

## RESEARCH ARTICLE

# Pathogenic budding yeasts isolated outside of clinical settings

Dana A. Opulente<sup>1,2</sup>, Quinn K. Langdon<sup>1</sup>, Kelly V. Buh<sup>1</sup>, Max A. B. Haase<sup>1,2</sup>, Kayla Sylvester<sup>1,2</sup>, Ryan V. Moriarty<sup>1,2</sup>, Martin Jarzyna<sup>1</sup>, Samantha L. Considine<sup>1</sup>, Rachel M. Schneider<sup>1,2</sup> and Chris Todd Hittinger<sup>1,2,\*</sup>†

<sup>1</sup>Laboratory of Genetics, Genome Center of Wisconsin, Wisconsin Energy Institute, J. F. Crow Institute for the Study of Evolution, University of Wisconsin-Madison, Madison, WI 53706, USA and <sup>2</sup>DOE Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, WI 53706, USA

\*Corresponding author: 425-G Henry Mall, 4102 Genetics/Biotechnology Center, Madison, WI 53706-1580. Tel: +1(608)262-1069; E-mail: [cthittinger@wisc.edu](mailto:cthittinger@wisc.edu)

**One sentence summary:** Here we describe the isolation of more than 50 strains of budding yeast opportunistic pathogens from natural settings, suggesting that they have more complex ecology than is currently appreciated.

**Editor:** Richard Calderone

†Chris Todd Hittinger, <http://orcid.org/0000-0001-5088-7461>

## ABSTRACT

Budding yeasts are distributed across a wide range of habitats, including as human commensals. However, under some conditions, these commensals can cause superficial, invasive, and even lethal infections. Despite their importance to human health, little is known about the ecology of these opportunistic pathogens, aside from their associations with mammals and clinical environments. During a survey of approximately 1000 non-clinical samples across the United States of America, we isolated 54 strains of budding yeast species considered opportunistic pathogens, including *Candida albicans* and *Candida* (*Nakaseomyces*) *glabrata*. We found that, as a group, pathogenic yeasts were positively associated with fruits and soil environments, whereas the species *Pichia kudriavzevii* (syn. *Candida krusei* syn. *Issatchenkia orientalis*) had a significant association with plants. Of the four species that cause 95% of candidiasis, we found a positive association with soil. These results suggest that pathogenic yeast ecology is more complex and diverse than is currently appreciated and raises the possibility that these additional environments could be a point of contact for human infections.

**Keywords:** pathogen; *Candida albicans*; *Candida glabrata*; ecology; *Candida tropicalis*; wild yeasts

## INTRODUCTION

Budding yeasts of the subphylum Saccharomycotina are distributed across a wide range of habitats (Kurtzman, Fell and Boekhout 2011; Buzzini, Lachance and Yurkov 2017; Opulente et al. 2018). These habitats can range from wild niches, including soil, flowers, and trees, to environments where they are considered commensals, such as in insects and animals, including the human microbiota (Kurtzman, Fell and Boekhout 2011; Gabaldón

and Fairhead 2018). However, under rare circumstances, these commensal yeasts can cause candidiasis, which is an infection caused by yeasts often assigned to the genus *Candida* and considered opportunistic pathogens (Gabaldón, Naranjo-Ortiz and Marcet-Houben 2016). While there are rare reports of several other Saccharomycotina species being the agents of candidiasis, including *Saccharomyces cerevisiae* (Strope et al. 2015), the species *Candida albicans*, *Candida* (*Nakaseomyces*) *glabrata*, *Candida parapsilosis*, and *Candida tropicalis* are responsible for approximately

Received: 20 December 2018; Accepted: 24 April 2019

© The Author(s) 2019. Published by Oxford University Press on behalf of FEMS. All rights reserved. For permissions, please e-mail: [journals.permissions@oup.com](mailto:journals.permissions@oup.com)

