isolation, species identification, coding and storage

Brown colonies were streaked for isolation on a fresh Niger seed agar plate in order to collect pure single colonies. Isolates were then examined by microscopy for assessing the yeast morphology and capsule presence, tested for urease activity and the ability to grow at 37°C and assimilate *myo*-inositol as a carbon source.

The cryptococcal species was identified by inoculating a fresh colony onto canavanine-glycine-bromothymol blue agar differential medium (Kwon-Chung and Bennett 1992).

A code identifying the country, the place of origin, the type of sample and the tree code number was assigned to each isolate, which were then suspended and stored in a vial containing 3 mL of sterile distilled water at room temperature.

Molecular analyses

Genomic DNA was extracted as previously reported (Viviani *et al.* 1997). Molecular type and mating type of all isolates was determined by four multiplex PCRs specific for both *C. neoformans* and *C. gattii* as described elsewhere (Cogliati *et al.* 2000; Esposto *et al.* 2004; Feng *et al.* 2013; Cogliati, D'Amicis and Tortorano 2015). Molecular types were assigned according to the standard nomenclature of the ISHAM working group for genotyping of *C. neoformans* and *C. gattii* (Meyer *et al.* 2009). Strains H99 (VNI-αA), JEC20 (VNIV-**a**D), JEC21 (VNIV-αD), IUM 96–2828 (VNII-**a**A), WM 626 (VNII-αA), WM779 (VGIV-αC), NIH312 (VGIII-αB), NIH191 (VGIII-**a**C), WM201 (VGI-αB) and IUM 00–5363 (VGII-**a**B) were used as reference strains.

Mating assay

A mating assay was performed to test the fertility of selected environmental isolates from the survey. The isolates were streaked onto a 90-mm plate containing 20 ml Murashige-Skoog agar medium (0.44% Murashige-Skoog basal medium; Sigma-Aldrich, St. Louis, MO, USA; and 4% agar in distilled water) and then mixed with a tester strain of the opposite mating type. Co-cultures were incubated at 25°C in the dark for at least 3–4 weeks and checked periodically for the formation of hyphae and basidiospores. Basidiospores were collected by cutting a square of the agar on which hyphae were produced (avoiding to touch the yeast colony edge), and transferring it to a tube containing 2 mL sterile distilled water. After gently stirring the tube, the supernatant was transferred to a new tube and checked microscopically for the presence of basidiospores and the absence of yeast cells and hyphae. A 100 μl volume of the spore suspensions were plated on Sabouraud dextrose agar and incubated at 37°C for 48 h. Ten single colonies grown on the plate were collected and processed for molecular typing. A nearly 1:1 ratio of MAT**a** and MATα spores confirmed the successful mating between the two tested strains. The strain pairs JEC20 (VNIV-**a**D) and JEC21 (VNIV-αD), and H99 (VNI-αA) and IUM 96–2828 (VNII-**a**A) were used as tester strains.

Taxonomy

Although a new taxonomic classification of *C. neoformans*/*C. gattii* species complex has recently been proposed (Hagen *et al.* 2015), it is still under discussion. Therefore, in this study we continue to adopt the classical taxonomy which classifies the agents of cryptococcosis into two species, *C. neoformans* and *C. gattii*, and *C. neoformans* into two varieties, *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*.

Statistics

Differences between the percentages of colonized trees observed in different tree populations, months of isolation

and mean daily temperature ranges were statistically analyzed by χ^2 test using the online statistical calculator at www.vassarstats.net. The χ^2 test was also applied to compare the results obtained in different sampling areas in order to assess whether the results obtained in a specific area could have influenced the overall results.

RESULTS

Sampling distribution

The survey was performed during 2012–2015. A total of 302 sites in 12 countries were sampled, primarily in Italy ($n = 152$), followed by Spain ($n = 47$), France ($n = 27$), Turkey ($n = 19$), Croatia ($n = 18$), Portugal ($n = 11$), Germany ($n = 10$), Greece ($n = 7$), Cyprus ($n = 6$), Libya ($n = 3$) and Israel plus the Netherlands with one site each (Fig. 1).

