Fundamental niche prediction of the pathogenic yeasts *Cryptococcus neoformans* and *Cryptococcus gattii* in Europe

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Summary

Fundamental niche prediction of *Cryptococcus neoformans* and *Cryptococcus gattii* in Europe is an important tool to understand where these pathogenic yeasts have a high probability to survive in the environment and therefore to identify the areas with high risk of infection. In this study, occurrence data for *C. neoformans* and *C. gattii* were compared by MaxEnt software with several bioclimatic conditions as well as with soil characteristics and land use. The results showed that *C. gattii* distribution can be predicted with high probability along the Mediterranean coast. The analysis of variables showed that its distribution is limited by low temperatures during the coldest season, and by heavy precipitations in the driest season. *C. neoformans* var. *grubii* is able to colonize the same areas of *C. gattii* but is more tolerant to cold winter temperatures and summer precipitations. In contrast, the *C. neoformans* var. *neoformans* map was completely different. The best conditions for its survival were displayed in sub-continental areas and not along the Mediterranean coasts. In conclusion, we produced for the first time detailed prediction maps of the species and varieties of the *C. neoformans* and *C. gattii* species complex in Europe and Mediterranean area.

Introduction

The *Cryptococcus neoformans* and *Cryptococcus gattii* species complexes are two of the etiological agents of cryptococcosis, a life-threatening fungal disease affecting both immunocompromised and immunocompetent hosts. Blastospores and basidiospores produced by the fungus in the environment are likely the main infection propagules able to reach the pulmonary alveoli and cause an infection leading to fatal meningitis in most clinical cases (Heitman *et al.*, 2011). Our understanding of the ecology of these pathogens is still lacking even though several studies have shown the presence and survival of the yeasts in various environmental sources since 1951 (Emmons, 1951; Heitman *et al.*, 2011; Litvintseva *et al.*, 2011). In 2004 investigators at the British Columbia University started a large environmental survey after the onset of an unprecedented outbreak in the cases of cryptococcosis due to a previously rare genotype of *C. gattii* on Vancouver Island (British Colombia, Canada). The numerous samplings collected in the area clearly showed that the yeast had colonized some areas of the island and the near mainland of British Columbia, and the correlation between the occurrence sites and the bioclimatic variables revealed that the optimal niche for this fungus was along the South-Eastern coast of Vancouver Harbour (Bartlett *et al.*, 2012). The same approach was then followed in the USA to evaluate the expansion of the outbreak along the North Pacific Coast of the country (Byrnes and Marr, 2011). Environmental surveys were also carried out in South America, especially in Argentina, Brazil and Colombia (Granados and Castañeda, 2005; Costa *et al.*, 2009; Cattana *et al.*, 2014). In Europe, some limited environmental studies were performed in Italy, Portugal, Spain and the Netherlands (Montagna *et al.*, 1997; Romeo *et al.*, 2012; Chowdhary *et al.*, 2012; Colom *et al.*, 2012; Ferreira *et al.*, 2014). Only recently a European network on behalf of the ISHAM Working Group for Genotyping of *C. neoformans* and *C. gattii* (http://www.isham.org/workinggroups/Genotyping_neoformans_gattii), including researchers from 12 countries, performed a large environmental survey aiming to understand the distribution of *C. neoformans* and *C. gattii* in Europe and in the Mediterranean area (Cogliati *et al.*, 2016). Both pathogens were isolated from trees and related arboreal material all around the Mediterranean basin showing differences in trees species colonized by the two species. At present, no studies enabling prediction of the fundamental niche of *C. neoformans* and *C. gattii* in Europe had been performed. Prediction of the fundamental niche represents an important tool to understand where these yeasts have a high probability to survive in the environment and therefore to identify the areas where the risk to encounter these pathogens is high. In this study, we recorded the geographic coordinates of all environmental sites where *C. neoformans* and *C. gattii* were isolated from the environment in Europe and the Mediterranean basin based on the results reported in the literature and our database (Cogliati *et al.*, 2016). Occurrence data were compared with several bioclimatic conditions as well as with soil characteristics and land use. A prediction map of species distribution for *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans* and *C. gattii* was produced using the maximum entropy model (Phillips *et al.*, 2006).