95% of infections (Pfaller and Diekema 2007; Diekema et al. 2012; Gabaldón, Naranjo-Ortíz and Marcet-Houben 2016). Other non-hybrid yeast species recognized as opportunistic pathogens include *Pichia kudriazevii* (syn. *Candida krusei* syn. *Issatchenkia orientalis*), *Candida dublinensis*, *Candida orthopsilosis*, *Meyerozyma (Candida) guilliermondii*, and *Clavispora (Candida) lusitanae* (Kurtzman, Fell and Boekhout 2011). *Pichia kudriazevii* is the fifth leading cause of yeast infections, is resistant to fluconazole and has reduced susceptibility to other antifungal treatments (Pelletier et al. 2005). Infections by the other species considered opportunistic pathogens are rare, but each has caused multiple infections in humans (Pfaller et al. 2010; Gabaldón, Naranjo-Ortíz and Marcet-Houben 2016). These nine species have been considered by leading taxonomists to be the most important budding yeast opportunistic pathogens (Kurtzman, Fell and Boekhout 2011).

While most budding yeast opportunistic pathogens are currently classified in the genus *Candida*, these pathogenic species belong to several phylogenetically distinct clades and are genetically diverse (Gabaldón, Naranjo-Ortíz and Marcet-Houben 2016). Despite this diversity, one commonality is that little is known about their ecology; it is unclear whether their primary ecological niche is endothermic animals and clinical settings, or whether they are adapted to additional environments. While disease-causing species have been recovered from a variety of habitats, such as food, air, and clothing, these rare isolates are generally interpreted as contamination, rather than being sourced from an environmental yeast habitat, such as soil, trees, flowers, or insects (Van Uden, Faia and Assis-Lopes 1956; Di Menna 1958; Kurtzman, Fell and Boekhout 2011; Bensasson et al. 2019). Therefore, it is unknown whether these species are able to survive for extended periods of time outside of clinical settings and endothermic animals. In other words, do these species have ecological niches beyond endothermic animals as commensals and clinical settings as pathogens? If so, do different species or groups have different habitats?

Understanding the ecology of pathogenic yeasts is critical to human health for multiple reasons. First, mortality from infections by these yeasts remains high, and candidiasis is the fourth most common hospital-associated bloodstream infection (Morgan et al. 2005; Gabaldón and Fairhead 2018; Revie et al. 2018). Second, in addition to the nine already considered important opportunistic pathogens, *Candida auris* recently emerged as a multi-drug resistant opportunistic pathogen, with additional strains exhibiting resistance to many anti-fungal drugs (Dolande et al. 2017; Revie et al. 2018). Finally, it is unclear whether there might be environmental reservoirs that could act as contact points for their primary hosts, or whether the niches of pathogenic yeasts are indeed exclusively endothermic animals and clinical settings (Daszak, Cunningham and Hyatt 2000).

Based on previous literature, we would predict opportunistic pathogenic yeasts are not present in wild (i.e. non-endothermic, non-clinical) habitats. Therefore, we would expect that opportunistic pathogenic yeasts would not be isolated through enrichment protocols from wild habitats. In general, yeast enrichment protocols are predicted to select against yeasts that are in low abundance within an environment through repeated bottleneck events and through competition with yeasts that are in higher abundance in the environment and better adapted to culturing outside of their hosts (Fingerman, Dombrowski and Sniegowski 2002; Sampaio and Gonçalves 2008; Sylvester et al. 2015). Contrary to this expectation, we found opportunistic pathogenic yeasts isolated from multiple non-clinical environments across the United States of America. We hypothesize that these opportunistic pathogenic yeasts have niches outside of

clinical and endothermic environments and are associated with specific environmental substrates. Furthermore, we hypothesize that pathogenic yeasts that are more commonly found in nature and only rarely cause candidiasis (i.e. *P. kudriazevii*, *C. dublinensis*, *C. orthopsilosis*, *M. guilliermondii*, and *Cl. lusitanae*) differ from those yeasts that are known to cause 95% of candidiasis (i.e. *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*). To test these hypotheses, we used data collected during a survey of approximately 1000 non-clinical, natural substrates, from which we isolated over 50 yeast strains from species considered to be opportunistic pathogens.

