

Social wasps are a *Saccharomyces* mating nest

Irene Stefanini^{a,1}, Leonardo Dapporto^{b,1}, Luisa Berná^c, Mario Polsinelli^d, Stefano Turillazzi^{d,e}, and Duccio Cavalieri^{a,d,2}

^aCentre for Research and Innovation, Fondazione Edmund Mach, 38010 Trento, Italy; ^bInstitut de Biologia Evolutiva, Consejo Superior de Investigaciones Científicas-Universitat Pompeu Fabra, ES-08003 Barcelona, Spain; ^cUnidad de Biología Molecular, Institut Pasteur de Montevideo, Montevideo 11400, Uruguay; ^dDepartment of Biology, University of Florence, 50019 Florence, Italy; and ^eCentro di Servizi di Spettromeria di Massa, University of Florence, 50100 Florence, Italy

Edited by Jeffrey P. Townsend, Yale University, New Haven, CT, and accepted by the Editorial Board December 9, 2015 (received for review August 18, 2015)

The reproductive ecology of *Saccharomyces cerevisiae* is still largely unknown. Recent evidence of interspecific hybridization, high levels of strain heterozygosity, and prion transmission suggest that outbreeding occurs frequently in yeasts. Nevertheless, the place where yeasts mate and recombine in the wild has not been identified. We found that the intestine of social wasps hosts highly outbred *S. cerevisiae* strains as well as a rare *S. cerevisiae* × *S. paradoxus* hybrid. We show that the intestine of *Polistes dominula* social wasps favors the mating of *S. cerevisiae* strains among themselves and with *S. paradoxus* cells by providing a succession of environmental conditions prompting cell sporulation and spores germination. In addition, we prove that heterospecific mating is the only option for European *S. paradoxus* strains to survive in the gut. Taken together, these findings unveil the best hidden secret of yeast ecology, introducing the insect gut as an environmental alcove in which crosses occur, maintaining and generating the diversity of the ascomycetes.

yeasts | *Saccharomyces cerevisiae* | *Saccharomyces paradoxus* | hybrids | social wasps

Since the birth of agriculture, the budding yeast *Saccharomyces cerevisiae* has flourished in human-made fermented products (1). However, insects such as social wasps have been recently shown to host *S. cerevisiae* in their intestine and spread them in the wild (2). For a long time it was agreed that the mating of *S. cerevisiae* spores mostly occurs between spores belonging to the same ascus (self-fertilization/inbreeding) and that outbreeding (mating of spores belonging to different asci) is a very uncommon event (3). However, several recent findings have called this hypothesis into question. Evidence of interspecific hybridization (4–6), a high level of strain heterozygosity (7, 8), and prion transmission (9) have suggested that outbreeding could occur more frequently than previously estimated (9).

We calculated the outbreeding rate from the heterozygosity level at polymorphic sites in three genes selected as able to reproduce the topology generated with the genomes of *S. cerevisiae* (10). Calculation of the outbreeding rate was carried out only on diploid strains for which the sequences of the three genes were available ($n = 34$; *SI Appendix, Table S1*), and was based on a modified model, accounting for the possibility of diploid individuals to derive either from intra-ascus mating or from outcrossing (11). Isolates from wasp gut were more likely to have originated from outbreeding compared with strains isolated from other sources (Fig. 1*A*). There are two possible reasons that could have led to this situation: either wasps prefer to feed on mated yeasts or the insect intestine makes yeast mating more likely.

If wasps prefer to feed on mated yeasts, a possibility suggested by the evidence that fruit flies are differentially attracted by *S. cerevisiae* strains (12), we should have inferred almost the same outbreeding rate for strains isolated from wasp intestines and grapes, although this was not the case (Fig. 1*A*). The second possibility is supported by previous findings reporting dissolution of yeast ascospores in the *Drosophila melanogaster* intestine (13). Nevertheless, this finding by itself does not demonstrate a role of the insects in favoring outbreeding: spore dissolution is necessary but not sufficient for outbreeding to occur. Indeed, dissolved spores

should germinate into viable cells and mate with cells from other strains, rather than self diploidizing (14).

