2012

Prezygotic Isolation Between Saccharomyces Cerevisiae and Saccharomyces Paradoxus Through Differences in Mating Speed and Germination Timing

Helen A. Murphy
William & Mary, hamurphy@wm.edu

Clifford W. Zeyl

Recommended Citation

Murphy, Helen A. and Zeyl, Clifford W., Prezygotic Isolation Between Saccharomyces Cerevisiae and Saccharomyces Paradoxus Through Differences in Mating Speed and Germination Timing (2012). *Evolution*, 66(4), 1196-1209.
10.1111/j.1558-5646.2011.01516.x

doi:10.1111/j.1558-5646.2011.01516.x

PREZYGOTIC ISOLATION BETWEEN *SACCHAROMYCES CEREVISIAE* AND *SACCHAROMYCES PARADOXUS* THROUGH DIFFERENCES IN MATING SPEED AND GERMINATION TIMING

Helen A. Murphy[1,2] and Clifford W. Zeyl[3]

[1]*Department of Biology, The College of William and Mary, Williamsburg, Virginia 23187–8795*

[2]*E-mail: hamurphy@wm.edu*

[3]*Wake Forest University, Winston-Salem, North Carolina 27109*

Received September 10, 2009
Accepted November 4, 2011
Data Archived: Dryad doi:10.5061/dryad.708bb5kr

Although prezygotic isolation between sympatric populations of closely related animal and plant species is well documented, far less is known about such evolutionary phenomena in sexual microbial species, as most are difficult to culture and manipulate. Using the molecular and genetic tools available for the unicellular fungus *Saccharomyces cerevisiae*, and applying them to *S. paradoxus*, we tested the behavior of individual cells from sympatric woodland populations of both species for evidence of prezygotic isolation. First, we confirmed previous observations that vegetative cells of both species mate preferentially with *S. cerevisiae*. Next, we found evidence for mate discrimination in spores, the stage in which outcrossing opportunities are most likely to occur. There were significant differences in germination timing between the species: under the same conditions, *S. paradoxus* spores do not begin germinating until almost all *S. cerevisiae* spores have finished. When germination time was staggered, neither species discriminated against the other, suggesting that germination timing is responsible for the observed mate discrimination. Our results indicate that the mechanisms of allochronic isolation that are well known in plants and animals can also operate in sexual microbes.

KEY WORDS: **Allochronic isolation, mating speed, reproductive isolation, spore germination, wild yeast.**

Reproductive isolation can prevent gene flow between sympatric species, maintaining their genetic isolation (Coyne and Orr 2004). Where hybrids between sympatric species have low fitness or fertility, prezygotic isolation can prevent mistakes in mate choice and the associated fitness cost of wasted reproductive effort. It can also play a role in the process of speciation. Prezygotic isolating mechanisms can take many forms, including differences in signals that allow individuals to recognize appropriate mates. Examples include mating calls in crickets (Mendelson and Shaw 2002), song and plumage color in birds (Saetre et al. 1997; Uy et al. 2008), body color in fish (Seehausen and van Alphen 1998), and pheromones in moths (McElfresh and Millar 2001). Other mechanisms may take the form of allochronic isolation: differences in the timing of life-history traits that lead to isolation between populations or species. Examples include flowering time (Ellis et al. 2006), spawning synchronization in algae (Clifton 1997) and corals (Knowlton et al. 1997), diel activity in moths (Devries et al. 2008), and migration and breeding in salmon (Quinn et al. 2000).

Although such reproductive isolating mechanisms have received attention in animals and plants, far less is known about

prezygotic isolation in fungi (Kohn 2005). The diversity of fungal life cycles, which can uncouple meiosis from syngamy and cell fusion from karyogamy, may hold interesting mechanisms of prezygotic isolation not found in other organisms. In a meta-analysis of the few studies that have been done, *Homobasidiomycota* tended to exhibit enhanced premating isolation in sympatry, whereas *Ascomycota* did not (Le Gac and Giraud 2008). Many of the species included in the analysis were parasitic, and their distinctive demographics and population structures make comparisons to other multicellular organisms difficult.

Unicellular sexual eukaryotes, many of which are fungal, are a particularly interesting group that has received almost no attention. For many plants and animals, sex is a necessary risk of reproduction, and numerous gametes may reduce the fitness cost of a mistake in mate choice. In multicellular fungi, an individual can mate with multiple partners, including itself, and without necessarily fusing nuclei, again reducing the cost of choosing a postzygotically incompatible partner. For example, genetically diverged, sympatric isolates of *Neurospora* exhibited a pattern of prezygotic reproductive isolation (Dettman et al. 2003). By contrast, for a single cell, mating not only interrupts mitotic reproduction, but nuclear fusion risks permanently trapping its lineage in a sterile hybrid. This may have little population-level effect but would be expected to impose strong selection on individual cells to avoid such a fate. Prezygotic isolation has been evolved in laboratory populations of *Saccharomyces cerevisiae*, a unicellular, free-living fungus, when strong selection and a high encounter rate were imposed (Leu and Murray 2006). However, the life cycle of *Saccharomyces* yeasts is unknown in nature and it is unclear whether such prezygotic isolation would be expected in natural populations. Our research focuses on two *Saccharomyces* species, *S. cerevisiae* and *S. paradoxus*.

They can be collected in woodlands and cultured in the laboratory, making them an ideal system for ecological and evolutionary studies (Replansky et al. 2008). These yeasts have a haplodiplontic life cycle; asexual growth can occur in both the haploid and diploid vegetative phase. Under nutrient limiting conditions, diploid cells undergo meiosis forming four stress-resistant haploid spores encased in an ascus. When conditions become favorable, spores germinate and become metabolically active cells once again. Mating occurs when haploid cells of opposite mating type (*a* and α) fuse. Woodland isolates of both species are homothallic, meaning that haploid cells can switch mating types after budding off a daughter cell. Because those cells are then compatible mates, the haploid stage is probably transient in natural populations; *S. paradoxus* and woodland *S. cerevisiae* isolates are invariably diploid. Despite the strong cost to hybridization, these yeasts can mate across species boundaries in the laboratory, suggesting the compatibility of the mating systems and leaving open the question of how they may avoid each other in nature.

Initial studies of the mating dynamics of *S. cerevisiae* and *S. paradoxus* have yielded intriguing results. First, in an analysis of mating behavior of four sympatric woodland strains of *S. cerevisiae* and *S. paradoxus*, Murphy et al. (2006) found that prezygotic reproductive isolation may exist in the form of differential mating kinetics. In cell-to-cell mate choice trials of vegetative cells, they found *S. cerevisiae* mated with itself significantly more than would be expected if mate choice were random. Surprisingly, *S. paradoxus* also mated significantly more with *S. cerevisiae*. *Saccharomyces cerevisiae* mated faster than *S. paradoxus*, and was more likely to mate overall; the mating propensities accounted for the outcome of the mate choice trials. It appeared that when an *S. paradoxus* cell was offered both *S. paradoxus* and *S. cerevisiae* cells as potential partners, the *S. cerevisiae* cell, with its high mating propensity, controlled the interaction resulting in an inappropriate mating. Prezygotic isolation in nature may be a simple result of these mating propensity differences. Although interesting, these results were based on only two strains per species and may simply be strain effects rather than a species effect.

Second, Maclean and Greig (2008) performed mate choice trials on five pairs of *S. paradoxus* and *S. cerevisiae* strains, including those used by Murphy et al. (2006). When given the choice between their own strain and the other species, spores of both species were more likely to mate with their own strain than would be expected if mating occurred randomly. Furthermore, they found that *S. cerevisiae* was choosier than *S. paradoxus*. They hypothesized that these results were due to differences in germination timing. Although the results showed a clear pattern of prezygotic isolation, there was no evidence of species-level mating discrimination. Their study assayed only five individual interspecific pairings, leaving open the possibility of strain effects or more importantly, that *Saccharomyces* species tend to inbreed in the spore stage regardless of the origin of the other potential mating partner.