## METHODS

### Sample collection, yeast isolation and quality control

Yeasts were collected throughout the United States from multiple substrates by members of the Hittinger Lab, as well as citizen scientists (Table S1, Supporting Information). Statistical analyses did not detect bias in pathogenic isolates coming from any individual collector ( $\chi^2 = 0.072$ ,  $P$ -value = 1, Table S2, Supporting Information) or among individuals isolating yeasts from samples ( $\chi^2 = 1.63$ ,  $P$ -value = 1, Table S3, Supporting Information). All samples were collected using sterile bags and did not come into contact with the humans collecting them. Samples were processed by different members of the lab using published yeast enrichment and isolation protocols (Sylvester et al. 2015). Negative controls were included to ensure no contamination had occurred. After enrichment, yeast species were identified by internal transcribed spacer (ITS) sequencing, as previously described (Sylvester et al. 2015). Table S1 (Supporting Information) contains GenBank accessions for ITS sequences.

### Statistical analyses

We removed strains from our analyses that were isolated from areas that are highly trafficked by humans, such as samples that were isolated from compost piles. In some cases, we isolated the same opportunistic pathogen out of a single sample; we collapsed multiple strains isolated from the same sample (including when isolated at different temperatures) to one representative strain since the strains may be closely related. In other words, for all analyses, a species was only counted once per sample, even if it was isolated multiple times from a sample. For analyses where we tested for associations between species and substrates, we removed any species that was isolated fewer than three times since there would be a lack of power to detect associations. In other words, any species that we isolated only once or twice was dropped from analyses that tested for associations between species and substrates.

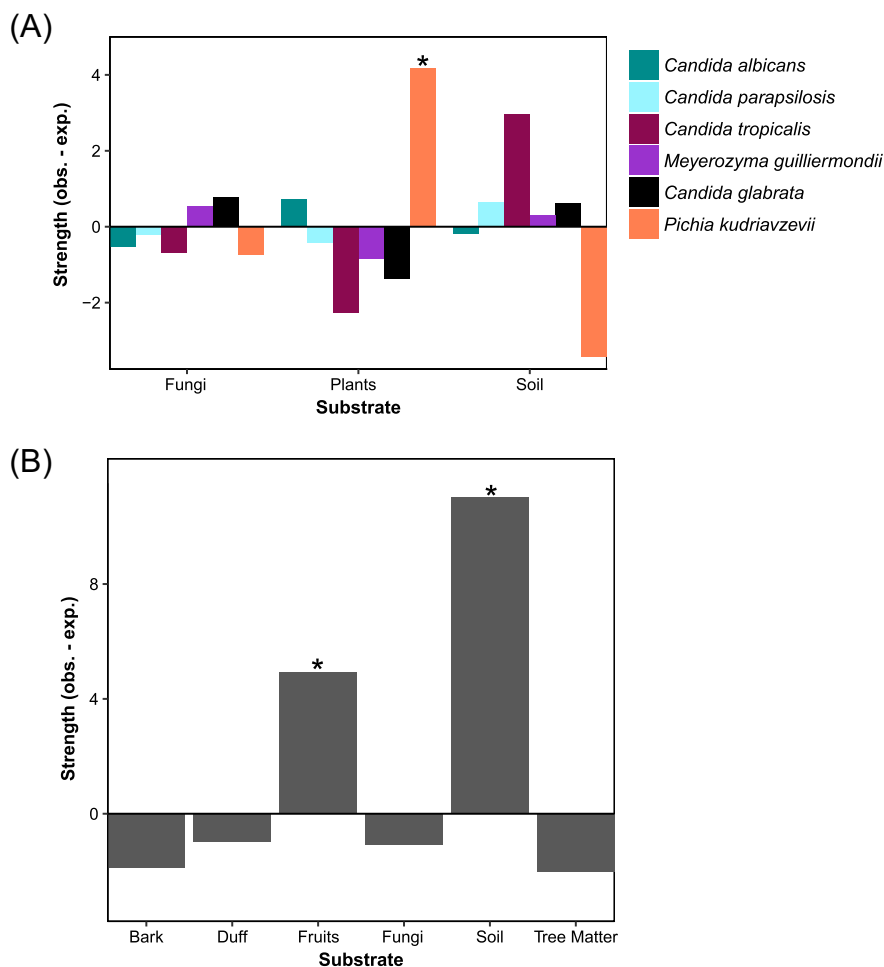
In order to determine whether there were associations between substrates and species, we permuted species and substrate presence/absence matrices ( $n = 1000$  permutations) using a swap algorithm. Permutations were performed using the R package *vegan* (v. 1.6–2). To determine whether there was a positive association between species and substrates, we counted the number of times a given species was isolated from a given substrate. For example, *C. tropicalis* was isolated seven times from soil. These values were also calculated for the permuted data, and the mean of the permuted counts was used to determine the expected isolation count. The strength of each association