Potentially, fruit flies could deliver dissolved spores onto ripe fruits, where the yeasts could meet other strains already present (15), but in this case we should have found the highest outbreeding rate for strains isolated from grape skin, rather than for those isolated from wasp gut. To justify the high outbreeding rate calculated for wasp isolates, several capital conditions should have been satisfied, either before wasp uptake or during the passage through the insect intestine: the simultaneous presence of different strains in the same environment and environmental changes allowing yeast cell sporulation, spore germination, and mating outside the ascus. The alimentary canal of adult insects, furnishing chemical–physical characteristics varying from the foregut to the hindgut (i.e., pH and oxygen availability) (16) and hosting for prolonged periods yeast cells collected from the wide range of environments where the wasps feed (2), is a good candidate for satisfying these conditions and allowing yeast mating. We tested the hypothesis of *S. cerevisiae* (Sce) crossing within the insect intestine by feeding *Polistes dominula* wasps five different Sce strains, each one recognizable by auxotrophic profile and genetic markers (*SI Appendix, Tables S2 and S3*). To prevent external introduction of yeast strains or exchange between insects, the wasps were kept individually separated in natural hibernation conditions. The Sce populations present within the intestine were investigated after a hibernation period lasting at least 2 mo (60 d) and compared with the population observable by

Significance

Despite the widespread interest on *Saccharomyces cerevisiae*, its wild lifestyle is far from being completely understood, with one of the most resounding examples being its sexual attitude. We show that the intestine of social wasps favors the mating of *Saccharomyces* strains by providing a succession of environmental conditions prompting sporulation and germination. We also demonstrate that the insect intestine favors hybridization of *S. cerevisiae* and *Saccharomyces paradoxus*. Although *S. paradoxus* survives in wild environments and rarely mates with *S. cerevisiae*, we discover that two European *S. paradoxus* strains cannot survive the wasp's intestinal environment but can be rescued through interspecific hybridization with *S. cerevisiae*. These findings are introducing insects as environmental alcoves in which yeast cells can meet and mate.

Author contributions: I.S., L.D., M.P., S.T., and D.C. designed research; I.S. and L.D. performed research; I.S., L.D., L.B., and D.C. analyzed data; and I.S., L.D., L.B., M.P., S.T., and D.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. J.P.T. is a guest editor invited by the Editorial Board.

Freely available online through the PNAS open access option.

¹I.S. and L.D. contributed equally to this work.

²To whom correspondence should be addressed. Email: duccio.cavalieri@unifi.it.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1516453113/-DCSupplemental.

hybrid (*SI Appendix, Fig. S7*). This could indicate that the strains face several sporulation-germination cycles while residing in the insect intestines. As inoculated diploid cells were able to hybridize (i.e., E324 and W303), they should have sporulated and then germinated before mating. Both RSY strains may be able to form hybrids, but the isolation of hybrids of RSY269 only indicates that after the mating, another sporulation event occurred within the insect intestines. Heterozygous *UME6* deletion strains, generated by the mating of RSY269 with one of the other strains inoculated to the wasps, could also have sporulated, but we were not able to isolate them because of their reduced germination ability (19).

We can conclude that the social wasp intestine provides *S. cerevisiae* with an environment favoring outbreeding and likely represents a highly selective environment for its “wild cousin” *S. paradoxus*. The Spa strains used were selected to represent the European *S. paradoxus* population (22). We are aware that the use of two *S. paradoxus* strains from the European clade of the *paradoxus* tree (22) could potentially lead to genotype-specific observations, favored by peculiar adaptations to a given environment (26). However, the common “mittel-European” geographic origin of the yeast strains and the wasps used in this study is coherent with the aim to mimic the ecological settings under investigation. Even if the evidence that the tested *S. paradoxus* were able to survive in the insect intestine only as hybrids with *S. cerevisiae* could be biased by the genotype of the selected strains, the lack of reports on the isolation of pure Spa strains from this environment in natural settings could indicate a general lower fitness of the pure strains compared with the interspecific hybrids. Further studies with strains and insects from other geographical areas will be needed to generalize our findings. However, the reported ability of the yeast strains studied here to sporulate, germinate, and mate, giving rise to intra- and interspecific hybrids, represents a milestone in our knowledge regarding the natural history of *Saccharomyces*, elucidating how their evolution depends on interaction with insects, favoring the formation of new genetic combinations. Ultimately, the high rate of outbreeding in insect intestine can provide a key to explaining the growing evidence for the existence of a high level of diversity and mosaicism in yeast populations (27). This diversity could be instrumental to surviving and thriving both in the wasps’ intestine and in the novel environmental microenvironments where insect-borne *Saccharomyces* cells will land, thanks to the hybrid fitness phenomenon (28, 29).

