Experimental evolution of species recognition

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Summary

Sex with another species can be disastrous, especially for organisms that mate only once, like yeast [1-3]. Courtship signals, including pheromones, often differ between species and can provide a basis for distinguishing between reproductively compatible and incompatible partners [4-6]. Remarkably, we show that the baker's yeast *Saccharomyces cerevisiae* does not reject mates engineered to produce pheromones from highly diverged species, including species that have been reproductively isolated for up to 100 million years. To determine if effective discrimination against mates producing pheromones from other species is possible, we experimentally evolved pheromone receptors under conditions that imposed high fitness costs on mating with cells producing diverged pheromones. Evolved receptors allowed both efficient mating with cells producing the *S. cerevisiae* pheromone and near-perfect discrimination against cells producing diverged pheromones. Sequencing evolved receptors revealed that each contained multiple mutations that altered the amino acid sequence. By isolating individual mutations, we identified specific amino acid changes that dramatically improved discrimination. However, the improved discrimination conferred by these individual mutations came at the cost of reduced mating efficiency with cells producing the *S. cerevisiae* pheromone, resulting in low fitness. This trade-off could be overcome by simultaneously introducing separate mutations that improved mating efficiency alongside those that improved discrimination. Thus, if mutations occur sequentially, the shape of the fitness landscape may prevent evolution of the optimal phenotype [7,8] - offering a possible explanation for the poor discrimination of receptors found in nature.

Results and Discussion

The interactions between mating pheromones and their receptors regulate two important components of reproductive success: mating efficiency, the coordination of sexual behaviours between partners resulting in successful mating; and mate discrimination, the ability to distinguish between post-zygotically compatible and incompatible partners [3]. When a population contains only compatible partners, receptors should evolve to maximize mating efficiency regardless of mate discrimination, thereby maximising reproductive success. But if a population also contains post-zygotically incompatible partners (e.g. different species), then responding to their sexual signals or mating with them will reduce reproductive success. Selection then should optimize both mating efficiency, to maximize mating with compatible partners, and mate discrimination, to ignore signals from incompatible partners [4].

Pheromones and their receptors are necessary for *Saccharomyces cerevisiae* mating, which occurs when haploid cells of opposite mating types (MAT**a** and MATα) pair and fuse to form a diploid zygote [9]. Each mating type secretes different mating pheromones: MAT**a** cells produce **a**-pheromones and MATα cells produce α-pheromones. These pheromones bind to G-protein coupled receptors (the α-pheromone receptor Ste2p or the **a**-pheromone receptor Ste3p) on the surface of the mating partner and trigger the yeast pheromone response, which ultimately results in zygote formation [10]. Haploid cells locate mating partners by polarizing their growth in the direction of the highest pheromone concentration [11,12]. Mutations in either pheromones or receptors can alter mating efficiency [13-15], and pheromone-receptor specificity has been proposed as a possible mechanism for mate discrimination between those species whose pheromone peptide sequences differ [3,16]. Attraction to pheromones produced by incompatible partners is particularly costly for yeast because each cell can mate only once; zygote inviability or sterility is equivalent to death for the mating haploids. Distantly related species, which generally produce pheromones with different peptide sequences (Fig. 1, Table S1), do not form viable mixed-mating-type zygotes with *S. cerevisiae* [18,19]. However, it is not known if the failure of different species to form viable zygotes is due to successful discrimination against heterospecific pheromones, or whether other pre-zygotic or post-zygotic incompatibilities prevent viable hybrid zygote production.

We have isolated the effects of pheromone-receptor interactions on mating success by expressing **a**-pheromones and α-pheromones from 17 different species in *S. cerevisiae*, allowing us to determine not only whether *S. cerevisiae* is capable of mating with cells expressing these different pheromones, but also how efficiently it does so. We expressed each predicted heterospecific mature pheromone as a single heterologous-pheromone-encoding unit within the dominant *S. cerevisiae* proprotein (*MFα1* [20] or *MFA1* [21]) under the endogenous promoter and terminator (Fig. S1). Each heterologous-pheromone-producing strain was tested for its ability to mate with *S. cerevisiae* cells of the opposite mating type (Fig. 1, Fig. S2). We found that *S. cerevisiae* pheromone receptors are capable of coordinating mating with pheromones from distantly related species. *S. cerevisiae* was able to mate with cells secreting most of the tested pheromones identified from species that diverged following the whole-genome duplication event which occurred approximately 100 million years ago [22] but only a single pheromone from species that diverged prior to duplication. Remarkably, the ability of heterospecific **a**-pheromones to promote efficient mating was generally all-or-nothing: **a**-pheromones that induced mating did so at high efficiency and generally contained a conserved four amino acid motif (FWDP) which is crucial for **a**-pheromone activity in *S. cerevisiae* [23]. Some heterospecific **a**-pheromones worked as well as the native *S. cerevisiae* **a**-pheromones. In contrast, α-pheromones showed a more graded distribution of efficiency. Heterospecific α-pheromones generated lower mating efficiencies than native *S. cerevisiae* α-pheromones; only two heterospecific α-pheromones allowed mating at >1% of the conspecific levels.