**Figure 1.** Opportunistic pathogenic species of yeasts in the subphylum Saccharomycotina are found throughout the United States of America on a variety of non-clinical substrates. (A) Map displaying where opportunistic pathogens were isolated. The colors represent the species isolated, and the shapes of the points represent the substrate from which they were isolated. (B) Bar graph of unique strains of each species.

was calculated by subtracting the observed values from the average expected values (from the permuted data). A positive association between a substrate and a species would have a positive value for the strength of association; the larger the value, the stronger the association with the substrate. We calculated the binomial confidence intervals to determine significant positive associations using the R package *Hmisc* (v. 4.1-1) and corrected for multiple tests across associations with Benjamini-Hochberg corrections. We also calculated whether, in general, pathogenic yeasts were isolated from specific substrates more often using the same method described above. In this analysis, we included all species, regardless of whether they were only

isolated once or twice. Finally, to detect whether there were differences between pathogens that cause 95% of candidiasis infections and all other opportunistic yeast pathogens, we categorized them into two groups: Group 1 consisted of the yeasts that cause 95% of infections (i.e. *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*), whereas the other opportunistic pathogenic yeasts (i.e. *P. kudriavzevii*, *C. dublinensis*, *C. orthopsilosis*, *M. guilliermondii*, and *Cl. lusitaniae*) were classified into Group 2. After this classification, we tested for associations between these groups and substrates using the methods described above. Additionally, we tested for differences in isolation numbers on both plants and soil using pairwise  $\chi^2$  analyses and corrected for multiple



**Figure 2.** Pathogenic yeast species are associated with non-clinical/non-endothermic environments. (A) *Pichia kudriavzevii* was significantly associated with plants ( $P_{\text{adj.}} = 0.018$ ). Bar graphs representing the strength (difference between observed and expected counts) of associations between pathogenic species and isolation substrates. Positive values indicate cases where the observed number of isolations were greater than random expectations without associations (the average of the permuted data). The strength indicates how strong of an association there was between species and substrates. (B) As a group (i.e. using all species we isolated, but only a single isolate per environmental sample), opportunistic pathogens were associated with soil ( $P_{\text{adj.}} < 0.001$ ) and fruits ( $P_{\text{adj.}} = 0.021$ ). Bar graph representing the strength of association between pathogenic yeasts as a group and specific isolation environments.

tests with Benjamini-Hochberg corrections. We did not include the fungi substrate since there were not many isolates to compare between the two groups. All analyses were done in the statistical language R (v. 3.3.0). All graphs were made using the R package *ggplot2* (v. 2.2.1).

## RESULTS/DISCUSSION

### Multiple non-clinical isolations of known pathogenic yeasts

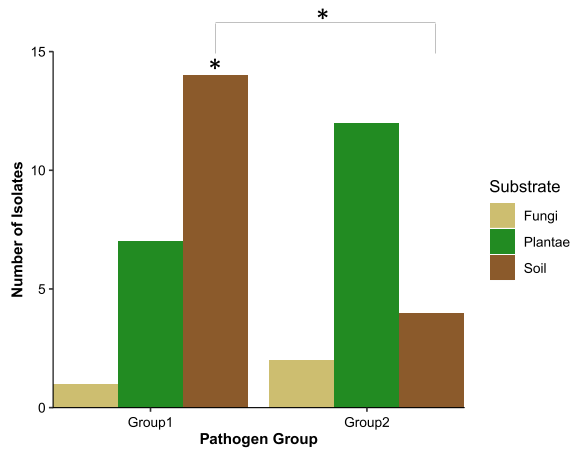
We extensively sampled non-clinical substrates across the United States of America to enrich for and isolate yeasts. In total, we collected approximately 1000 samples and have isolated about 5000 strains of yeasts. Across our sampling regime, we isolated 54 strains of species that are considered opportunistic pathogens (Fig. 1, Table S1, Supporting Information). Recently and independently, three strains of *C. albicans* were isolated from oak bark in New Forest in the United Kingdom (Bensasson *et al.* 2019). Collectively, these results suggest that pathogenic yeast