Trees

Samples were collected from 3765 trees representing more than 100 different genera (Table S1, Supporting Information). Most of the trees were *Eucalyptus* (37%), *Olea* (olive tree, 14%), *Pinus* (13%), *Quercus* (oak, 5%), *Ceratonia* (carob tree, 3%), *Prunus* (2%) and *Platanus* (plane tree, 2%). The highest percentage of sampled trees was in Italy (43%), followed by Turkey (12%), Cyprus (10%), Libya (9%), Spain (6.5%) and Greece (6%). A total of 188 trees (5%) were colonized by *C. neoformans* or *C. gattii*. Colonized trees were found in Cyprus, France, Greece, Italy, Libya, Portugal, Spain and Turkey, with the highest percentage of positive trees recorded in Spain and Greece (16.7% and 16.8%, respectively). The percentage of colonized trees relative to each tree genus was the following: *Ceratonia* ($n = 130$, 27.7%), *Platanus* ($n = 79$, 10.1%), *Prunus* ($n = 96$, 9.4%), *Olea* ($n = 536$, 9.3%), *Pinus* ($n = 515$, 4.7%), *Eucalyptus* ($n = 1392$, 3.7%) and *Quercus* ($n = 179$, 1.1%). Sporadic positive samples were also found on *Aesculus hippocastanum*, *Carpinus betulus*, *Juglans nigra*, *Juniperus* spp., *Gleditsia triacanthos* and *Pyrus communis*.

Samples

A total of 6436 samples were collected from trunk hollows (62%), bark (11.5%), leaves (8%), flowers (1.3%), soil under trees (16%) and other samples (1.2%) that included fruits, decaying wood, debris near trees or bird excreta on trees. The majority of the samples, 44.4% and 9.6%, were collected in Italy and Greece, respectively. A total of 220 samples (3.4%) were positive. Colony-forming units per sample were variable and ranged from 1 to 100. *Cryptococcus neoformans* or *C. gattii* was recovered from cultures of trunk hollow swabs, bark scrapings, soil and decaying