Results

The results showed that the distribution of *C. gattii* can be predicted with high probability (AUC = 0.954) in the Southern part of Europe, namely along the Southeastern coast of Spain, Southern Italy, Greece and some areas along the coast of Northern Africa (Fig. 1A). The analysis of the variables showed that the distribution of this species is mainly limited by two variables: minimum temperature in the coldest season and summer rainfall. Response curves of *C. gattii* for the variables 'minimum temperature in the coldest month' and 'numbers of days below 0°C' showed that the former directly correlated and the latter was inversely correlated and that the probability of presence for *C. gattii* drastically dropped when exposed to environments with a minimum temperature in winter of few degrees below 0°C
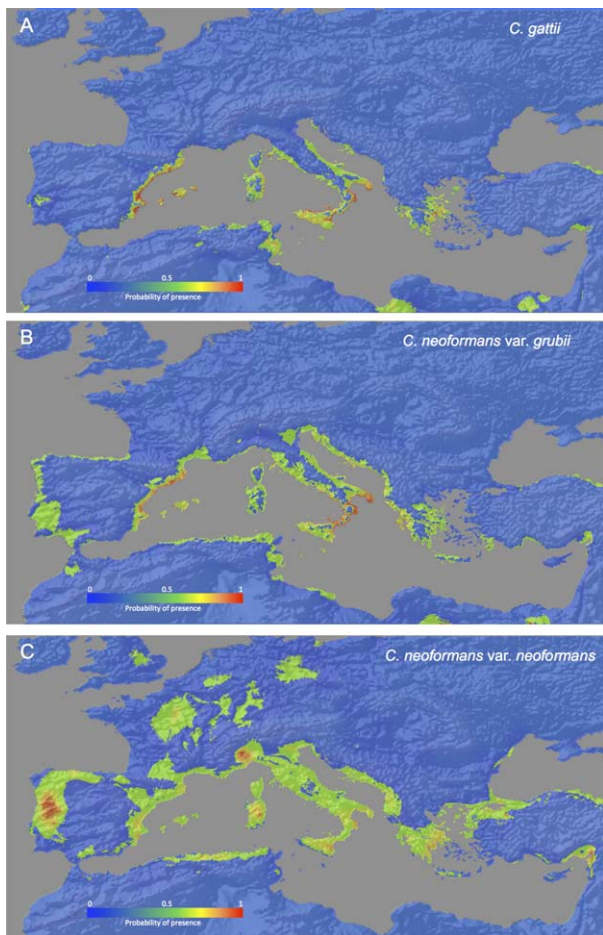
Fig. 1. Niche modelling performed by MaxEnt software using the most relevant bioclimatic layers. Predicted distribution maps for *C. gattii* (A), *C. neoformans* var. *grubii* (B) and *C. neoformans* var. *neoformans* (C) are shown. Different colours represent different probability of presence of the yeasts. [Color figure can be viewed at wileyonlinelibrary.com]

and with more than 5 days of frost (Figs. 2 and 3). Similarly the response curve of the variable 'summer rainfall' quickly dropped to very low probability of presence values as soon as the rainfall increased beyond 100 mm and reached the probability 0 above 250 mm (Fig. 4). By the analysis of *C. gattii* occurrence data with soil characteristic a high correlation was observed for two features included in the layer 'dominant use of soil'. Both the areas covered by the Mediterranean 'macchia' and where the cultivation of olive trees is practiced, displayed a logistic probability of presence >0.9 (Fig. 5).