The goal of the present study was to determine whether prezygotic isolation exists in the form of mating discrimination among natural yeast populations. We completed an exhaustive mating analysis including within-species dynamics of a large sample of woodland isolates from sympatric populations to address this question.

The two sympatric woodland populations used in this study are described in detail elsewhere (Kuehne 2005); we will briefly summarize them here. In eastern North America, *S. cerevisiae* exhibits a clonal population structure, with three main haplotypes dispersed among numerous woodlands, with some woodlands and even single trees containing multiple haplotypes. Microsatellite divergence was detected within haplotypes that was associated with woodland collection site. To distinguish between the haplotypes in this study, we arbitrarily label them genetic group A, B, and C. Second, *S. paradoxus* exhibited two genetically isolated

populations within North America: a large recombining population found throughout the sampled woodlands and a smaller clonal population that appeared to have migrated from Europe and whose range is limited to a few woodland sites (Kuehne et al. 2007). Following the convention of Kuehne et al. (2007), we refer to the migrant population as genetic group A and the large recombining population as genetic group B. The two *S. paradoxus* genetic groups within North America exhibit their own nonrandom mating dynamics (H. A. Murphy and C. W. Zeyl, unpubl. ms.) and will not be discussed here. Rather, we focus on the species-level interactions between populations of *S. cerevisiae* and *S. paradoxus* that are known to inhabit the same woodlands.

Materials and Methods

COLLECTION OF STRAINS

The strains and sampling method are described in detail elsewhere (Sniegowski et al. 2002; Kuehne 2005; Kuehne et al. 2007). Briefly, oak flux (or surrounding soil) was sampled in a number of woodlands in eastern North America and passed through a series of media that favored growth of *Saccharomyces* yeasts. From each environmental sample, one putative yeast colony was isolated and crossed with known tester strains to determine species membership. Later genetic analyses on nine loci confirmed species identity. All isolates were stored in 15% glycerol at −80°C. For the current study, we chose six strains available from each population to be analyzed.

MEDIA

For growth and mating, we used synthetic oak exudate (SOE), a medium developed to loosely approximate oak exudate (Murphy et al. 2006) that contains 1% sucrose, 0.5% fructose, 0.5% dextrose, 0.15% peptone, 0.1% yeast extract, and 2% agar for solid medium. For sporulation, we first grew cultures in Yeast Peptone Dextrose (YPD): 2% dextrose, 1% yeast extract, 2% peptone, and then transferred to sporulation plates: 1% potassium acetate, 0.005% zinc acetate, and 2% agar (Rose et al. 1990). For selection of transformants and identification of mated pairs, growth medium was supplemented with 150 μg/mL G418, 50 μg/mL CloNat, or 400 μg/mL hygromycin, as appropriate.

CONSTRUCTION OF YEAST STRAINS

The natural isolates were all homothallic; to perform mating assays, heterothallic strains were required. The natural isolates were transformed using a standard lithium acetate procedure (Gietz and Woods 2002) or a modified protocol for low transformation efficiency strains (Gerke et al. 2006) with KanMX4 (Wach et al. 1994), NatMX4, or HygMX4 (Goldstein and McCusker 1999) cassettes targeted to the HO gene. Successful transformants were sporulated and the resulting tetrads dissected. From each natural isolate, stable *a* and α kanamycin-resistant strains from the same ascus were identified and stored in 15% glycerol. These haploid strains were then transformed with a different cassette to construct isogenic strains with different antibiotic markers. Diploids homozygous for an antibiotic resistance were created by mating *a* and α strains, which preserved the original genetic composition. Two complete sets of strains with different antibiotic resistances were created; transformations were polymerase chain reaction verified (Tables 1 and 2).

SPORE VIABILITY

Diploid colonies from the mate choice trials described below were sporulated. Asci were digested using a standard zymolyase procedure (Rose et al. 1990) and dissected on SOE plates. For each original colony, approximately 32 spores were laid out in a grid-like pattern. The plates were incubated at 30°C for 72 h, when the numbers of colonies visible to the naked eye were recorded. For most strain combinations, the spore viability was assayed for two to five orginal zygotes. However, for some strain combinations of interspecies hybrid mating, only one zygote was assayed due to poor sporulation. The data were analyzed using a one-way nested analysis of variance (ANOVA) with planned comparisons in JMP Version 8.0.1 (SAS Institute Inc., Cary, NC).

MATE CHOICE TRIALS: VEGETATIVE CELLS

Liquid cultures were grown overnight in 5-mL SOE at 30°C in a shaking incubator. Small amounts (approximately 10 μL of 1:100 dilution) of each of three cultures were placed in separate drops on an SOE plate and used as the source pools to set up mate choice trials. Using a micromanipulator and Zeiss Axioskop FS microscope, approximately 16–20 trials were set up at marked locations on each plate. For each trial, a focal cell (either *a* or α) was placed in contact in a triangle formation with two cells of the other mating type, each with a different antibiotic resistance; one cell was derived from the same strain as the focal strain and the other from a different strain. Mating plates were incubated at 30°C for 3–4 h, after which for each trial, the budding haploid cell was separated from the zygote and moved to a marked location on the plate. Once colonies formed approximately two days later, the plates were replica plated to antibiotic plates to determine that cells had mated. To account for possible effects of plate or assay date, for all strain combinations, mate choice trials were performed on multiple plates over multiple days. Only trials that contained budding haploid cells were used for analysis, as this ensured a choice between two viable mating partners.

Because the mating types of the cells were known, all trials offered the focal cell two partners of opposite mating type and thus were informative. Therefore, if mating occurred randomly, 50% of the time the focal cell would have mated with the partner

Table 1. *Saccharomyces cerevisiae* strains. Genetic groups A, B, and C refer to the three haplotype found in the woodland population of *S. cerevisiae*. The letters were assigned arbitrarily to indicate genetically similar isolates. Two sets of transformants were created from the original isolates: one with kanamycin resistance and one with either hygromycin (H) or CloNat (N) resistance. In the latter case, the letters next to strain numbers indicate resistance.

Species/ haplotype[a]	Collection location	Original strain[b]	*ho*::*KAN MATa*	*ho*::*KAN MATα*	*ho*::*HYG/ NAT MATa*	*ho*::*HYG/ NAT MATα*	Diploid *ho*:: *HYG/NAT*	Diploid *ho*:: *HYG/NAT*
Sc/A (1)	Buck Hill Falls, PA	YPS670	YPS3060[c]	YPS3062[c]	YPS3332[c] N	YPS3340[c] N	YMZ32	YMZ33 N
Sc/A (2)	New Brunswick, NJ	YPS630	YPS2073	YPS2076	YMZ47	YMZ53 H	YMZ39	YMZ56 H
Sc/B (3)	New Brunswick, NJ	YPS615	YPS2057	YPS2058	YPS3343 N	YPS3326 N	YMZ34	YMZ35 N
Sc/B (4)	New Brunswick, NJ	YPS623	YPS2066	YPS2063	YMZ44 H	YMZ45 H	YMZ38	YMZ50 H
Sc/C (5)	Buck Hill Falls, PA	YPS681	YPS3137[c]	YPS3138[c]	YPS3317[c] N	YPS3318[c] N	YMZ30	YMZ31 N
Sc/C (6)	Media, PA	YPS133	YPS2067	YPS2070	YMZ52 H	YMZ10 H	YMZ36	YMZ55 H

[a]The number in parentheses refers to the label given to the strains in the figures of this article.
[b]As described in Sniegowski et al. (2002) and Kuehne (2005).
[c]As described in Murphy et al. (2006).

Table 2. *Saccharomyces paradoxus* strains. Genetic group A refers to migrants from Eurasia; B refers to original North American population.