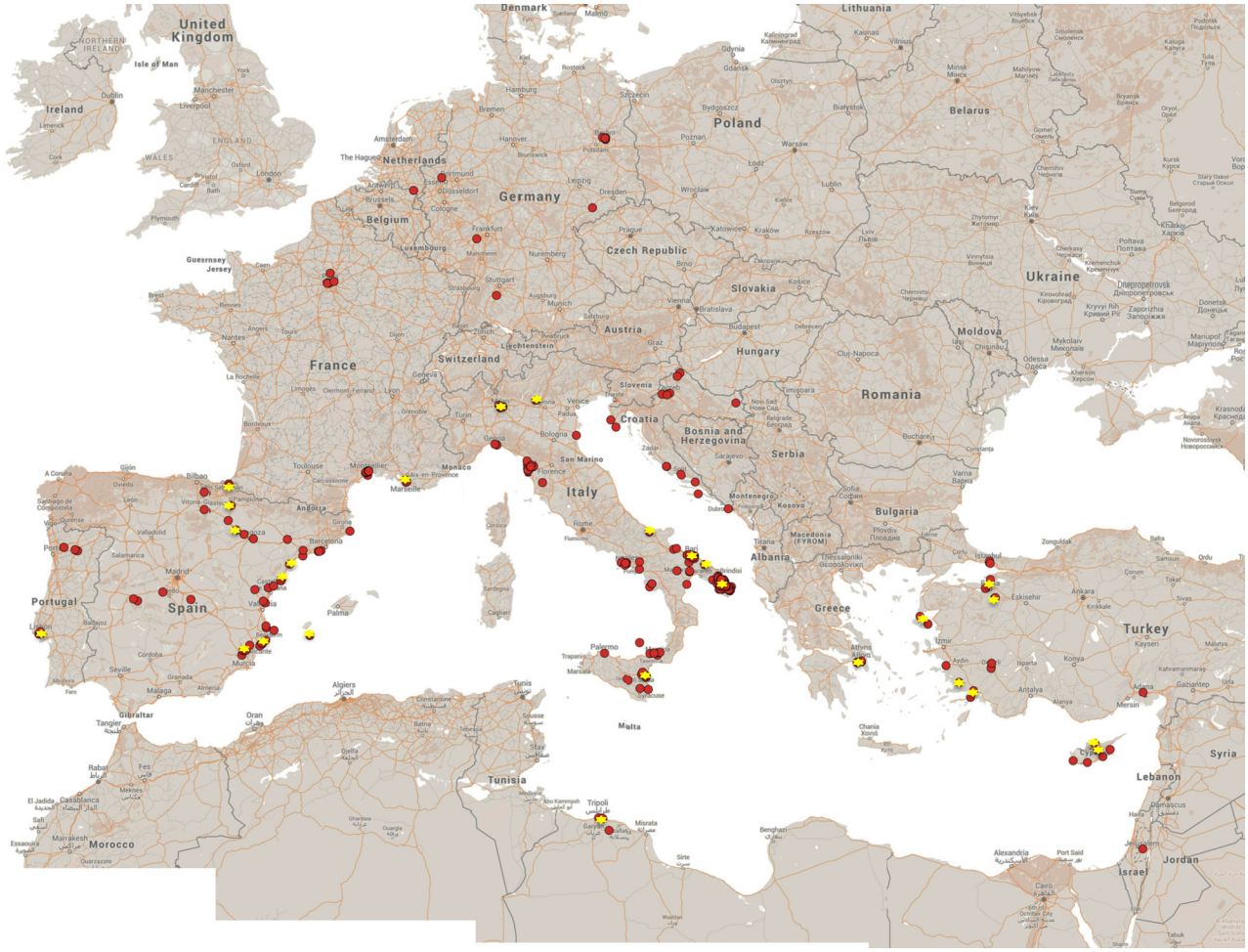

Figure 1. A map of the Mediterranean basin showing the sampling sites (red dots). Stars indicate the positive sites. The coordinates for each site were recorded and then plotted on the map using GoogleMaps (www.google.com).

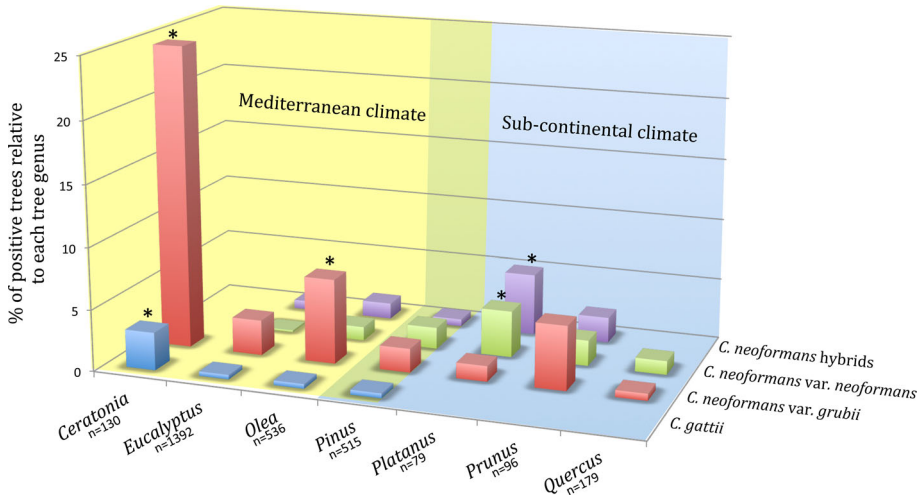

Figure 2. Percentage of colonized trees per tree genera. In the histogram, only the tree genera that are represented at least by 2% of the total sampled trees ($n = 1765$) have been included. The trees of genus *Pinus* can adapt to different climate and in the graphic are located in an overlapping zone. Asterisk indicates that the value is statistically higher ($P < 0.05$) compared to the values observed in the other tree populations. n = Number of sampled trees for each tree genus.

wood, whereas no isolates were recovered from leaves and flowers. Trunk hollows had a percentage positivity of 4.1% ($n = 3986$), bark of 3% ($n = 738$) and soil of 2.4% ($n = 1039$). In addition, nine samples from debris of decaying wood were positive.

Distribution of *C. neoformans* and *C. gattii* isolates

During the survey, 512 isolates from 188 trees were recovered representing 474 *C. neoformans* and 38 *C. gattii* isolates. The percentage of colonized trees relative to the two species was 4.6% for *C. neoformans* and 0.4% for *C. gattii* with a ratio of 13.5:1, respectively ($n = 3765$). *Cryptococcus neoformans* was isolated from 177 trees belonging mainly to the genera *Eucalyptus*, *Olea* (olive trees), *Ceratonia* (carob trees) and *Pinus*, but also from *Aesculus*, *Carpinus*, *Juglans*, *Juniperus*, *Gleditsia*, *Platanus*, *Prunus*, *Pyrus* and *Quercus* (Fig. 2). The trees colonized by *C. neoformans* isolates were found in Cyprus, France, Greece, Italy, Libya, Portugal, Spain and Turkey. *Cryptococcus gattii* was isolated from 13 trees belonging to four different genera: five trees of *Eucalyptus*, four trees of *Ceratonia* (carob trees), two trees of *Olea* (olive trees) and two trees of *Pinus pinea* (Fig. 2). The trees were distributed in Spain (Alicante, Tarragona and Mendivil in Navarra), Italy (Bari and Catania) and Greece (Athens and Salamina Island). One carob tree in Spain and one olive tree in Italy were colonized by both *C. neoformans* and *C. gattii*.

A comparison of the percentage of positive trees for each tree genus in the different sampling areas was also performed in order to exclude the influence of the results obtained in a specific area on the overall analysis. No statistical difference was observed ($P > 0.05$) between the percentage of positive trees in the different sampling areas confirming that the differences observed are likely associated with the tree species considered.