The predicted area for *C. neoformans* var. *grubii* distribution (AUC = 0.930) extended to all coasts of Spain and Italy, the Southern coast of France, the coasts of Croatia, Albania, Greece and Southern Turkey and several areas in Northern Africa (Fig. 1B). As found for *C. gattii*, winter minimum temperature and summer rainfalls were the variables with the major weight in the analysis. The response curve
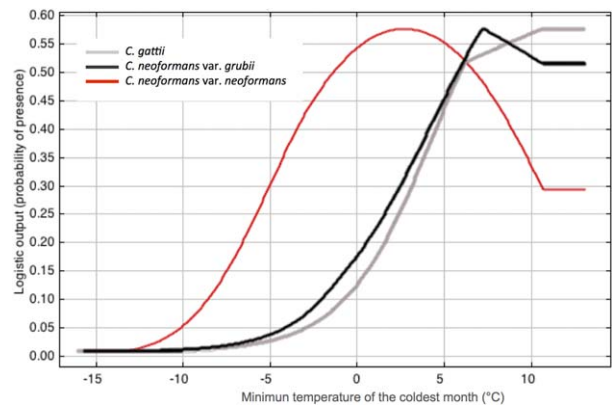

Fig. 2. Probability of presence predicted by the model for *C. gattii*, *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*, depending on minimum temperature recorded during the coldest month. [Color figure can be viewed at wileyonlinelibrary.com]

for winter temperature tolerance was slightly higher than that observed for *C. gattii* and the summer rainfall tolerance was up to 450 mm (Figs. 2–4). No significant correlation was found when soil data were analysed.

In contrast, the prediction map of *C. neoformans* var. *neoformans* was different. The best conditions for its survival were displayed in sub-continental areas and not along the coasts (AUC = 0.935). A high probability was predicted in Central Portugal, Northern Italy, Central Sicily and Central Sardinia, the Paris Basin, Greece, the Bosphorus area, the Adana province, and the sub-continental strip near the Mediterranean coast of Algeria (Fig. 1C). A great difference, with respect to the other two taxa, was observed when analysing the response curve for minimum winter temperature (Fig. 2). The lowest probability of presence for *C. neoformans* var. *neoformans* was predicted at minimum temperature around −10°C, whereas the maximum peak of probability was around 3°C, at higher temperatures the probability started to
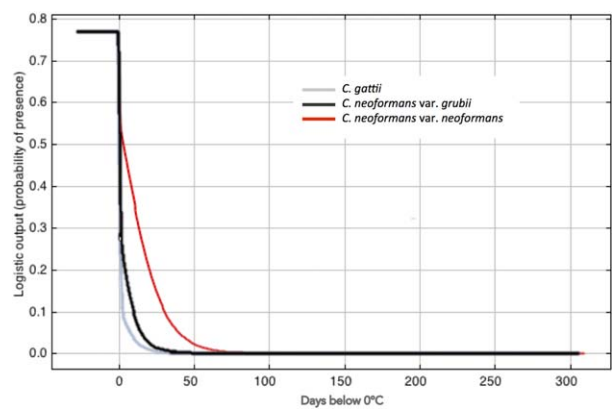

Fig. 3. Probability of presence predicted by the model for *C. gattii*, *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*, depending on number of frost days recorded during the year. [Color figure can be viewed at wileyonlinelibrary.com]
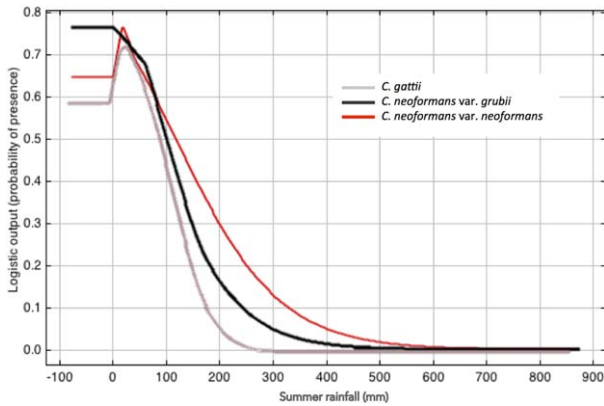
Fig. 4. Probability of presence predicted by the model for *C. gattii*, *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*, depending on rainfall recorded during the driest month. [Color figure can be viewed at wileyonlinelibrary.com]

decrease. Interestingly, in the areas where the winter minimum temperature is around 6°C the three taxa have the same probability to be present. Also the response curve for the variable 'days below 0°C' showed that *C. neoformans* var. *neoformans* can potentially tolerate environments with a number of frost days up to 50 (Fig. 3). The same trend was observed for the variable 'summer rainfall' where it showed a tolerance of up to 550 mm (Fig. 4).