Species/genetic group[a]	Collection location	Original strain[b]	*ho*::*KAN MATa*	*ho*::*KAN MATα*	*ho*::*NAT MATa*	*ho*::*NAT MATα*	Diploid *ho*::*KAN*	Diploid *ho*::*NAT*
Sp/A (1)	Buck Hill Falls, PA	YPS642	YPS3421	YPS3422	YPS3385	YPS3386	YMZ16	YMZ17
Sp/A (2)	Tuscarora Forest, PA	YPS744	YMZ1	YMZ2	YPS3430	YPS3431	YMZ18	YMZ19
Sp/A (3)	Buck Hill Falls, PA	YPS644	YMZ26	YMZ27	YMZ24	YMZ25	YMZ29	YMZ28
Sp/B (4)	Buck Hill Falls, PA	YPS664	YPS3399[c]	YPS3400[c]	YPS3417[c]	YPS3418[c]	YMZ11	YMZ12
Sp/B (5)	Buck Hill Falls, PA	YPS646	YPS3420[c]	YPS3419[c]	YPS3405[c]	YPS3406[c]	YMZ14	YMZ13
Sp/B (6)	Buck Hill Falls, PA	YPS668	YPS3392	YMZ72	YPS3395	YPS3396	YMZ73	YMZ15

[a]The number in parentheses refers to the label given to the strains in the figures of this article.
[b]As described in Sniegowski et al. (2002) and Kuehne (2005).
[c]As described in Murphy et al. (2006).

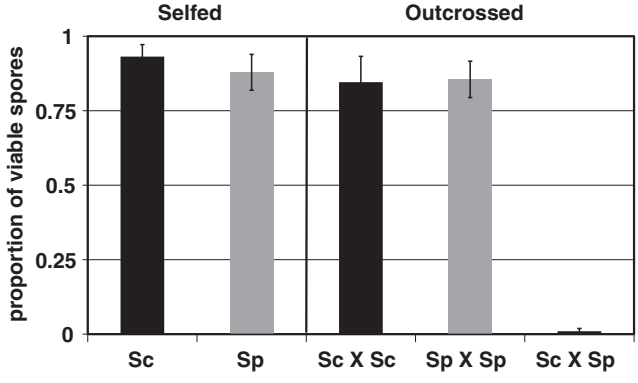

Figure 1. **Spore viability y-axis: proportion of visible colonies after 72 h of growth. The number of strain combinations in each category is 6, 6, 14, 6 (only within *S. paradoxus* genetic group strain combinations were analyzed), and 36 in order from Sc to Sc × Sp. Selfing refers to spore viability of a strain when inbred; outcrossing refers to spore viability of matings between strains. Hybrid spore viability is significantly lower than within-species spore viability ($P < 0.0001$).**

of the same strain. If the focal cell mates with the alternative strain less than 50% of the time, then it has a bias either toward mating with itself or against the alternative strain. The opposite is true if the focal cell mates with the alternative strain more than 50% of the time.

MATE CHOICE TRIALS: SPORES

Cultures were grown for 24–48 h in 3-mL YPD in a shaking incubator at 30°C, then spread on sporulation plates and incubated for 24–48 h. Asci were partially digested using a standard zymolyase procedure. Small amounts of digested asci (approximately 10 μL of 1:100 dilution) from two strains with different antibiotic resistances were placed in separate drops on an SOE plate and used a source pools for the mate choice trials. Using a micromanipulator and Zeiss Axioskop FS microscope, approximately 24–32 trials were set up at marked locations on each plate. For each trial, two spores from one strain and a third spore from the other strain were placed in contact in a triangle formation. After incubation at 30°C for approximately 6 h, the zygote and unmated cell were separated. Once colonies formed approximately two days later, the mating plates were replica plated to antibiotic plates to determine that spores had mated. As with vegetative cells, for all strain combinations, mate choice trials were performed on multiple plates and days with independent sporulated cultures. Only trials in which the unmated spore formed a colony were used for analysis, as this indicated that both potential partners had been viable spores.

Because the mating types of the spores were unknown, not all trials were informative (see Fig. 4A). If mating occurred randomly, then 66% of the time the focal spore would have mated with the partner from the alternative strain. If the focal spore mates with the alternative strain less than 66% of the time, then it either has a bias toward mating with itself or a bias against the alternative strain. The opposite is true if the focal spore mates with the alternative strain more than 66% of the time.

MATE CHOICE TRIALS: ANALYSIS

To test for deviations from random mating between species, binomial distributions corresponding to the null hypothesis of random mating were compared with the 0.025, 0.5, and 0.975 quantiles of binomial distributions that were calculated using the open-source software R. In calculating those quantiles, mate choice results from vegetative cells and from spores were pooled across groups and strains for each species. The results were also analyzed by logistic regression using function glm in R (Gelman and Hill 2007). The model fitted to interspecific trials was

$$\begin{aligned}&\text{logit (probability of hybridization)}\\&= \beta_0 + \beta_{\text{stage}} + \beta_{\text{chooser.species}} + \beta_{\text{stage}*\text{chooser.species}} + \alpha_{\text{chooser.group}}\\&\quad + \alpha_{\text{option.group}} + \alpha_{\cdot\text{chooser.group}*\text{option.group}},\end{aligned}$$

where the function logit $(x) = x/(1 - x)$ transforms probabilities, which are constrained to lie between 0 and 1, to an unbounded scale.

For each species independently, the following model was fitted to the pooled results of intraspecific spore and vegetative cell trials:

$$\begin{aligned}&\text{logit (probability of hybridization)}\\&= \beta_0 + \beta_{\text{stage}} + \alpha_{\text{chooser.group}} + \alpha_{\text{option.group}}\\&\quad + \alpha_{\cdot\text{chooser.group}*\text{option.group}}.\end{aligned}$$

Coefficients β estimate the effects of binary predictors such as species identity or life cycle stage: $\beta_{\text{stage}} = 1$ indicates spore as opposed to vegetative cell and $\beta_{\text{species}} = 1$ indicates *S. paradoxus* choosing. Error terms α indicate the contributions to variation in mate choice of hierarchical categories such as genetic group within species and strain within genetic group.

STAGGERED MATE CHOICE TRIALS

These trials were similar to the spore mate choice trials, except that the pools of *S. cerevisiae* asci were put down up to 4 h later than *S. paradoxus* asci. All plates for all time points were begun at the same time by putting down pools of *S. paradoxus* asci and isolating from them all the spores necessary for the mate choice trials. For time point zero, the *S. cerevisiae* strain was put down at the same time as the *S. paradoxus* and set up as described in section "Mate Choice Trials:Spores." For all other time points, *S. cerevisiae* spores were put on the plate 1, 2, 3, or 4 h after the *S. paradoxus* spores and only then did the spores come into contact with one another.