Finally, the direct link between social insects and the yeast species biodiversity is relevant to human industry, as the genetic diversity generated in the wasp’s gut could favor adaptation to the ever-changing fermentative environment, as demonstrated by the evidence that several of the most successful industrial strains are interspecific hybrids (30). Thus, preserving the treasure potentially hidden in the gut of vineyard wasps could be relevant from both the ecological and biotechnological standpoints.

Materials and Methods

Determination of the Inbreeding of *S. cerevisiae* Strains. The 34 *S. cerevisiae* isolates analyzed in this study in terms of inbreeding rate are listed in *SI Appendix, Table S1*. This set of strains encompasses strains isolated from natural (wasp intestine, grapes), industrial (wine fermentation), and human-related (human feces) environments. Population genetics analysis was carried out on the polymorphic sites of three genes whose sequences are able to reproduce the structure of the tree constructed on the whole-genome sequences of *S. cerevisiae* strains, *EXO5*, *URN1*, and *IRC8* (10). Only sequences of (at least) diploid *S. cerevisiae* strains were included in the study, downloaded from SGD (*Saccharomyces* Genome Database) and from NCBI (National Centre for Biotechnology Information) (last access, May 2015). SNPs were identified by multiple alignment, and a unique sequence was generated by concatenating them, as previously described (10). Heterozygous sites

were maintained as ambiguous bases. The outbreeding rate was estimated for *S. cerevisiae* strains through an R-script calculating the Johnson’s outbreeding rate (11). Results were compared with inbreeding levels calculated with the *fstat()* function of the *adegenet* R package (31) and with *FSTAT* (32) and *RMES* (33). The model proposed by Johnson and coworkers is considered to be the most suitable for calculating the inbreeding rate of microorganisms such as *S. cerevisiae* and *S. paradoxus*, as it takes into account the possibility of diploid individuals deriving either from intra-ascus mating or from random outcrossing. We thus referred the text to the results of this calculation, even considering that the inbreeding levels calculated with *FSTAT*, *RMES*, and the *fstat()* function of the *adegenet* R package gave similar results. Differences between strains, grouped according to the isolation source (wasp intestines, grapes, wines), were evaluated by applying the Wilcoxon test [*wilcox.test()* function of the *stats* R package (34)].

In Vivo Experiments: Insect Feeding and Dissection, Yeast Isolation, and Identification.

Wasps were inoculated with two different types of strain mixes. The first, encompassing only *S. cerevisiae* strains, was made up of equal amounts of W303, BY4742 FOX3/GFP, E324 (17), RSY269, and RSY270 (18) strains (*SI Appendix, Table S2*). The second mix of strains encompassed both *S. cerevisiae* (the same as the first mix) and *S. paradoxus* strains CBS432 and Q95.3 (23). Before inoculation, the strains were grown separately overnight in rich medium [YPD: 1% (wt/vol) yeast extract, 2% (wt/vol) peptone, 2% (wt/vol) dextrose]. After the overnight preculture, strains were counted through a Bürker chamber and were diluted in sterile water to obtain a 6× (for the mix encompassing *S. cerevisiae* strains only) or 8× (for the mix encompassing *S. cerevisiae* and *S. paradoxus* strains) cell suspension. The mixes were prepared just before wasp feeding to avoid eventual mating of strains before inoculation. Each yeast mix, having a final cell concentration of 10^{10} cells/mL, contained the same amount of each strain (1.5×10^9 cells/mL and 1.25×10^9 cells/mL for the mix made up only of *S. cerevisiae* and for the mix made up of *S. cerevisiae* and *S. paradoxus*, respectively). To make the yeast solution more appealing to wasps, the mixes were supplemented with small amounts of sugar (sucrose, <0.1% wt/vol). Each wasp was fed individually: the wasp was collected with tweezers and 10 μ L cell mix (containing 10^8 yeast cells) was injected directly into its mouth with a pipette, both to avoid cell dispersion and to ensure the ingestion of all of the cells.