Discussion

This study predicted, for the first time, the European and Mediterranean areas where the two species complex of *C. neoformans* and *C. gattii* can potentially survive. The results showed that *C. gattii* is not able to tolerate low temperatures during winter season or to survive when the temperature drops below 0°C. In addition, areas with low
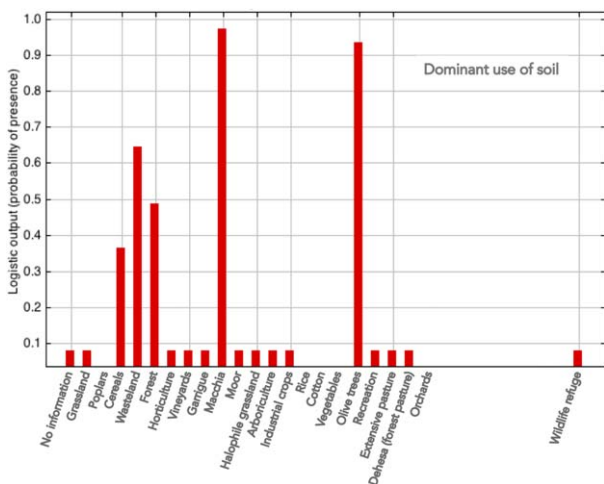

Fig. 5. Probability of presence predicted by the model for *C. gattii* depending on dominant use of soil. Each column represents a feature of the layer. [Color figure can be viewed at wileyonlinelibrary.com]

precipitation during summer are strongly correlated with the presence of the species. These conditions fit perfectly with Mediterranean climate with warm winters and dry summers. Similar results were obtained during the Vancouver environmental survey where the distribution in the environment of *C. gattii* isolates, belonging to molecular type VGII, was correlated with the areas surrounding the Vancouver Harbour (Mak *et al.*, 2010). This area is a microclimatic region protected from Northern winds by mountains and mitigated by the warm oceanic current of North Pacific, therefore the climate is classified as temperate warm similar to the Mediterranean one. In addition, winter low temperatures and summer precipitations were high predictive variables for modelling ecological niche of *C. gattii* in Vancouver, in agreement with the results obtained in this study. Correlation of *C. gattii* distribution with the Mediterranean climate is also confirmed by the high predictive value observed in the area where olive tree cultivation and Mediterranean macchia are present. Olive and carob trees are typical Mediterranean trees and they were recently recognized as an important ecological niche for *C. gattii* (Colom *et al.*, 2012; Cogliati *et al.*, 2016). It is interesting to note that cultivation of olive trees could be used as indicator of climatic changing and in particular of global warming (García-Mozo *et al.*, 2016). In the past, this tree was able to grow only in the Mediterranean climate conditions, but its distribution in Europe is now expanding also in areas where the cultivation was not possible due to cold winters. The progressive warming of the area neighbouring the original site is therefore allowing the colonization by olive trees and future expansion of the cultivations could correspond to a potential expansion of the fundamental niche of *C. gattii* (Robert *et al.*, 2015).

A similar map was produced for *C. neoformans* var. *grubii* that is potentially able to colonize the same areas of *C. gattii*, but it is more tolerant of cold winter temperatures and higher summer precipitations and therefore the predicted distribution area is wider than that predicted for *C. gattii*.