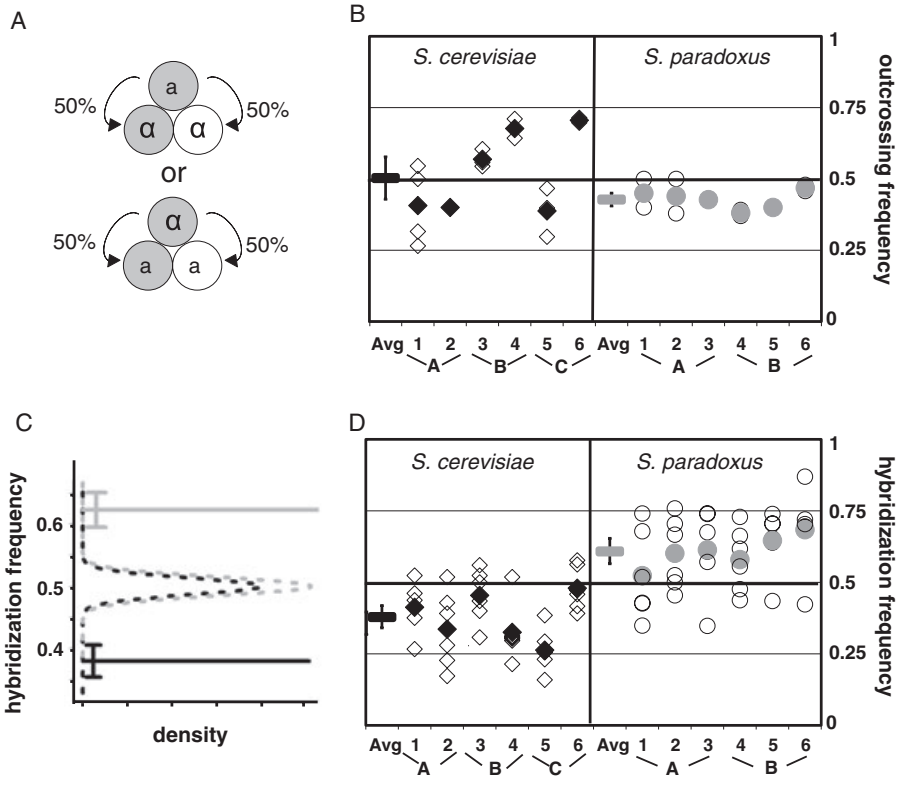

Figure 2. **Mate choice in vegetative cells (A) vegetative mate choice trial. A focal cell of one mating type is given the option of mating with two cells of opposite mating type one from its own strain and one from a different strain/species. Because the mating type of all cells in the trial is known, if mating is random, over numerous trials, the focal cell should mate with each of the choices 50% of the time. (B) Within-population mating. Y-axis is the proportion of outcrossing events out of the total number of mate choice trials conducted for a given strain combination. Each strain was given the choice between (1) mating with a partner of its own strain or (2) an alternative partner of the same population. Each *S. cerevisiae* strain was tested against a random subset of strains in its population and each *S. paradoxus* strain was tested against the other two strains of its genetic group (A and B); for each species, there were 758 and 566 trials, respectively, for an average of 47 trials per strain combination. Average responses for choosing strains are given by black diamonds for *S. cerevisiae* and gray circles for *S. paradoxus*; results of the individual strain combinations are open symbols. On the left of each panel is the average for the given species ±2 SEM (the proportion of hybridization for each pairwise combination was considered one datapoint). Random mating is represented by the black line. (C) Binomial probability density functions for interspecies trials. The curves represent the expected (null) distribution of hybridized trials for the number of trials performed, if mating is random. The vertical bars represent the median (with 2.5% and 97.5% binomial quantiles at the base of the line) of the actual observations. Black line—*S. cerevisiae*; gray line—*S. paradoxus*. (D) Interspecies mating. Y-axis is the proportion of hybridization events out of the total number of mate choice trials conducted for a given strain combination. All *S. cerevisiae* strains were tested against all *S. paradoxus* strains, and vice versa. There were 2724 mate choice trials over the 72 mating combinations, for an average of 35 trials per combination. Symbols as described in B.**

GERMINATION TIMING

Strains were sporulated as described above. Ten to 20 spores from each strain were placed on an SOE plate and incubated at 30°C; germination and budding were monitored every half hour for 510 min. As soon as germination was visible for a spore, the time was recorded; monitoring continued through the appearance of the first bud projection. For each strain, the assay was performed on multiple sporulated cultures, and to avoid possible variation among batches of medium/plates, all strains were assayed on the same batch of plates. Spores that germinated, but did not bud within the 540 min but subsequently formed visible colonies were recorded as having budded in 570 min. Although 570 min is a conservative estimate, there was no practical way to determine how long budding actually took. Most such delayed germination/budding occurred in *S. paradoxus* strains. The data were analyzed using ANOVA in JMP 8.0.1. There were two stages to the cell cycle (germination and budding), two species, five genetic groups (three nested in *S. cerevisiae* and two nested in *S. paradoxus*), 12 strains (three nested in each S. *paradoxus* genetic group and two nested in each *S. cerevisiae* genetic group), and anywhere from 15 to 40 (average 29) replicates of each strain at each stage depending on how many spores were viable.

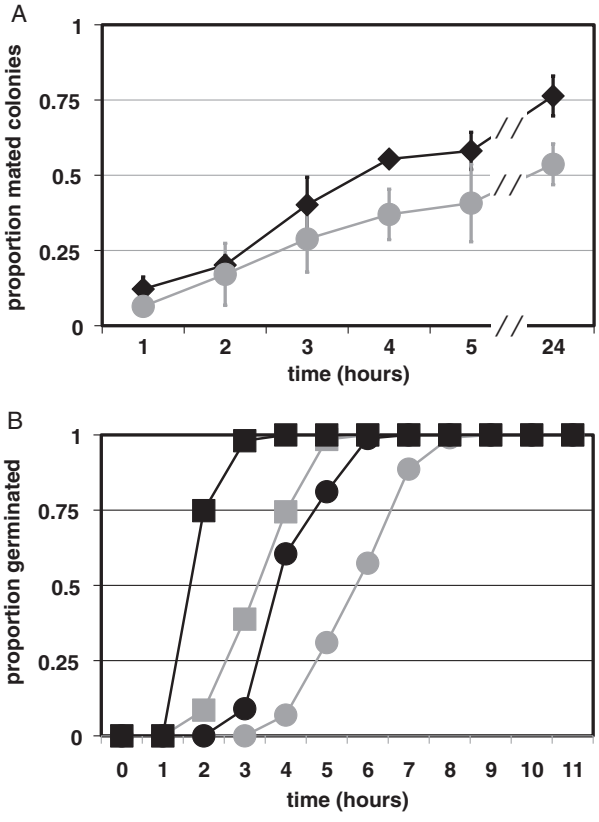

Figure 3. **Mating speed and germination time in *Saccharomyces*. Black represents *S. cerevisiae*; gray represents *S. paradoxus*. (A) Mating time in vegetative cells. For each strain, equal numbers of *a* and α cells were combined on a mating plate and the proportion of mated cells was monitored over time. Y-axis: proportion of population sample that had mated. For each species, the proportion of mated cells was averaged over all strains (± 2 SEM). (B) Spore germination. Time required to germinate and bud was monitored for spores of each strain. Y-axis: cumulative proportion of observed spores that germinated (squares) or projected a bud (circles) in a given 1-h time interval; strain results were averaged to obtain species results.**

MATING PROPENSITY

Cultures were grown overnight in 5-mL SOE in a shaking incubator, centrifuged, washed, resuspended in H_2O, and briefly sonicated to break up any clumps. For each strain, equal numbers of *a* and α cells with different antibiotic resistances were combined, poured onto 60-mm SOE plates, and dried in a sterile flow hood. Mating plates were incubated at 30°C and sampled every hour for 5 h, as well as once at 24 h. Each mating plate was sampled three times at each time point by scraping up small amounts of culture from three locations on the plate and suspended in water. The samples were diluted and spread onto an SOE plate; the number of colonies per plate was variable due to the inexact nature of scraping up cells from the mating plate and ranged anywhere from 60 to 250 colonies. Forty-eight hours later, the sample plates were replica plated to double antibiotic plates to determine the proportion of mated colonies. To minimize variation due to experimental error, the mating propensity assay was performed on all isolates at the same time. Data were analyzed using a nested two-way ANOVA in JMP 8.0.1. There were five time points (1–5 h), two species, 12 strains (six nested in each species), and three replicates of the measure of proportion of mated colonies for each strain at each time point. Three of the plates were not counted due to contamination.

Results

As with other interspecies crosses of *S. cerevisiae* and *S. paradoxus* (Naumov 1987, 1996), among our strains, spores derived from hybrids were almost completely inviable (Fig. 1; $P < 0.0001$), thus verifying the cost to mismating.