Prevalence and distribution of molecular types

The map in Fig. 3 shows the distribution of molecular types found around the Mediterranean basin. The prevalent molecular type was VNI with 330 isolates from 129 trees distributed in all seven countries that yielded positive samples. Molecular type VNII was only isolated from two trees in Tripoli, Libya, whereas 107 VNIV isolates were recovered from 27 trees located in Greece, Italy, Spain and Turkey. In addition, 35 AD-hybrids (molecular type VNIII) were isolated from 26 trees in Greece, Libya and Turkey. VGI was the prevalent molecular type for *C. gattii* with 37 isolates obtained from 12 trees. One isolate from a Spanish *Ceratonia siliqua* tree was identified as VGIV. Trees colonized by two molecular types, VNI and VGI (two trees), or VNI and VNIV (four trees), or VNI and VNIII (three trees), were also found.

Prevalence and distribution of mating types

Both mating type **a** and mating type α were found among *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans* and *C. gattii* isolates collected during the survey. The mating type allelic pattern αA was the prevalent mating type (327 isolates) among *C. neoformans* var. *grubii*, whereas the mating type **a**A was identified in seven isolates, six from Spain and one from Italy. *Cryptococcus neoformans* var. *neoformans* αD mating type was recovered in Italy (71 isolates), Spain (one isolate) and Turkey (6 isolates), whereas mating type **a**D was found only in Greece (29 isolates). The 35 AD-hybrid isolates presented two different mating-type allelic patterns, three were heterozygous αAD**a** and 32 were homozygous αAAα. One olive tree in Italy and four carob trees in Spain were colonized by both αA and **a**A isolates, whereas two *Eucalyptus* trees in Greece were colonized by both αA and **a**D isolates. Coexistence of αA and αD as well as αA and AD-hybrids was observed in Italy (two trees) and Turkey (three trees), respectively.

Regarding *C. gattii*, ten trees were colonized by mating type αB strains, two trees in Italy by mating type **a**B and one in Spain by mating type αC. One of the two Italian **a**B isolates shared the same olive tree with *C. neoformans* var. *grubii* **a**A and αA strains. Similarly, in Spain one *C. gattii* αB strain coexisted with one *C. neoformans* var. *grubii* αA strain in a carob tree.

Results of mating assays

Seven mating assays were performed to test the fertility of the isolates that originated from the trees where two opposite mating type isolates shared the same niche. Five assays were intra-variety mating assays between **a**A and αA isolates, whereas the other two were inter-variety assays between αA and **a**D isolates.