We next tested whether increased receptor-pheromone specificity could evolve in response to selection against inviable hybrids. Laboratory evolution experiments have repeatedly demonstrated that selection against hybridization can promote pre-zygotic reproductive isolation between different populations [24]. However, these studies have provided few clues as to the reproductive traits that prevent mating or their genetic bases. Without an *a priori* hypothesis for the mechanism of pre-zygotic isolation, evolution experiments usually rely on high levels of genome-wide variation in the hope of capturing differences in appropriate reproductive traits. As a result, it has been very difficult to identify causal mutations [25]. Here, we restricted genetic variation to the α-pheromone receptor *STE2* only. We transformed a MAT**a** strain lacking the chromosomal copy of *STE2* with one of five pools of centromeric plasmids containing a copy of *STE2* in which the open reading frame contained random mutations (low mutation rate: L1, L2, L3; high mutation rate: H1, H2). These MAT**a** cells were then subjected to one of two selective regimes (Fig. S3). In the *compatible-only* regime, the MAT**a** cells were allowed to mate with an equal number of post-zygotically compatible MATα cells producing the conspecific *S. cerevisiae* α-pheromone WHWLQLKPGQPMY. In the *mixed* regime, the MAT**a** cells were offered these compatible MATα cells mixed with a 9-fold excess of incompatible MATα cells expressing the most efficient heterospecific-α-pheromone WHWLRLDPGQPLY (Fig. 1). Post-zygotic compatibility was determined by dominant drug resistance cassettes linked to the MAT loci (Fig. S3): two drugs were used simultaneously to select only zygotes formed between MAT**a** cells and MATα cells expressing the conspecific pheromone, and these double-resistant zygotes provided

MAT**a** cells for the next mating cycle. Five cycles were carried out before the response to selection was measured.

Mating efficiency increased under both the *compatible-only* and the *mixed* selection regimes (Fig. 2), likely attributable to selection for increased sensitivity to the low α-pheromone levels secreted by our experimental strains (approximately 50% of wildtype levels, data not shown). When presented with only one type of producer (either conspecific or heterospecific), evolved lines from both regimes showed 6× improvements in mating efficiency with conspecific-α-producers relative to pre-selection strains (Fig. 2A, Table S2). In contrast, evolved lines from the two regimes differed in mating efficiency with heterospecific-α-producers: *compatible-only* lines were 8.7× higher than pre-selection, while *mixed* lines were only 2.7× higher than pre-selection (Fig. 2B). Thus, selection against mating with heterospecific-α-producers in the *mixed* regime specifically prevented improved mating efficiency with heterospecific-α-producers but not conspecific-α-producers, suggesting an improved ability to discriminate between pheromone types in the *mixed* lines.

To directly evaluate the ability to discriminate between pheromone types, we presented lines with a mixture of conspecific and heterospecific-α-producers (Fig. 2C). Both treatments showed improved mating with conspecific-α-producers, but *mixed* lines were 3.1× worse at mating with heterospecific-α-producers than were *compatible-only* lines. Calculating the number of matings with conspecific-α-producers relative to each mating with a heterospecific-α-producer allowed us to compare the ability of each line to discriminate between pheromone types independently of overall mating efficiency (Fig. 2C). We found that mate discrimination had increased 3.9× in *mixed* lines but decreased 1.9× in the *compatible-only* lines, resulting in a 7.6× difference in mate discrimination between the two regimes.