VEGETATIVE CELLS

Our results generally support a previous report that vegetative cells of both species tend to mate with vegetative *S. cerevisiae* cells (Murphy et al. 2006), although individual strain combinations varied (Fig. 2C,D; Table 3). This could simply result from a tendency of *S. cerevisiae* to inbreed and *S. paradoxus* to outcross, rather than discrimination against potential *S. paradoxus* partners, but intraspecific mate choice trials do not support this explanation. We tested it by allowing vegetative cells to choose between potential mates of their own strain and a different strain either from their own genetic group or from a different genetic group, but from the same species. In these assays, mating preferences varied among strain combinations (Table 4). Most *S. cerevisiae* strains did discriminate based on the genetic group (haplotype) of the alternative partner, but with no trend toward inbreeding: three strains tended to choose genetic groups other than their own, whereas the other three preferred mates of their own genetic group (Fig. 2B). In *S. paradoxus*, there were specific pairwise interactions among strains (Table 5) and even a tendency toward inbreeding (Fig. 2B, right panel). When the alternative partner was from the other genetic group, *S. paradoxus* strains mated in idiosyncratic patterns (data not shown, H. A. Murphy and C. W. Zeyl, unpubl. ms.). We conclude that the tendency of vegetative cells of both species to mate with *S. cerevisiae* is not explained by *S. cerevisiae* inbreeding or by *S. paradoxus* outcrossing.

Next, we determined whether mating propensity in all 12 strains could explain the preference of vegetative cells of both species for *S. cerevisiae* mates. *Saccharomyces cerevisiae* mated more quickly (Fig. 3A, Table 6) and was more likely to mate overall than *S. paradoxus* (one-way nested ANOVA at 24-h time point, $F = 14.67$, $P = 0.004$, df = 1, 9). There was significant variation among *S. paradoxus* strains, and the difference between the species was smaller than in previously reported assays (Murphy

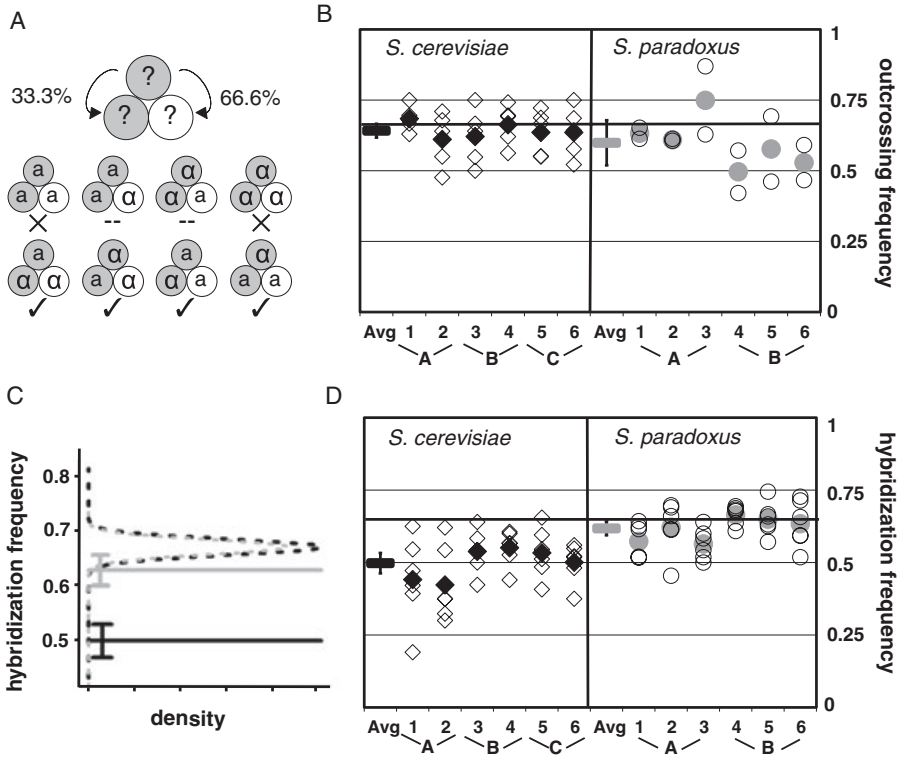

Figure 4. **Mate choice in spores (A) spore mate choice trial. Two spores of one strain (focal strain) and one spore of another strain/species were placed together; mating types of the spores were unknown. There were eight possible combinations of mating types and strains (shown in bottom two rows of panel). Two of the combinations could not lead to mating because all spores were of one mating type (denoted by X) and two required outcrossing because the alternative strain was the only compatible mating partner (denoted by –). The final four combinations allowed a choice to be made (denoted by √). If mating occurred randomly, over numerous trials, the focal strain should outcross 66% of the time (four out of six successful matings would be outcrossed). (B) Within-population mating. Y-axis is the proportion of outcrossing events out of the total number of mate choice trials conducted for a given strain combination. Each *S. cerevisiae* strain was tested against the other six strains in its species; there were 754 trials for an average of 25 trials per strain combination. Each *S. paradoxus* strain was tested against the other two strains of its genetic group (A and B); there were 273 mate choice trials for an average of 23 trials per strain combination. Symbols as described in 2B. (C) Binomial probability density functions for interspecies trials. The curves represent the expected (null) distribution of hybridized trials for the number of trials performed, if mating is random. The vertical bars represent the median (with 2.5% and 97.5% binomial quantiles at the base of the line) of the actual observations. Black line—*S. cerevisiae*; gray line—*S. paradoxus*. (D) Interspecies mating. Y-axis is the proportion of hybridization events out of the total number of mate choice trials conducted for a given strain combination. All *S. cerevisiae* strains were tested against all *S. paradoxus* strains, and vice versa. There were 2202 mate choice trials over the 72 strain combinations, for an average of 30 trials per combination. Symbols as described in 2B.**

et al. 2006). However, *S. cerevisiae* consistently mated more quickly and was more likely to mate, which may explain the results of the mate choice trials.

SPORES

Spores of both species were more likely to mate with a spore of their own strain than with one of the other species, as reported by Maclean and Greig (2008) for fewer strain combinations. *Saccharomyces cerevisiae* was by far the more discriminating species, but the overall hybridization frequency of *S. paradoxus* spores was significantly less than would result from random mating (Fig. 2B,C, right panel; Table 3). Hybridization was detected more often for spores of both species than for vegetative cells, as indicated by the positive logistic regression coefficient $\beta_{spore} = 0.48$ (Table 3). This does not mean, however, that spores were less discriminating. Because the mating types of spores in mating trials were unknown, if mating were random we would have observed a hybridization rate of two of three among spores, a frequency 16.7% higher than among randomly mating vegetative cells (see Materials and Methods). We therefore compare the observed effect of the spore stage on hybridization probability with the null hypothesis of a probability 17% higher than that for vegetative cells. A logistic regression coefficient can be converted to an effect of a unit change in the predictor variable on the probability of an observation by dividing the coefficient by 4 (Gelman and Hill 2007); here, this is the increase in probability of hybridization

Table 3. Logistic regression of mate choice in interspecies trials. Coefficients β (and standard errors) are effects of chooser species (*S. paradoxus* = 1, *S. cerevisiae* = 0) and life cycle stage (spore = 1, vegetative cell = 0) on probability of hybridization. Error terms α (and standard errors) attribute variation to group and strain within-group identities of choosers and options, and their interactions.

Effect	Coefficient (standard error)	Inference
Intercept	$\beta_0 = -0.48$ (0.09)	
Species choosing	$\beta_{species} = 0.98$ (0.13)	Hybridization is more likely when *S. paradoxus* is choosing
Life cycle stage	$\beta_{stage} = 0.48$ (0.08)	Outcrossing is detected more often among spores than vegetative cells
Species×stage interaction	$\beta_{species\ \times stage} = -0.47$ (0.12)	Choosing as a spore increases hybridization probability less in *S. paradoxus* than in *S. cerevisiae*
Variance component		Inference
Genetic group choosing	$\sigma_{chooser.group} = 0.04$	Very little variation in mate choice among genetic groups
Genetic group of alternative	$\sigma_{alternative.group} = 0.00$	
Choosing×alternative group interaction	$\sigma_{chooser.group \times alternative.group} = 0.08$	
Strain choosing	$\sigma_{chooser.strain} = 0.12$	Strains vary in behavior as choosers, and some pairwise combinations are slightly more likely to hybridize than others
Alternative strain	$\sigma_{alternative.strain} = 0.05$	
Choosing×alternative strain interaction	$\sigma_{chooser \times alternative} = 0.09$	

Table 4. Logistic regression of mate choice among *S. cerevisiae* groups. Notation is as in Table 3.