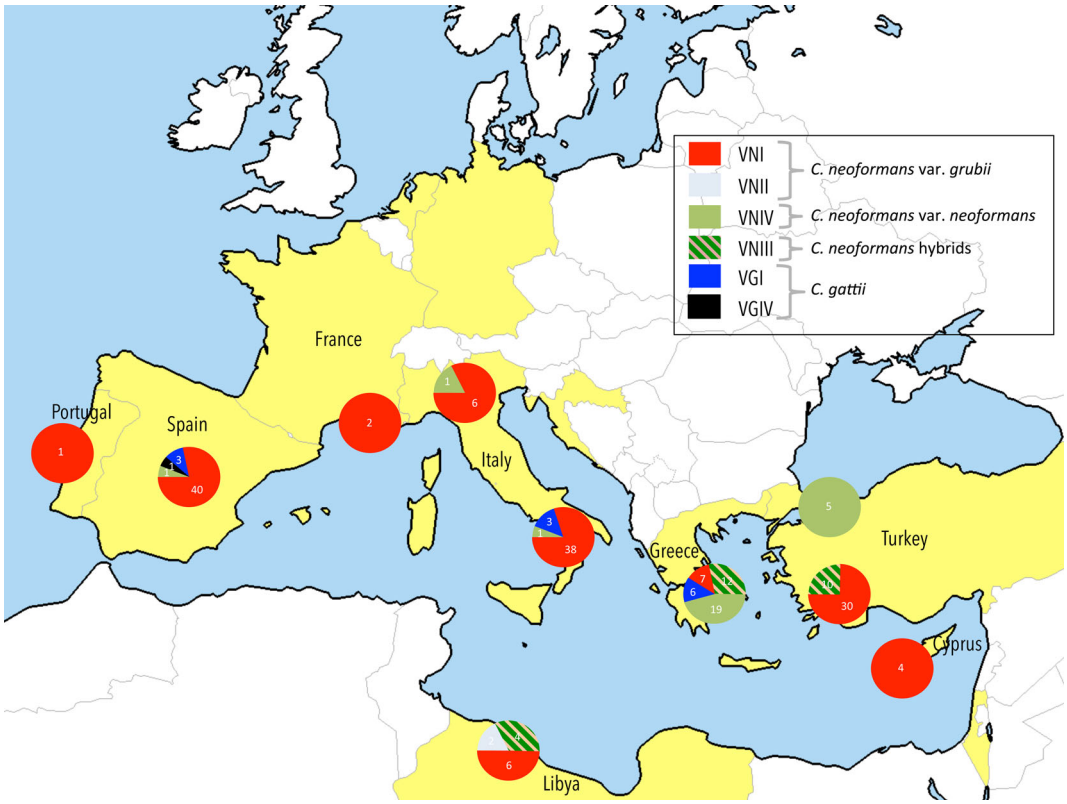

Figure 3. Distribution and prevalence of *C. neoformans* and *C. gattii* molecular types around the Mediterranean basin. Numbers inside the circles indicate the numbers of positive trees. Participating countries are presented in yellow.

All intra-variety assays produced filaments and basidiospores. In addition, molecular analysis showed that the progeny included both **a**A and αA mating types in a Mendelian ratio (Fig. 4). In contrast, none of the inter-varietal matings was fertile after 4 weeks of observation.

Seasonality and daily mean temperature

Data of seasonality are presented in Fig. 5. Samples were collected monthly, year round, by each of the collaborating groups. The highest number of samples was recorded in July (19.9%) followed by September (19.4%) and October (14.4%). The lowest number of samples was recorded in February (0.8%) and January (1.6%). The percentage of trees colonized by *C. neoformans* showed a significant increase ($P < 0.05$) in March (22%), April (6.5%), September (9.2%) and December (8.1%) when compared to the overall percentage of colonization (4.6%) observed for this species. In the other months, the value was lower with a minimum recorded in February (no positive trees) and August (1.1%, $P < 0.05$). *Cryptococcus gattii* colonization of trees was higher in March (1.7%, $P < 0.05$), April (3.3%, $P < 0.05$) and May (0.8%, $P > 0.05$) than that observed in overall tree population (0.4%). No *C. gattii* positive trees were found in January, February, August, October and December.

Samples were collected within a range of daily mean temperatures of 7°C–32°C. Depending on the mean temperature of the sampling day, the data relative to each sampling were placed in one of five temperature ranges: <10°C, 11°C–15°C, 16°C–20°C, 21°C–26°C and >26°C. The percentage of colonized trees relative to each temperature range was calculated for both the *Cryptococcus* species and displayed in a histogram (Fig. 6). The results showed that, although *C. neoformans* was recovered in all temperature ranges with no statistically difference ($P > 0.05$), *C. gattii* was recovered only when the mean daily temperature was above 10°C.

The analyses were repeated for the different sampling areas and, after comparison, the results were not significantly different ($P > 0.05$).

DISCUSSION

This study is the first European collaborative prospective environmental survey investigating the distribution of the *C. neoformans*/*C. gattii* species complex. Although the survey was not exhaustive, it covered a wide territory of Europe including a large part of the Mediterranean coast as well as some continental areas. This first environmental survey generated an immense quantity of data, which are now available for detailed follow-up analysis in future studies.

The results showed that both *C. neoformans* and *C. gattii* are present in the Mediterranean environment in association with trees. *Cryptococcus neoformans* is more prevalent than *C. gattii* with a 13-fold higher percentage of colonized trees. Our findings suggest that trees could represent an important environmental niche and a stable reservoir for both species, and that bird excreta could represent a secondary and temporary niche especially for *C. neoformans*. The association of trees with *Cryptococcus* is also implicated by the findings of the yeasts as infectious or colonizing agents in animals reported from other parts of the world, such as koalas, squirrels, monkeys and parrots whose lives revolve around trees. Goats have the habit of eating tree barks and they also develop cryptococcosis (Roussilhon, Postal