The importance of the role of rainfall in the distribution of *C. neoformans* and *C. gattii* could be explained by a different ability to compete with other species that proliferate during wet periods. This hypothesis is in agreement with a recent study carried on in Zambia reporting that the different distribution of the two *Cryptococcus* species complexes was also strongly associated to a different fungal microbiome (Vanhove *et al.*, 2016). A high recovery of *C. neoformans* from the environment during rainy period was also observed in Colombia in two different studies (Granados and Castaeda, 2005; Vélez and Escandón, 2016)

Low temperature tolerance seems to be the main variable influencing the prediction of fundamental niche for *C. neoformans* var. *neoformans*. The increased difference observed when comparing response curves for this variable showed that this cryptococcal yeast has potentially a

higher ability, with respect to the other two taxa, to colonize sub-continental areas where winters are more severe than along the Mediterranean coasts. In contrast, in this latter climate zone *C. neoformans* var. *neoformans* has a lower probability of presence than *C. neoformans* var. *grubii* and *C. gattii*. *In vitro* studies carried out by other investigators (Martinez *et al.*, 2001) showed that *C. neoformans* var. *grubii* is able to tolerate higher temperatures better than *C. neoformans* var. *neoformans*, whereas, by our recent experiments, the latter can better grow at low temperatures (data presented at the 10th International Conference on *Cryptococcus* and Cryptococcosis, Foz do Iguaçu, Brazil, 2017. Abstr. EP3). Both these experimental data support the distribution maps predicted by this study.

The prediction of the species distribution for *C. neoformans* var. *neoformans* was also influenced by the summer rainfall variable, which showed the highest response curve revealing that this cryptococcal yeast is able to tolerate environments with a humid climate. For this reason, the geographical region with the highest probability of presence was predicted in central Portugal where summers are particularly rainy due to wet Atlantic currents. Future environmental surveys will elucidate the actual presence of the pathogen in this region.

This study showed that in Europe there are bioclimatic conditions for co-existence of the two *C. neoformans* varieties and *C. gattii* confirming that hybridization may occur in the environment. This is in agreement with the high rate of inter-varietal AD-hybrids reported in Europe (Viviani *et al.*, 2006) as well as the isolation of rare inter-species hybrids (Bovers *et al.*, 2006; Bovers *et al.*, 2007). In our analysis, a prediction of AD-hybrids distribution was not possible due to the scarce number of environmental isolates presently available. Future investigations including a larger number of isolates will allow the drawing of a map reporting the regions where hybridization has the highest probability to occur. The control of hybrid distribution is crucial since hybridization could produce the emergence of high virulent strains (Cogliati *et al.*, 2012) with unexpected impacts on human public health.

Autochthonous cases of cryptococcosis due to *C. gattii* described in literature are in agreement with the predicted distribution map of this species with most of the cases reported in Southern Italy, Greece and Spain (Velegraki *et al.*, 2001; Colom *et al.*, 2003; Iatta *et al.*, 2012; Hagen *et al.*, 2012). *Cryptococcus* var. *neoformans* clinical isolates were primarily reported from Denmark, France, Germany, Northern Italy, Portugal and Spain confirming the presence of this pathogen in continental and sub-continental European areas (Cogliati, 2013). With regard to *C. neoformans* var. *grubii*, clinical isolates were reported throughout the continent despite the more restricted distribution area predicted in this study. The higher virulence observed for *C. neoformans* var. *grubii* compared to *C.*

neoformans var. *neoformans* and *C. gattii* could explain the higher prevalence of cryptococcosis cases due to this cryptococcal yeast also in areas where its probability of presence is low (Barchiesi *et al.*, 2005).

This study represents the first attempt to find the optimal bioclimatic conditions able to support the survival of *C. neoformans* and *C. gattii* species complexes in Europe, although a higher number of isolates and a more capillary distribution of samplings is needed to draw a more detailed picture of the relationship existing between these pathogens and their environment. The continuous monitoring of environmental and clinical isolates in Europe and in Mediterranean areas combined with statistical model for species distribution prediction will elucidate how *Cryptococcus* and cryptococcosis epidemiology is evolving and will provide a valid tool for early outbreaks detection.

Experimental procedures

Occurrence data

Data concerning the environmental presence of *C. gattii* and *C. neoformans*, in Europe and in the Mediterranean basin, were obtained by literature (Supporting Information Data, Table S1) and from our database (Cogliati *et al.*, 2016). Environmental samples included bird excreta, soil, air and arboreal sources, as well as veterinary samples obtained from domestic animals (dogs, cats, goats) or stationary wild animals (squirrels). Each occurrence point was geospatially referenced and listed in a datasheet reporting the geographical coordinates, species and variety. In the surveyed geographical area, a total of 84 occurrence points were recorded for *C. neoformans* var. *grubii*, 26 for *C. neoformans* var. *neoformans* and 21 for *C. gattii*. Distribution of the occurrence points is shown in the map in Fig. 6.