Effect	Coefficient (standard error)	Inference
Intercept	$\beta_0 = 0.01$ (0.12)	Outcrossing is detected more often among spores than among vegetative cells
Life cycle stage	$\beta_{stage} = 0.59$ (0.11)	
Variance component		Inference
Genetic group choosing	$\sigma_{chooser.group} = 0.00$	No variation in outcrossing rates is attributable to group identity.
Genetic group of alternative	$\sigma_{alternative.group} = 0.00$	
Choosing×alternative group interaction	$\sigma_{chooser.group \times alternative.group} = 0.00$	
Strain choosing	$\sigma_{chooser.strain} = 0.16$	Outcrossing frequencies vary among strains and strain combinations
Alternative strain	$\sigma_{alternative.strain} = 0.14$	
Choosing×alternative strain interaction	$\sigma_{chooser \times alternative} = 0.10$	

associated with switching from vegetative cells to spores. Thus, we estimate the spore effect to be approximately 12%, below the null expectation. A strongly negative interaction term indicates that the stronger avoidance of hybridization by spores than by vegetative cells is less pronounced in *S. paradoxus* than in *S. cerevisiae*.

Unlike the previous study on spore mating, we tested the behavior of spores within their own species. *Saccharomyces cerevisiae* and group A *S. paradoxus* spores mated randomly within their genetic groups (Fig. 4B), and in the case of *S. cerevisiae*, among genetic groups as well. Random mating by both *S. cerevisiae* and *S. paradoxus* group A in intraspecific trials indicates that the lack of hybridization in interspecies trials was due to mating discrimination rather than an overall tendency of those *Saccharomyces* spores to inbreed.

Saccharomyces paradoxus group B unexpectedly discriminated more strongly against *S. paradoxus* from the other genetic group than it did against *S. cerevisiae* (compare the right

Table 5. Logistic regression of mate choice among *S. paradoxus* groups. Notation is as in Table 3.

Effect	Coefficient (standard error)	Inference
Intercept	$\beta_0 = 0.10$ (0.16)	Outcrossing is detected more often among spores than vegetative cells
Life cycle stage	$\beta_{stage} = 0.38$ (0.18)	
Variance component		Inference
Genetic group choosing	$\sigma_{chooser.group} = 0.00$	Outcrossing rates do not vary among groups, but some pairwise combinations of groups show higher outcrossing rates than do other pairs of groups
Genetic group of alternative	$\sigma_{alternative.group} = 0.00$	
Choosing×alternative group interaction	$\sigma_{chooser.group \times alternative.group} = 0.30$	
Strain choosing	$\sigma_{chooser.strain} = 0.00$	Outcrossing probability does not vary among strains, but some strain pairings outcross more often than others
Alternative strain	$\sigma_{alternative.strain} = 0.00$	
Choosing×alternative strain interaction	$\sigma_{chooser \times alternative} = 0.27$	

Table 6. Mating propensity ANOVA. Proportion of mated colonies at each time point was analyzed. Strain was designated a random effect. Note that *S. cerevisiae* mates more (species effect) and mates more quickly (species × time effect).

Source	SS	MS	df	*F* value	*Pr>F*
Species	0.568	0.568	1	7.69	0.0196
Time	4.330	1.083	4	65.17	<0.0001
Strain (species)	0.741	0.074	10	4.45	0.0003
Species×time	0.169	0.042	4	2.54	0.0541
Strain (species)×time	0.667	0.017	40	2.01	0.0019
Residual	1.017	0.008	118	–	–

SS, sums of squares; MS, mean square.

panels of Fig. 4B,D). The spores of this group, like its vegetative cells, tended to inbreed in intraspecific trials. Logistic regression indicated that they were 7% more likely to hybridize with *S. cerevisiae* than to outcross with group A *S. paradoxus* (proportion of hybrids: 0.53 vs. 0.65; $F = 16.99$, $P = 0.054$, df = 1, 2; strain and strain × species effects were not significant, $P = 0.63$, $P = 0.39$, respectively).

GERMINATION TIMING

We tested the hypothesis that the observed pattern of mating in interspecies spore mate choice trials was due to a difference in germination timing. We estimated germination times for 163 spores from the six strains of *S. paradoxus* and a total of 158 spores from the six strains of *S. cerevisiae*. *Saccharomyces cerevisiae* spores germinated and budded significantly faster than those of *S. paradoxus* (Fig. 3B,Table 7). There was significant variation among strains, but not between the genetic groups. When specifically comparing *S. paradoxus* groups A and B, there was no significant difference (planned contrast, $P = 0.364$). *Saccharomyces paradoxus* spores did not begin germinating until approximately 75% of all *S. cerevisiae* spores already had, and even then, it was only a small fraction (6%) that did. Rather, *S. paradoxus* germination coincided with the appearance of the first *S. cerevisiae* bud projections.

STAGGERED GERMINATION

If different germination timing is indeed the mechanism by which the spores avoid mismating, then delaying the germination of *S. cerevisiae* may increase the frequency of interspecies matings. We tested this idea with mate choice trials in which *S. cerevisiae* was placed on the mating plate at the same time as *S. paradoxus*, as well as 1, 2, 3, and 4 h later (Fig. 5). There was an overall effect of time (repeated measures nested two-way ANOVA, $F = 3.01$, df = 4, 38, $P = 0.03$), as well as interaction between time and species ($F = 3.24$, df = 4, 38, $P = 0.02$), but no significant effect of species or strain combination ($F = 2.16$, df = 1, 14.5, $P = 0.16$ and $F = 1.67$, df = 12, 38, $P = 0.11$, respectively). When placed down at the same time, both species were able to discriminate, as expected. With a 1-h delay of the faster germinator, *S. cerevisiae*, both strains were still able to discriminate properly. But a 2-h delay of *S. cerevisiae*, which should synchronize the germination time of the two species, led to random mating. After a 3-h delay, *S. paradoxus* could discriminate again, and with a 4-h delay, both species had recovered the ability to discriminate against inappropriate partners. Thus, synchronizing spore germination appeared to randomize mating (contrast: [0, 1, 3, 4 h] vs. 2 h; $P = 0.003$).

Table 7. Germination timing. Time to germination and time to first bud projection for each spore was analyzed. Strain was designated a random effect.

Source	SS	MS	df	*F* value	*Pr>F*
Species	1299413	1299413	1	188.1	0.0056
Cell cycle	2527309	2527309	1	876.2	0.0003
Genetic Group (species)	24067.9	8022.65	3	0.3912	0.7639
Strain (species, genetic group)	159664	22809.2	7	5.8685	0.0163
Species×cell cycle	3380	3380	1	1.172	0.3705
Genetic group (species)×cell cycle	8875.12	2958.4	3	0.784	0.5368
Strain (species, genetic group)×cell cycle	27206.8	3386.7	7	1.671	0.1132
Residual	1437196.7	2326	619	–	–

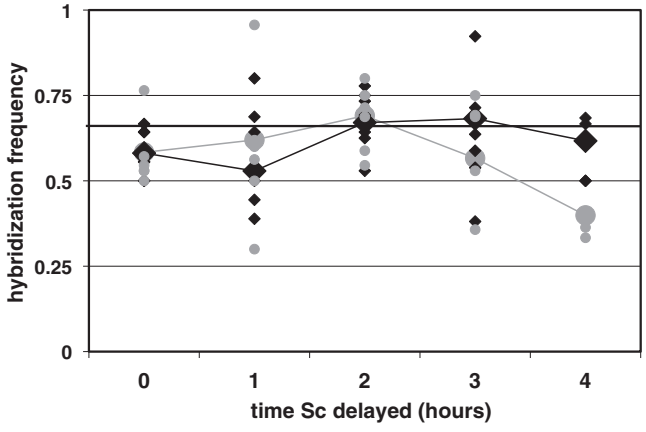

Figure 5. Staggered germination spore mate choice black diamonds—*S. cerevisiae* is "chooser" strain; gray circles—*S. paradoxus* is "chooser" strain. Large symbols represent average species response. Y-axis is the proportion of hybridization events; x-axis is time in hours that *S. cerevisiae* spores were included in the mate choice trials after *S. paradoxus*. Seven random strain combinations were analyzed; the experiment included a 4-h delay for only three of them. For each strain combination, one mating plate was prepared per time point for each species, and each plate resulted in 9–23 successful trials (each mating plate is represented by one datapoint on graph).