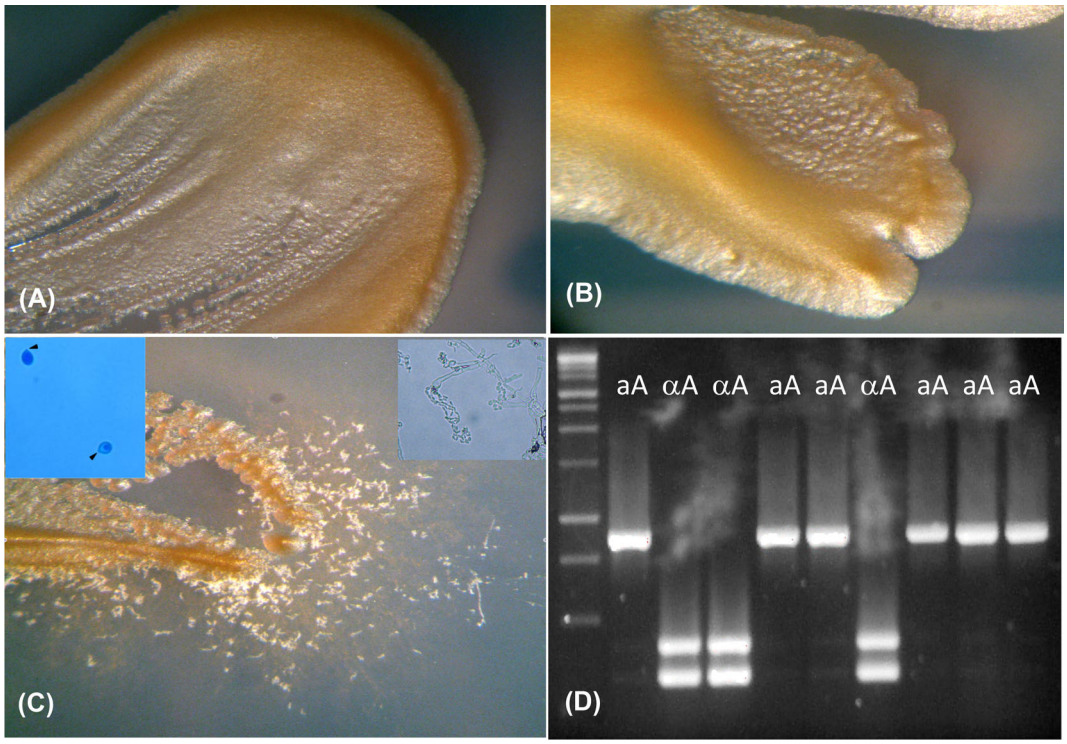

Figure 4. Results of one representative mating assay performed on Murashige Skoog agar medium. One VNI-αA isolate and one VNI-**a**A isolate which shared the same niche in the same tree was used for crossing. (**A**) VNI-αA culture. (**B**) VNI-**a**A culture. (**C**) Filament production in the mixed culture (VNI-αA × VNI-**a**A). The upper-right inset shows a basidium with long chains of basidiospores. The upper-left inset shows basidiospores collected in water suspension. Arrows indicate the stalk-like ends of the basidiospores. (**D**) Multiplex PCR to determine the mating type allelic pattern of the progeny. Each lane represents DNA from a single basidiospore culture. Molecular Ladder: 100 bp DNA ladder (Promega Italia, Milano, Italy).

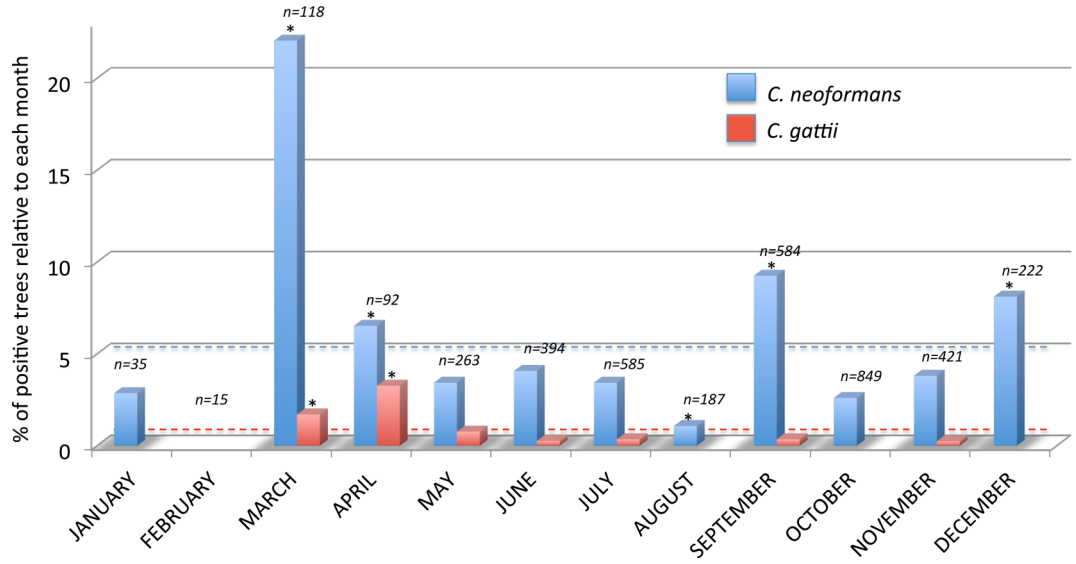

Figure 5. Percentage of trees colonized by *C. neoformans* and *C. gattii* recorded in each month of the year. The blue and red dashed lines represent the overall percentage of colonization observed for *C. neoformans* and *C. gattii*, respectively. Asterisk indicates that the value is statistically different from the mean ($P < 0.05$) compared to the values observed in the other months. n = Number of sampled trees for each month.

and Ravisse 1987; López-Martínez and Castañón-Olivares 1995; Torres-Rodríguez *et al.* 1999; Krockenberger, Canfield and Malik 2003; Stilwell and Pissarra 2014; Iatta *et al.* 2015; Maestrale *et al.* 2015).