ecology is more complex and diverse than is currently appreciated and raises the possibility that these additional environments could be a point of contact for infections.

The strains we isolated came from 40 different samples; in 14 cases, multiple strains of a single species were isolated from a single sample, often from different isolation temperatures (Table S1, Supporting Information). Once those were collapsed into a single representative species for each sample, we had 40 unique strains, 55% of which were from the four species that cause 95% of candidiasis infections (Fig. 2). The species isolated most was *P. kudriavzevii*, making up 25% ( $n = 10$ ) of our isolates of opportunistic pathogenic species. *P. kudriavzevii* has been previously isolated from non-clinical settings several times across a wide range of environments, including fermentations, soil, and fruits (Kurtzman, Fell and Boekhout 2011; Douglass *et al.* 2018), and its prevalence in our environmental isolations is consistent with previous studies.

### Associations between pathogenic yeasts and non-clinical environments

While *P. kudriavzevii* and *M. guilliermondii* have been often isolated from non-clinical/ectothermic environments (Brandão *et*



**Figure 3.** Pathogenic yeasts that cause 95% of candidiasis (Group 1) differed with other pathogenic yeasts (Group 2). Group 1 yeasts were positively associated with soil environments ( $P_{\text{adj}} = 0.024$ ,  $n = 14$ ), and Group 1 yeasts were isolated more often from soil than Group 2 yeasts ( $P = 0.036$ ,  $\chi = 5.56$ ). Bar graphs representing the counts of Group 1 and Group 2 species isolated from different natural substrates. Group 1 included *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. parapsilosis*. Group 2 included *P. kudriavzevii*, *M. guilliermondii*, *Cl. Lusitaniae*, and *C. orthopsilosis*. Both groups contained a single representative isolate per environmental sample.

al. 2017; Moubasher, Abdel-Sater and Zeinab 2018), the other species we isolated have only been rarely isolated from non-clinical settings. It is unknown whether these species are actively growing in these environments, but these non-clinical environments could potentially act as a secondary niche for these species. Furthermore, these environments could be an additional source of contact between these species and their primary hosts, potentially as the organism passes between hosts. To determine whether pathogenic yeasts were associated with particular non-clinical environments, we classified our samples in two ways. We used a more general description of whether each strain was isolated from plants, soil, or fungi; and then, when the specific information was available, a more specific description of the substrate (e.g. fruits, leaves, sand) was given. This method was used to determine whether specific parts of a substrate were more important for pathogenic yeasts than others.

Samples varied significantly in terms of the number of species isolated from an environment (Table S1, Supporting Information). Across all species with multiple isolation events, species were not limited to a single substrate. For example, *C. albicans* was isolated from fruits, soil, and plant matter. *Pichia kudriavzevii* was significantly associated with isolation from plants ( $P_{\text{adj}} = 0.0185$ ) (Fig. 2A, Table S4, Supporting Information). We detected no other significant associations among substrates at the general descriptive level for the other species in our data.