Discussion

In both *S. cerevisiae* and *S. paradoxus*, each mating type produces a mating type specific pheromone, as well as receptors for the pheromone produced by opposite mating type (Herskowitz 1988); these pheromones show very little divergence between the species, although the receptors are slightly less well conserved (see Supplementary Material). *Saccharomyces cerevisiae* has been shown to discriminate among mating partners based on the amount of pheromone being produced (Jackson and Hartwell 1990a,b), suggesting the possibility of sexual selection in yeast (Pagel 1993; Smith and Greig 2010).

Very little is known about the life cycle of *Saccharomyces* yeasts in nature, as its growth in nature has not been observed. Population genetic and genomic analyses of *S. paradoxus* indicate that sexual cycles are rare, compared to mitotic reproduction, and that populations are highly inbred (Koufopanou et al. 2006; Tsai et al. 2008). Based on data from laboratory studies, it is thought that *Saccharomyces* yeasts exist predominantly in the diploid stage (Knop 2006) and are dispersed by insect vectors (Replansky et al. 2008). From genomic data alone, it is impossible to determine whether the inbreeding is behavioral, resulting from a preference of haploid cells for closely related mates, or an effect of highly structured populations in which potential mates are closely related because dispersal rates are low. Our results suggest the latter, as we observed no consistent preference in either species for mates from their own strain. Recent work has shown that when *S. cerevisiae* is ingested by *Drosophila*, vegetative yeast cells are destroyed, while spores pass through the digestive tract unharmed and able to mate randomly with higher rates of outcrossing (Reuter et al. 2007). This evidence, combined with observations of spores mating immediately after germination (Guilliermond 1905; Winge and Laustsen 1937), suggest that outcrossing opportunities are likely to arise in the spore stage of the life cycle after a dispersal event. However, because not all spores mate and asci often contain only three viable spores leaving one spore unmated, haploid vegetative cells may occasionally encounter each other, although this is probably rare. We assayed the mating dynamics between sympatric woodland populations of *S. cerevisiae* and *S. paradoxus* in both spores and vegetative cells and found evidence for strong differences in mating characteristics that could lead to prezygotic isolation between the species in nature. These differences are in mating and germination timing, not unlike the mechanisms of allochronic isolation observed in multicellular organisms (Coyne and Orr 2004).

First, the mating behavior of vegetative cells was assayed in individual cell-to-cell mate choice trials. Our results showed that both species usually mate with *S. cerevisiae*, probably due to differences in mating propensity, confirming a previous observation based on two strains of each species (Murphy et al. 2006). As previously suggested, these timing differences may mediate a form of prezygotic isolation in which faster maters mate with

each other, leaving the slower maters available only to each other. This form of prezygotic isolation has evolved in experimental laboratory populations in as few as 26 sexual cycles (Leu and Murray 2006). However, what is known of the life history of both *S. cerevisiae* and *S. paradoxus* suggests that most encounters would be between spores rather than vegetative cells. If differences in spore germination timing are the main mechanism by which hybridization is avoided, the abundant variation in mating preferences that we observed among vegetative cells of *S. cerevisiae* may play little or no role in most mating behavior, and therefore be effectively neutral, or pleiotropic effects of alleles selected for their effects on other traits.

Second, the mating behavior of spores was assayed in spore-to-spore mate choice trials. Our results agree with those of Maclean and Greig (2008): spores mate with conspecific spores more than would be expected if mating occurred randomly, thus exhibiting a clear pattern of prezygotic isolation. In intraspecific mate choice trials, where spores chose between potential partners of their own strain of a different haplotype, some strains preferred or avoided others, but in neither species did spores tend to inbreed. Therefore, their tendency in interspecific trials not to hybridize is not the byproduct of a preference for close relatives, but rather an active discrimination against heterospecific spores.

Unlike vegetative haploid cells, which constitutively produce pheromones and, upon sensing pheromone of the opposite mating type, increase their own production to begin the mating process (Bardwell 2004), dormant spores do not produce pheromones. Therefore, if spores of one species germinate before those of the other species, they will sense pheromone only from conspecifics. If such spores do not sense any pheromone upon germination (i.e., no appropriate mating partners), they will proceed through the cell cycle, effectively making mating impossible until mitotic reproduction is complete (Wittenberg and La Valle 2003). When the slower species begins germinating, it will also detect pheromone from conspecifics and be protected from mating with the wrong species, as those cells will be in the process of budding (Maclean and Greig 2008). To test this hypothesis, we investigated the amount of time it took for spores from the different strains to germinate and found very significant differences between the species, as well as strong synchronization within each species. *Saccharomyces paradoxus* spores did not begin germinating until almost all *S. cerevisiae* had finished. When *S. paradoxus* began germinating, *S. cerevisiae* already had visible bud projections, suggesting that the cells had committed to mitotic reproduction and were past the point in the cell cycle where mating would be possible (Kron and Gow 1995). To determine whether these differences in germination timing were indeed responsible for the observed prezygotic isolation in the spore mate choice trials, we delayed the germination time of *S. cerevisiae* to coincide with *S. paradoxus* germination. We found that a delay of 1 h did not change either species' ability to discriminate; however, a delay of 2 h led to random mating by both species. After a 3-h delay, *S. paradoxus* had recovered its ability to discriminate and after a 4-h delay, both species were able to discriminate again. These results support the hypothesis that differences in germination timing underlie spore mate discrimination between these two populations.

Do both species experience, and react similarly to, similar conditions in nature? Although we cannot perform an experimental test, we can address this issue with other data. First, these strains were all isolated from oak flux; in some instances, both species were isolated from the same tree, suggesting that they experience similar conditions. Second, they can ferment the same sugars and use nutrients similarly, and they grow at similar rates (data not shown). Finally, in the laboratory, both species are induced to sporulate and germinate by the same media and conditions. Isolation via germination timing in nature is therefore plausible. We acknowledge that we cannot conclude from our data that selection acted on the described mating traits for the purpose of reproductive isolation. The classic signature of displacement is greater prezygotic isolation in sympatry than in allopatry, but testing for this would require a much more fine-scale knowledge of yeast biogeography than is currently realistic.

The prezygotic isolation we have documented here is incomplete, whether because selection against hybridization is patchy or inconsistent, or because it is still evolving. Our knowledge of yeast population biology still lags behind the vast resources of *S. cerevisiae* cell biology and genetics, which contributed to this study the ability to manipulate genotypes, mating types, and individual cells, but a recent surge of interest in natural populations, and the growing number of genome sequences, offer growing potential to connect the cellular and molecular details with the genetic structure and evolutionary history of yeast populations. This study is a step toward the genetic dissection of speciation in the largely neglected microbial eukaryotes.

ACKNOWLEDGMENTS

We thank P. Sniegowski and H. Kuehne for access to strains, and P. Sniegowski, J. N. Jasmin, and two anonymous reviewers for comments on an earlier version of the manuscript. This work was supported by National Science Foundation-DEB 0820969 to CWZ and HAM.