Wasp Treatment and Dissection. Healthy and parasite-infected wasps were caught before hibernation and were inoculated with 10^8 yeast cells suspended in water, with the addition of sugar. Control wasps were fed with sugared water. Treated wasps were kept separate from each other in 6-well plates at seasonal temperatures. Wasp survival was scored twice a week. After 2 or 4 hibernation months (for *S. cerevisiae* only or for *S. paradoxus* and *S. cerevisiae* mixes, respectively), the surviving wasps were washed twice with water to avoid external contamination and then dissected in sterile conditions in sterile Petri dishes, using sterile clamps under a stereomicroscope. When possible, crop and gut were analyzed separately. Crop and gut contents were suspended in 100 μ L sterile water and plated at low density (yielding a maximum of 10 colonies per plate) on rich solid medium (YPD, 2% Agar) supplemented with penicillin and streptomycin to prevent bacterial growth (35). Plates were kept at 27 °C for at least 2 d, and then colonies were further isolated. *Saccharomyces sensu stricto* strains were identified by means of ITS1-4 RFLP (4); *S. cerevisiae* and *S. paradoxus* strains were identified by means of *MET2* RFLP (36). Isolated *S. cerevisiae* strains were identified by means of their auxotrophies, by spotting 5 μ L water cell suspension on YNB [0.67% (wt/vol) YNB with ammonium sulfate, without amino acids, 2% (wt/vol) dextrose, 2% (wt/vol) agar] lacking specific amino acids or bases (*SI Appendix, Table S2*) and confirmed by means of strain-specific DNA amplification (*SI Appendix, Table S3*).

ACKNOWLEDGMENTS. We thank R. Strich [UMDNJ (University of Medicine and Dentistry of New Jersey) School of Osteopathic Medicine] and J. Ramírez (Universidad de Extremadura) for providing the nongerminating and nonsporulating *S. cerevisiae* strains. We are also grateful to J.-L. Legras [INRA (Institut National de la Recherche Agronomique)] and A. Luchetti (University of Bologna) for their insight on population genetics and Vivienne Frankell for manuscript proofreading. This work was supported by the Integrated Programme Agreement “METAFODLABS,” reference number S116/2012/537723, funded by the Autonomous Province of Trento and by the research office of the Autonomous Province of Trento.

1. Cavalieri D, McGovern PE, Hartl DL, Mortimer R, Polsinelli M (2003) Evidence for *S. cerevisiae* fermentation in ancient wine. *J Mol Evol* 57(Suppl 1):S226–S232.

2. Stefanini I, et al. (2012) Role of social wasps in *Saccharomyces cerevisiae* ecology and evolution. *Proc Natl Acad Sci USA* 109(33):13398–13403.