This study reveals a difference in the association of the two *Cryptococcus* species with specific trees. In particular, *Ceratonia* (carob tree) and *Olea* (olive tree), two tree genera typical for the Mediterranean region, together with *Eucalyptus* trees produced the highest number of positive samples. However, when the values were normalized as a percentage of the positive tree genus and stratified for each of the *Cryptococcus* species and varieties, the results showed that *Ceratonia* is an important niche for both

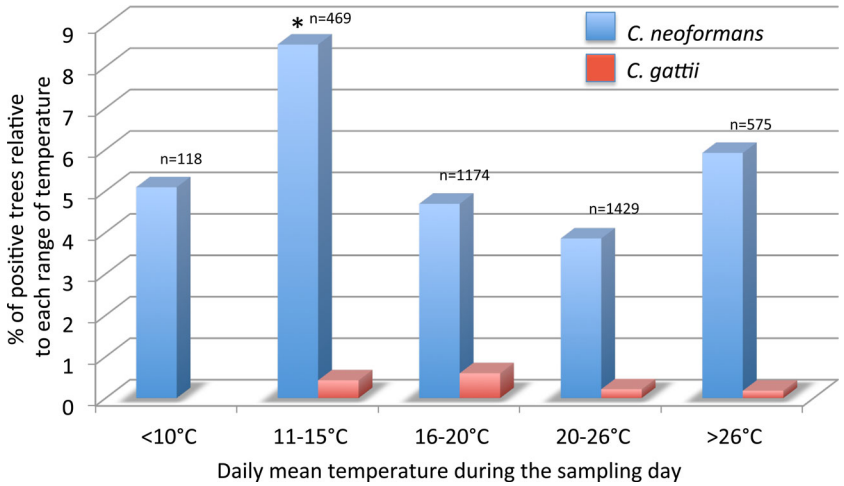

Figure 6. Percentage of trees colonized by *C. neoformans* and *C. gattii* in relation to the daily average temperature during the sampling days. Asterisk indicates that the value is statistically higher ($P < 0.05$) compared to the values observed in the other groups. n = Number of sampled trees for each range of temperatures.

C. gattii and *C. neoformans* var. *grubii*, but not for *C. neoformans* var. *neoformans* and AD-hybrids, since the latter were not recovered from these trees. In addition, *C. gattii* was recovered only from trees typical for the Mediterranean climate (*Ceratonia*, *Olea*, *Eucalyptus* and *Pinus pinea*), whereas *C. neoformans* var. *grubii* colonized 12 different tree genera confirming the ability of this pathogen to adapt to different environments and, hence, contributing to its global distribution. The importance of *Ceratonia siliqua* as a niche for *C. gattii*, shown in the present survey, is in agreement with the data reported by previous environmental studies carried out in Spain (Colom *et al.* 2012; Linares *et al.* 2015). In contrast, *C. neoformans* var. *neoformans* and AD-hybrids showed a preference to colonize trees typical of the subcontinental climate, such as *Platanus*, *Prunus* and *Quercus*. This could reflect the ability of *C. neoformans* var. *neoformans* to tolerate lower temperature better than *C. neoformans* var. *grubii* and *C. gattii* as previously shown by other authors (Martinez, Garcia-Rivera and Casadevall 2001). However, the survey has the limit that the samples were not collected with a rigorous randomized method and therefore the conclusions here reported must be considered as an attempt to describe the observed data and a starting point for future more extensive studies.

Although the two *C. neoformans* varieties and *C. gattii* have differences in tree preference, the climatic zones do not have sharp boundaries and they overlap along the entire Mediterranean basin. Therefore, in the Mediterranean environment, the different *C. neoformans* and *C. gattii* populations are continuously in contact with each other. This is confirmed by the finding that isolates belonging to different species or varieties shared a niche of the same tree, as well as by the presence of hybrids in the same area.