LITERATURE CITED

Bardwell, L. 2004. A walk-through of the yeast mating pheromone response pathway. Peptides 26:339–350.

Clifton, K. E. 1997. Mass spawning by green algae on coral reefs. Science 275:1116–1118.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Massachusetts.

Dettman, J. R., D. Jacobson, E. Turner, A. Pringle, and J. W. Taylor. 2003. Reproductive isolation and phylogenetic divergence in Neurospora: comparing methods of species recognition in a model eukaryote. Evolution 57:2721–2741.

Devries, P. J., G. T. Austin, and N. H. Martin. 2008. Diel activity and reproductive isolation in a diverse assemblage of Neotropical skippers (*Lepidoptera: Hesperiidae*). Biol. J. Linn. Soc. 94:723–736.

Ellis, A. G., A. E. Weis, and B. S. Gaut. 2006. Evolutionary radiation of "stone plants" in the genus *Argyroderma* (Aizoaceae): unraveling the effects of landscape, habitat, and flowering time. Evolution 60:39–55.

Gelman, A., and J. Hill. 2007 Data analysis using regression and multilevel/hierarchical models. Cambridge Univ. Press, New York.

Gerke, J. P., C. T. L. Chen, and B. A. Cohen. 2006. Natural isolates of *Saccharomyces cerevisiae* display complex genetic variation in sporulation efficieny. Genetics 174:985–997.

Gietz, R., and R. Woods. 2002. Transformation of yeast by the Liac/Ss Carrier Dna/Peg method. Meth. Enzymol. 350:87–96.

Goldstein, A., and J. McCusker. 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast 15:1541–1553.

Guilliermond, M. 1905. Recherches sur la germination des spores et la conjugation chez les levures. Revue générale de botanique 509:337–376.

Herskowitz, I. 1988. Life cycle of the budding yeast *Saccharomyces cerevisiae*. Microbiol. Rev. 52:536–553.

Jackson, C., and L. Hartwell. 1990a. Courtship in *Saccharomyces cerevisiae*-an early cell-cell interaction during mating. Mol. Cell. Biol. 10:2202–2213.

Jackson, C., and L. Hartwell. 1990b. Courtship in *Saccharomyces cerevisiae*: both cell types choose mating partners by responding to the strongest pheromone signal. Cell 63:1039–1051.

Knop, M. 2006. Evolution of the *hemiascomycete* yeasts: on life styles and the importance of inbreeding. BioEssays 28:696–708.

Knowlton, N., J. L. Maté, H. M. Guzmán, R. Rowan, and J. Jara. 1997. Direct evidence for reproductive isolation among the three species of the *Montastraea annularis* complex in Central America (Panamá and Honduras). Marine Biol. 127:705–711.

Kohn, L. M. 2005. Mechanisms of fungal speciation. Annu. Rev. Phytopathol. 43:279–308.

Koufopanou, V., J. Hughes, G. Bell, and A. Burt. 2006. The spatial scale of genetic differentiation in a model organism: the wild yeast *Saccharomyces paradoxus*. Phil. Trans. R. Soc. Lond. B 361:1941–1946.

Kron, S. J., and N. A. Gow. 1995. Budding yeast morphogenesis: signalling, cytoskeleton and cell cycle. Curr. Opin. Cell Biol. 7:845–855.

Kuehne, H. A. 2005. Genetic population structure and biogeography of natural *Saccharomyces* populations. Ph.D. diss., Department of Biology, University of Pennsylvania, Philadelphia.

Kuehne, H. A., H. A. Murphy, C. A. Francis, and P. D. Sniegowski. 2007. Allopatric divergence, secondary contact, and genetic isolation in wild yeast populations. Curr. Biol. 17:407–411.

Le Gac, M., and T. Giraud. 2008. Existence of a pattern of reproductive character displacement in *Homobasidiomycota* but not *Ascomycota*. J. Evol. Biol. 21:761–772.

Leu, J. Y., and A. W. Murray. 2006. Experimental evolution of mating discrimination in budding yeast. Curr. Biol. 16:280–286.

Maclean, C. J., and D. Greig. 2008. Prezygotic reproductive isolation between *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*. BMC Evol. Biol. 8:1.

McElfresh, J. S., and J. G. Millar. 2001. Geographic variation in the pheromone system of the Saturniid moth *Hemileuca eglanterina*. Ecology 82: 3505–3518.

Mendelson, T. C., and K. L. Shaw. 2002. Genetic and behavioral components of the cryptic species boundary between *Laupala cerasina* and *L. kohalensis* (*Orthoptera: Gryllidae*). Genetica 116:301–310.

Murphy, H. A., H. A. Kuehne, C. A. Francis, and P. D. Sniegowski. 2006. Mate choice assays and mating propensity differences in natural yeast populations. Biol Lett. 2:553–556.

Naumov, G. I. 1987. Genetic basis for classification and identification of the ascomycetous yeasts. Stud. Mycol. 30:469–475.

Naumov, G. I. 1996. Genetic identification of biological species in the *Saccharomyces sensu stricto* complex. J. Ind. Microbiol. 17:295–302.

Pagel, M. 1993. Honest signalling among gametes. Nature 363:539–541.

Quinn, T. P., M. J. Unwin, and M. T. Kinnison. 2000. Evolution of temporal isolation in the wild: genetic divergence in timing of migration and breeding by introduced chinook salmon populations. Evolution 54:1372–1385.

Replansky, T., V. Koufopanou, D. Greig, and G. Bell. 2008. *Saccharomyces sensu stricto* as a model system for evolution and ecology. Trends Ecol. Evol. 23:494–501.

Reuter, M., G. Bell, and D. Greig. 2007. Increased outbreeding in yeast in response to dispersal by an insect vector. Curr. Biol. 17:R81–R83.

Rose, M. D., F. Winston, and P. Hieter. 1990. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Saetre, G.-P., T. Moum, S. Bures, M. Kral, M. Adamjan, and J. Moreno. 1997. A sexually selected character displacement in flycatchers reinforces premating isolation. Nature 387:589–592.

Seehausen, O., and J. J. van Alphen. 1998. The effect of male coloration on female mate choice in closely related Lake Victoria cichlids (*Haplochromis nyererei* complex). Behav. Ecol. Sociobiol. 42:1–8.

Smith, C., and D. Greig. 2010. The cost of sexual signaling in yeast. Evolution 64:3114–3122.

Sniegowski, P. D., P. G. Dombrowski, and E. Fingerman. 2002. *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* coexist in a natural woodland site in North America and display diffferent levels of reproductive isolation from European conspecifics. FEMS Yeast Res. 1:299–306.

Tsai, I. J., D. Bensasson, A. Burt, and V. Koufopanou. 2008. Population genomics of the wild yeast *Saccharomyces paradoxus*: Quantifying the life cycle. Proc. Natl. Acad. Sci. USA 105:4957–4962.

Uy, J. A. C., R. G. Moyle, and C. E. Filardi. 2008. Plumage and song differences mediate species recognition between incipient flycatcher species of the Solomon Islands. Evolution 63:153–164.

Wach, A., A. Brachat, R. Pohlmann, and P. Philippsen. 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast 10:1793–1808.

Winge, Ö., and O. Laustsen. 1937. On two types of spore germination, and on genetic segregations in *Saccharomyces*, demonstrated through single-spore cultures. Comptes rendus des travaux du Laboratoire Carlsberg: Series physiologique 22:99–117.

Wittenberg, C., and R. La Valle. 2003. Cell-cycle-regulatory elements and the control of cell differentiation in the budding yeast. BioEssays 25: 856–867.

Associate Editor: P. Turner

Supporting Information

The following supporting information is available for this article:

Figure S1: MFA1
Figure S2: STE3
Figure S3: MFAlpha1
Figure S4: STE2

Supporting Information may be found in the online version of this article.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.