Due to the smaller sample sizes at the specific descriptive levels for our substrates, we did not test for associations between them and species. However, at this level, our opportunistic pathogen isolates collectively exhibited significant associations with fruits ( $P_{\text{adj.}} = 0.021$ ) and soil ( $P_{\text{adj.}} < 0.001$ ) (Fig. 2B, Table S5, Supporting Information). The association between fruits and our isolates was most likely driven by our *P. kudriavzevii* isolates, which were, in our more general analysis, positively associated with plants. This association could be the result of animals acting as a vector to transport these opportunistic pathogenic species to fruits they regularly visit. The association with soil was mostly driven by *C. tropicalis*, but other species

also contributed; 39% ( $n = 7$ ) of our soil isolates were *C. tropicalis*. Our results, combined with the independent isolation of three strains of *C. albicans* from oaks in the United Kingdom (Bensasson et al. 2019), suggest that these alternative niches could potentially act as a secondary contact site between these pathogenic yeasts and their human hosts.

### The most prevalent candidiasis agents are associated with soil

The ecological complexity of these opportunistic pathogens may go beyond species level differences, so we assessed whether yeasts might vary by pathogenicity as well. One notable ecological difference between prevalent causes of candidiasis (Group 1) and the important minor contributors (Group 2) is that some of the yeasts in Group 2 are frequently found in non-clinical settings (e.g. *P. kudriavzevii* and *M. guilliermondii*). Therefore, we hypothesized that there would be differences in ecological associations between these two groups, so we tested for associations between each group and our isolation environments. We found that Group 1 was associated with soil environments ( $P_{\text{adj}} = 0.024$ , Fig. 3, Table S6, Supporting Information). However, we did not detect associations between Group 2 and any specific substrate. We also quantified whether there were significant differences in isolation numbers for each substrate between Group 1 and Group 2. We found a significant difference in isolation numbers on soil ( $P_{\text{adj.}} = 0.036$ ,  $\chi = 5.56$ , Fig. 3, Table S7, Supporting Information). Collectively, these results suggest that there are ecological differences between these groups. These results further suggest that those pathogenic yeasts more commonly found in the environment are found across many different niches.

## CONCLUSIONS

We isolated several opportunistic pathogenic species of yeasts from the subphylum Saccharomycotina from multiple non-clinical, non-endothermic environments across the United States of America. Among those strains isolated, over 50% represent the opportunistic pathogens that cause 95% of candidiasis. These species, *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. glabrata*, have been rarely isolated from non-clinical settings, and when they have been isolated from the environment, they have usually been interpreted as contaminants. Our extensive isolations from natural settings challenge this assumption, suggesting that opportunistic pathogens can persist in alternative niches and that their ecologies may be more complicated than is currently assumed. Non-clinical environments could be a short-term habitat as these yeasts are passed between their predominant hosts, endothermic animals.

## SUPPLEMENTARY DATA

Supplementary data are available at [FEMSyr](https://www.femsyr.com) online.

## ACKNOWLEDGMENTS

We thank the following citizen scientists or lab members for collecting samples or generating preliminary results: Aaron G. Barton, Amanda Beth Hulfachor, Russell L. Wrobel, Leslie Shown, The Zasadil Family, EmilyClare P. Baker, Drew T. Doering, Angela Sheddan, Sarah Wright, Bill Saucier, Bill Vagt, Annette Opulente and S. Smead and family. This material is based upon work supported by the National Science Foundation under Grant

Nos. DEB-1253634 (to CTH), DEB-1442148 (to CTH) and DGE-1256259 (Graduate Research Fellowship to QKL); funded in part by Lakeshore Nature Preserve Student Engagement Grants (to MJ and RMS); and funded in part by the DOE Great Lakes Bioenergy Research Center (DOE Office of Science BER DE-SC0018409 to Timothy J. Donohue). QKL was also supported by the Predoctoral Training Program in Genetics, funded by the National Institutes of Health (5T32GM007133). CTH is a Pew Scholar in the Biomedical Sciences and Vilas Faculty Early Career Investigator, supported by the Pew Charitable Trusts and Vilas Trust Estate, respectively.