Geographical area definition

The extension of the geographical area suitable for niche modelling analysis was determined on the basis of the occurrence data distribution. The area included all the Mediterranean basin and most of continental Europe occurring within the following coordinates: Northern limit latitude 54.470, Southern limit latitude 29.916, Western limit longitude −10.810 and Eastern limit longitude 37.441. The map resolution was 10 arc-minutes. The LandSerf software (v2.3, developed by Jo Wood, www.landserf.org) was used to manage the prediction maps generated by the niche modelling software and grid files conversion.

Datasets used as environmental layers for niche modelling

Global monthly temperature and rainfall grids, with a spatial resolution of 10 arc-minutes grid spacing, were downloaded
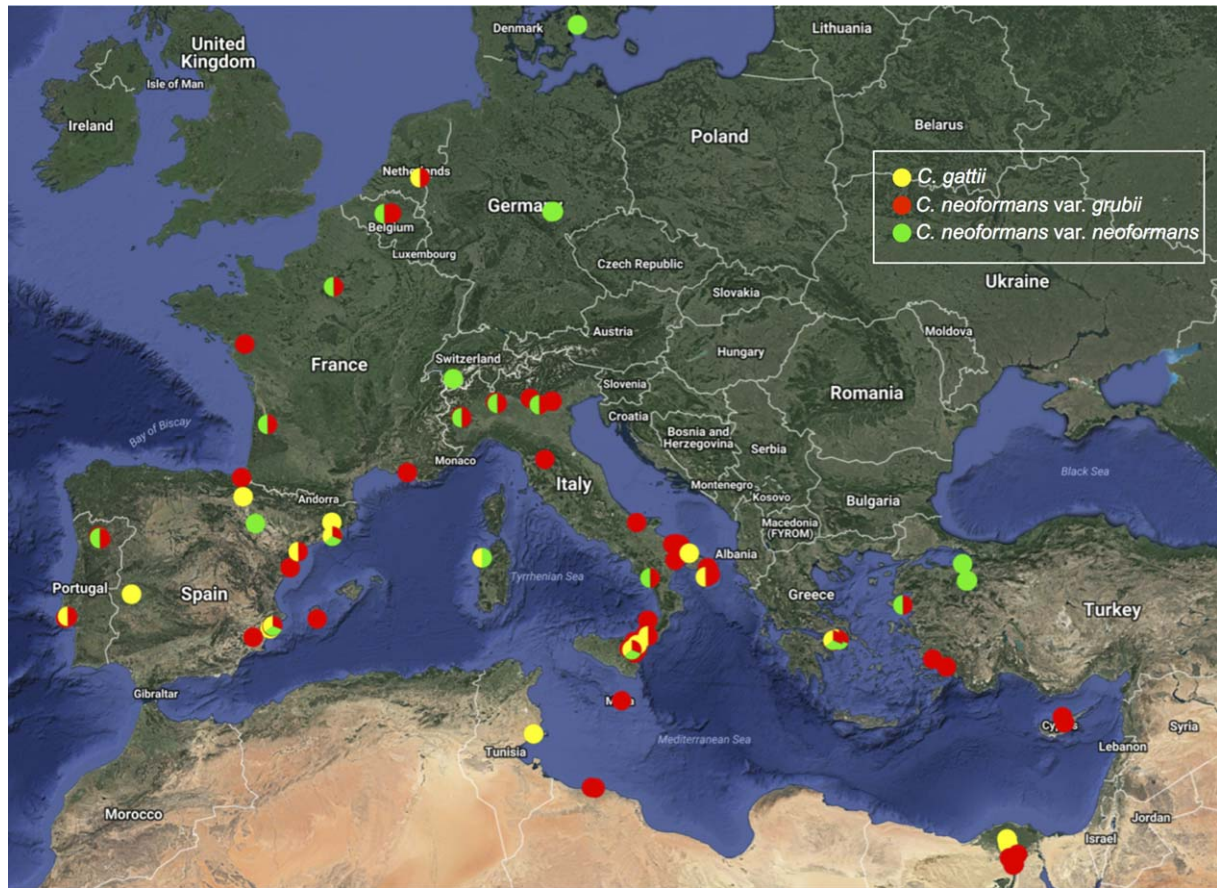
Fig. 6. Geographical distribution of the presence data observed for *C. gattii*, *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*. Sites where two or three different taxa are simultaneously present are indicated with a bicoloured or tricolour circle. [Color figure can be viewed at wileyonlinelibrary.com]