3. Cubillos FA, Vásquez C, Faugeron S, Ganga A, Martínez C (2009) Self-fertilization is the main sexual reproduction mechanism in native wine yeast populations. *FEMS Microbiol Ecol* 67(1):162–170.
4. Sebastiani F, Barberio C, Casalone E, Cavalieri D, Polsinelli M (2002) Crosses between *Saccharomyces cerevisiae* and *Saccharomyces bayanus* generate fertile hybrids. *Res Microbiol* 153(1):53–58.
5. Murphy HA, Zeyl CW (2010) Yeast sex: Surprisingly high rates of outcrossing between asci. *PLoS One* 5(5):e10461.
6. Gray JC, Goddard MR (2012) Sex enhances adaptation by unlinking beneficial from detrimental mutations in experimental yeast populations. *BMC Evol Biol* 12:43.
7. Burke MK, Liti G, Long AD (2014) Standing genetic variation drives repeatable experimental evolution in outcrossing populations of *Saccharomyces cerevisiae*. *Mol Biol Evol* 31(12):3228–3239, 10.1093/molbev/msu256.
8. Magwene PM (2014) Revisiting Mortimer's Genome Renewal Hypothesis: Heterozygosity, homothallism, and the potential for adaptation in yeast. *Adv Exp Med Biol* 781:37–48.
9. Kelly AC, Wickner RB (2013) *Saccharomyces cerevisiae*: A sexy yeast with a prion problem. *Prion* 7(3):215–220.
10. Ramazzotti M, Berná L, Stefanini I, Cavalieri D (2012) A computational pipeline to discover highly phylogenetically informative genes in sequenced genomes: application to *Saccharomyces cerevisiae* natural strains. *Nucleic Acids Res* 40(9):3834–3848.
11. Johnson LJ, et al. (2004) Population genetics of the wild yeast *Saccharomyces paradoxus*. *Genetics* 166(1):43–52.
12. Christiaens JF, et al. (2014) The fungal aroma gene *ATF1* promotes dispersal of yeast cells through insect vectors. *Cell Reports* 9(2):425–432.
13. Reuter M, Bell G, Greig D (2007) Increased outbreeding in yeast in response to dispersal by an insect vector. *Curr Biol* 17(3):R81–R83.
14. Ruderfer DM, Pratt SC, Seidel HS, Kruglyak L (2006) Population genomic analysis of outcrossing and recombination in yeast. *Nat Genet* 38(9):1077–1081.
15. Polsinelli M, Romano P, Suzzi G, Mortimer R (1996) Multiple strains of *Saccharomyces cerevisiae* on a single grape vine. *Lett Appl Microbiol* 23(2):110–114.
16. Engel P, Moran NA (2013) The gut microbiota of insects - diversity in structure and function. *FEMS Microbiol Rev* 37(5):699–735.
17. Mortimer RK, Romano P, Suzzi G, Polsinelli M (1994) Genome renewal: A new phenomenon revealed from a genetic study of 43 strains of *Saccharomyces cerevisiae* derived from natural fermentation of grape musts. *Yeast* 10(12):1543–1552.
18. Ramírez M, Ambrona J (2008) Construction of sterile *ime1*Δ-transgenic *Saccharomyces cerevisiae* wine yeasts unable to disseminate in nature. *Appl Environ Microbiol* 74(7):2129–2134.
19. Strich R, Khakhina S, Mallory MJ (2011) *Ume6p* is required for germination and early colony development of yeast ascospores. *FEMS Yeast Res* 11(1):104–113.
20. Akhtar M (1982) Studies on digestive enzymes of some orthopteroide insects. *Pakistan Entomol.* 4(1–2):33–38.
21. Morales L, Dujon B (2012) Evolutionary role of interspecies hybridization and genetic exchanges in yeasts. *Microbiol Mol Biol Rev* 76(4):721–739.
22. Liti G, et al. (2009) Population genomics of domestic and wild yeasts. *Nature* 458(7236):337–341.
23. Sweeney J, Kuehne H, Sniegowski P (2004) Sympatric natural *Saccharomyces cerevisiae* and *S. paradoxus* populations have different thermal growth profiles. *FEMS Yeast Res* 4(4-5):521–525.
24. Charron G, Leducq J-B, Bertin C, Dubé AK, Landry CR (2014) Exploring the northern limit of the distribution of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* in North America. *FEMS Yeast Res* 14(2):281–288.
25. Maclean CJ, Greig D (2008) Prezygotic reproductive isolation between *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*. *BMC Evol Biol* 8:1.
26. Smith EN, Kruglyak L (2008) Gene-environment interaction in yeast gene expression. *PLoS Biol* 6(4):e83.
27. Strobe PK, et al. (2015) The 100-genomes strains, an *S. cerevisiae* resource that illuminates its natural phenotypic and genotypic variation and emergence as an opportunistic pathogen. *Genome Res* 25(5):762–774.
28. Stelkens RB, Brockhurst MA, Hurst GD, Miller EL, Greig D (2014) The effect of hybrid transgression on environmental tolerance in experimental yeast crosses. *J Evol Biol* 27(11):2507–2519.
29. Piatkowska EM, Naseeb S, Knight D, Delneri D (2013) Chimeric protein complexes in hybrid species generate novel phenotypes. *PLoS Genet* 9(10):e1003836.
30. Plech M, de Visser JA, Korona R (2014) Heterosis is prevalent among domesticated but not wild strains of *Saccharomyces cerevisiae*. *G3 (Bethesda)* 4(2):315–323.
31. Paradis E (2010) *pegas*: An R package for population genetics with an integrated-modular approach. *Bioinformatics* 26(3):419–420.
32. Goudet J, Perrin N, Waser P (2002) Tests for sex-biased dispersal using bi-parentally inherited genetic markers. *Mol Ecol* 11(6):1103–1114.
33. David P, Pujol B, Viard F, Castella V, Goudet J (2007) Reliable selfing rate estimates from imperfect population genetic data. *Mol Ecol* 16(12):2474–2487.
34. R Core Team (2012) R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria).
35. Rose M, Winston F, Hieter P (1990) *Methods in yeast genetics—a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY.
36. Masneuf I, Hansen J, Groth C, Piskur J, Dubourdieu D (1998) New hybrids between *Saccharomyces sensu stricto* yeast species found among wine and cider production strains. *Appl Environ Microbiol* 64(10):3887–3892.