**Conflicts of interest.** None declared.

## REFERENCES

- Bensasson D, Dicks J, Ludwig JM et al. Diverse lineages of *Candida albicans* live on old oaks. *Genetics* 2019;**211**:277–88.
- Brandão LR, Vaz ABM, Santo LCE et al. Diversity and biogeographical patterns of yeast communities in antarctic, patagonian and tropical lakes. *Fungal Ecol* 2017;**28**:33–43.
- Buzzini P, Lachance M-A, Yurkov A. *Yeasts In Natural Ecosystems: Diversity*. Cham, Switzerland: Springer, 2017.
- Daszak P, Cunningham AA, Hyatt AD. Emerging infectious diseases of wildlife—threats to biodiversity and human health. *Science* 2000;**287**:443–449.
- Diekema D, Arbefeville S, Boyken L et al. The changing epidemiology of healthcare-associated candidemia over three decades. *Diagn Microbiol Infect Dis* 2012;**73**:45–48.
- Di Menna ME. *Candida albicans* from grass leaves. *Nature* 1958;**181**:1287.
- Dolande M, García N, Capote AM et al. *Candida auris*: antifungal multi-resistant emerging yeast. *Curr Fungal Infect Rep* 2017;**11**:197–202.
- Douglas AP, Offei B, Braun-Galleani S et al. Population genomics shows no distinction between pathogenic *Candida krusei* and environmental *Pichia kudriavzevii*: one species, four names. *PLOS Pathog* 2018;**14**:e1007138.
- Fingerman E, Dombrowski PG, Sniegowski PD. *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* coexist in a natural woodland site in North America and display different levels of reproductive isolation from European conspecifics. *FEMS Yeast Res* 2002;**1**:299–306.
- Gabaldón T, Fairhead C. Genomes shed light on the secret life of *Candida glabrata*: not so asexual, not so commensal. *Curr Genet* 2018;**65**:93–98.
- Gabaldón T, Naranjo-Ortiz MA, Marcet-Houben M. Evolutionary genomics of yeast pathogens in the Saccharomycotina. *FEMS Yeast Res* 2016;**16**:fow064.
- Kurtzman C, Fell JW, Boekhout T. *The Yeasts: A Taxonomic Study*. London, United Kingdom: Elsevier, 2011.
- Morgan J, Meltzer MI, Plikaytis BD et al. Excess mortality, hospital stay, and cost due to candidemia: a case-control study using data from population-based candidemia surveillance. *Infect Control Hosp Epidemiol* 2005;**26**:540–47.
- Moubasher AH, Abdel-Sater MA, Zeinab SM. Diversity of floricolous yeasts and filamentous fungi of some ornamental and edible fruit plants in Assiut area, Egypt. *Curr Res Environ Appl Mycol* 2018;**8**:135–61.
- Opulente DA, Rollinson EJ, Bernick-Roehr C et al. Factors driving metabolic diversity in the budding yeast subphylum. *BMC Biol* 2018;**16**:26.
- Pelletier R, Alarie I, Lagacé R et al. Emergence of disseminated candidiasis caused by *Candida krusei* during treatment with caspofungin: case report and review of literature. *Med Mycol* 2005;**43**:559–64.
- Pfaller MA, Diekema DJ, Gibbs DL et al. Results from the ARTEMIS DISK global antifungal surveillance study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. *J Clin Microbiol* 2010;**48**:1366–77.
- Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* 2007;**20**:133–63.
- Revie NM, Iyer KR, Robbins N et al. Antifungal drug resistance: evolution, mechanisms and impact. *Curr Opin Microbiol* 2018;**45**:70–76.
- Sampaio JP, Gonçalves P. Natural populations of *Saccharomyces kudriavzevii* in Portugal are associated with oak bark and are sympatric with *S. cerevisiae* and *S. paradoxus*. *Appl Environ Microbiol* 2008;**74**:2144–52.
- Strope PK, Skelly DA, Kozmin SG et al. The 100-genomes strains, an *S. cerevisiae* resource that illuminates its natural phenotypic and genotypic variation and emergence as an opportunistic pathogen. *Genome Res*. 2015;**25**:762–74.
- Sylvester K, Wang Q-M, James B et al. Temperature and host preferences drive the diversification of *Saccharomyces* and other yeasts: a survey and the discovery of eight new yeast species. *FEMS Yeast Res* 2015;**15**:fov002.
- Van Uden N, Faia MDM., Assis-Lopes L. Isolation of *Candida albicans* from vegetable sources. *Microbiology* 1956;**15**:151–53.