from the Worldclim database (http://www.worldclim.org/). Also 19 bioclimatic grids derived from the above variables were obtained from the same database (Supporting Information Data, Table S2). They represented annual trends, seasonality and extreme or limiting environmental factors. The data grids included annual mean temperature, mean diurnal range, isothermality, temperature seasonality, maximum temperature of warmest month, minimum temperature of coldest month, temperature annual range, mean temperature of wettest quarter, mean temperature of driest quarter, mean temperature of warmest quarter, mean temperature of coldest quarter, annual precipitation, precipitation of wettest month, precipitation of driest month, precipitation seasonality, precipitation of wettest quarter, precipitation of driest quarter, precipitation of warmest quarter and precipitation of coldest quarter.

An alternative bioclimatic set of grids was downloaded from the University of Alberta, Canada, (https://sites.ualberta.ca/~ahamann/data/climateeu.html) to confirm the results obtained with the WorldClim dataset. In addition to temperature and precipitation records, this database also contains different bioclimatic variables, such as annual and summer heat/moisture indexes, days below 0°C, days above 5°C, days below 18°C, days above 18°C, number of frost-free days, and snow precipitations (Supporting Information Data, Table S2). The data covered Europe and Turkey, but excluded the North African and Middle-Eastern regions.

Layers containing information about altitude, slope, dominant and secondary use of soil, and soil characteristics, were obtained from the European Soil Data Center (ESDAC, http://eusoils.jrc.ec.europa.eu, Supporting Information Data, Table S2). These data covered only European countries and Russia.

Niche modelling

The most probable fundamental ecological niche of *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans* and *C. gattii* was modelled using MaxEnt software version 3.3.3k developed at Princeton University, USA, (https://www.cs.princeton.edu/~schapire/maxent). The software is able to calculate the probability that the presence sites are randomly distributed in the different features of each layer compared to the actual distribution. Based on the number

of presence data available and the kind of features in the layers (categorical or continuous) the system can apply all the appropriate algorithms: linear, quadratic, product, threshold, hinge and discrete. The distribution area displayed on the prediction map is the maximum entropy model produced by the different algorithms and each cell of the grid contains the probability value to find the species in that site. The accuracy of the analysis is evaluated determining the area under the receiver operating characteristic curve (AUC), which is maximized when the value is near to 1.0 and is minimized when value is near 0.5 (Phillips *et al.*, 2006).

The software is able also to perform jackknife analysis that allows determining the weight of each variable in the analysis. In addition, response curves for each variable are generated to understand the relationship between the variable and the distribution of the species.

The main analysis was performed using WorldClim data since they included the whole geographical area covered by our survey. Temperature and rainfall layers were first compared with presence data to identify the main variables contributing to draw the prediction map, and the same analysis was also performed for bioclimatic data. The most relevant variables from both the analyses were then analysed all together to display the definitive map. The data from the University of Alberta were analysed as above and used to confirm the results obtained using WorldClim data. Soil layers were used as categorical variables and the results were interpreted to find relationships between the presence data and the features included in the grids. The analyses were repeated independently for *C. gattii*, *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*.

Acknowledgements

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. *C. neoformans* and *C. gattii* presence data recovered from the literature.

Table S2. Environmental variables analysed in the present study (main variables for Cryptococcus presence are highlighted).