Hybridization between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* is well documented in Europe by the identification of numerous AD-hybrids in clinical isolates which have a prevalence of about 30% (Viviani *et al.* 2006). In contrast, only a few hybrids have been isolated from pigeon droppings (Baró *et al.* 1999; Ferreira *et al.* 2014) due to the paucity of environmental studies carried out in Europe. This study reports for the first time the association of AD-hybrids with trees and their presence in the same area where the putative parental *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* isolates may coexist. This finding suggests that hybridization between the two *C. neoformans* varieties is occurring in the European environment and this may play an important role as a mechanism of evolution of this species (Cogliati, Lin and Viviani 2009; Li *et al.* 2012; Desnos-Ollivier *et al.* 2015). Although the inter-varietal mating assays carried out in this study did not succeed, this does not mean that the isolates studied are not compatible but probably means that the *in vitro* assay conditions here adopted were not optimal for hybridization. The high variability of conditions and substrates encountered in the environment may better favor this process.

The presence of *C. gattii* in Greece, Southern Italy and Spain confirms the previous results obtained in these geographical areas where the pathogen was isolated from both clinical and environmental sources (Montagna *et al.* 1997; Torres-Rodríguez *et al.* 1999; Velegraki *et al.* 2001; Colom *et al.* 2005; Viviani *et al.* 2006; Solla *et al.* 2008; Ropstad *et al.* 2011; Colom *et al.* 2012; Iatta *et al.* 2012; Romeo *et al.* 2012; Hagen *et al.* 2012a). The survey also confirmed that VGI is the prevalent *C. gattii* molecular type, which is in agreement with a previous analysis carried out by Hagen *et al.* (2012a) that identified an endemic VGI cluster in Mediterranean Europe. However, one isolate was identified as VGIV-αC (Linares *et al.* 2015) suggesting that other molecular types are also able to colonize the Mediterranean basin. Further molecular analysis of the isolates collected during this survey by MLST will elucidate the relationships and the clusters present in the European environment.

Cryptococcus neoformans VNI was the prevalent molecular type distributed all around the Mediterranean basin from Portugal to Libya confirming the ubiquitous presence of this pathogen in the region (Cogliati 2013). In this study, a high prevalence of the VNIV molecular type was found in Greece, where it represented the most common molecular type, and Northern Turkey, where it was the only molecular type present, whereas it occurred less frequently in Italy and Spain. On the basis of these results, it could be speculated that this molecular type is spreading from subcontinental areas of the South-Eastern Mediterranean towards the Western part of Europe. Further sampling and an accurate niche modeling analysis is in progress to corroborate the above hypothesis.

Our results showed that mating type **a** and α are present in the Mediterranean environment for both *C. neoformans* varieties and *C. gattii*. Interestingly, we found that the occurrence of two

strains with different mating types in the same tree is not rare, as already observed for *C. gattii* in Australia (Halliday *et al.* 1999); therefore, trees are possible niches to complete the sexual cycle. This hypothesis is supported by the observation that most of the isolates with different mating types sharing the same niche were able to produce filaments and recombinant basidiospores in the mating assays. In addition, other authors showed that *C. neoformans* and *C. gattii* are able to proliferate and mate *in vitro* in a *Cryptococcus-Arabidopsis* system (Xue *et al.* 2007).

These findings suggest that the current view of a clonal population structure observed for both *C. neoformans* and *C. gattii* could be due to the genotyping results being mainly obtained from clinical isolates and the relatively low number of available environmental isolates investigated (Cogliati 2013). The analysis of a larger number of environmental isolates might show a more relevant involvement of sexual reproduction in the evolution and propagation of the *C. neoformans/C. gattii* species complex. Our data are corroborated by previous studies reporting recombination among *C. neoformans* var. *grubii* (VNI and VNB) and *C. gattii* (VGI and VGII) populations isolated from the environment (Litvintseva *et al.* 2003; Saul, Krockenberger and Carter 2008; Carriconde *et al.* 2011).

A different trend can be observed with respect to seasonality depending on the *Cryptococcus* species considered. Sampling during different seasons did not greatly influence the recovery of *C. neoformans* although a peak of positive trees was observed during spring. Similarly, *C. gattii* recovery was more likely during spring and early summer, but was absent during the colder seasons. Both species were isolated less in August, which is the hottest and driest month in the Mediterranean area, suggesting the difficulty to cultivate these yeasts during such climatic conditions. A recent study carried out in Colombia reported a similar observation with a low probability to recover *Cryptococcus* from trees during the seasons with reduced rainfall (Noguera, Escandón and Castañeda 2015). When daily mean temperatures were considered, the results confirmed that *C. neoformans* can be recovered during a wide range of temperatures, whereas *C. gattii* seems to be absent or difficult to cultivate at temperatures below 10°C.

In conclusion, the present survey established a wide laboratory network that, for the first time, collected extensive information concerning the environmental distribution and ecology of the *C. neoformans/C. gattii* species complex in Europe and the Mediterranean area. The results represent the basis for future studies on environmental niches of *Cryptococcus* in Europe and an important step towards the comparison of clinical and environmental isolates.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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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.

Conflict of interest. None declared.

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