The phenotypic responses to selection we observed in both mating efficiency and mate discrimination were associated with changes in *STE2*, as ancestral strains transformed with evolved plasmid pools showed similar phenotypes to the evolved strains (Fig. 2D). To identify causal mutations underlying the observed responses to selection, we extracted plasmids from four isolates from each evolved line, sequenced them (Table S3), and tested the phenotype that one plasmid from each line generated in the ancestor. We found that while some plasmids were very similar to the unmutated *STE2* in both mating efficiency and discrimination, others showed highly exaggerated versions of the evolved phenotypes in each regime (Fig. 2E). Most plasmids contained multiple mutations. To determine how individual substitutions affected phenotype, we isolated every mutation from two plasmids from the *mixed* regime and tested their effects in the ancestor (Fig. 3A). Many of the mutations in evolved plasmids occurred in regions with known effects on receptor function (Fig. 4). The most common changes we detected, found in both *compatible-only* and *mixed* regimes, were previously shown to be associated with pheromone hypersensitivity, a trait that likely allowed more efficient mating at the low pheromone levels produced by our experimental strains. Premature stop codons were detected in one third of sequenced plasmids. These stop codons are predicted to result in truncation of the cytosolic tail of the receptor, and all predicted truncations occurred downstream from the endocytosis signal SINNDAKSS. Truncation of the cytosolic tail results in up to a 100× increase in pheromone sensitivity [28] possibly by preventing docking of Sst2p, a negative regulator of G-protein signalling which attenuates the pheromone response after ligand-induced activation [29]. Mutations at two residues in the third intracellular loop of Ste2p were also identified in multiple plasmids from both regimes: K239 and Q240. The third intracellular loop interacts directly with the Gα protein Gpa1p, and alanine substitutions at these two residues have previously been shown to result in pheromone hypersensitivity [30]. We demonstrated that the mutation Q240K increased mating efficiency 2.7× relative to the wildtype receptor (Fig. 3A). We also found large increases in mating efficiency associated with mutations near both the N-terminus (L8M) and the C-terminus (T411N). The importance of these residues to pheromone sensitivity is unknown, but we found that either of these mutations alone captured the high-efficiency/low-discrimination phenotype of the *compatible-only* lines.

Two residues that were mutated in multiple plasmids from the *mixed* lines were found to have strong effects on mate discrimination: F204 and T282. F204, located within the 2nd extracellular loop, is thought to interact directly with the ligand. Cells carrying receptors with the mutations F204S or F204C are sterile and 100× less sensitive to conspecific-α-pheromone [31,32]. We found that substituting the aromatic phenylalanine residue at this position with the aromatic residue tyrosine (F204Y) resulted in high discrimination against heterospecific-α-pheromone but also greatly reduced mating efficiency with cells producing conspecific-α-pheromone. Residue T282 is located within transmembrane domain 7. The mutation T282A shows a >10× lower sensitivity to the *S. kluyveri* α-pheromone relative to the wildtype receptor, despite wildtype-levels of binding affinity [33]. Thus, although it may not interact directly with the ligand, residue T282 is thought to play a role in activation of the pheromone response. We found that the mutations T282I and T282P allowed greatly improved discrimination against heterospecific-α-pheromone but, similar to F204Y, also showed reduced mating efficiency with conspecific-α-pheromone relative to the wildtype receptor (Fig. 3B). Mutations that improved discrimination against the heterospecific pheromone used in our evolution experiment also improved discrimination against other heterospecific pheromones (Fig. 2E).

For all single mutations, we observed a strong negative correlation between mating efficiency and mate discrimination (Fig. 3B). This result closely mirrors the previously observed negative relationship between pheromone hypersensitivity and the ability to discriminate between cells producing the conspecific-α-pheromone and cells producing no pheromone [12]. We did not find any single mutation that could capture the high-discrimination/high-efficiency phenotype of the *mixed* lines, but two substitutions could. In the plasmids we sequenced from the *mixed* regime, receptors containing mutations that improved discrimination (e.g. F204Y or T282I) always contained compensatory mutations that restored efficient mating (e.g. Q240K or T411N). Although we cannot rule out that single mutations that improve discrimination without compromising mating efficiency are possible, the observed cost of discrimination offers a potential explanation for the poor discrimination of wildtype *S. cerevisiae*. The high *STE2* mutation rate used in our evolution experiment was crucial to the discovery of the trade-off between discrimination and mating efficiency as it allowed multiple mutations to be selected simultaneously. At lower mutation rates, changes that improved discrimination alone would not have increased in frequency due to the associated cost of low mating efficiency. In nature, this cost of discrimination would prevent single mutations from reaching a high enough frequency that they would be combined with compensatory mutations by either subsequent mutation or recombination.

As well as confirming theoretical predictions of how species recognition evolves, and identifying the underlying mutations, our results offer two possible explanations for why wildtype *S. cerevisiae* exhibits poor pheromonal mate discrimination. If *S. cerevisiae* rarely or never has the opportunity to mate with species producing different pheromones (as in our *compatible-only* regime), then selection to avoid them will be weak or absent, and discrimination is not expected to evolve. Indeed, our results indicate that selection for efficient mating may actually result in weaker discrimination. However, even if *S. cerevisiae* frequently encounters other species during mating, mutations that increase pheromonal discrimination may not spread unless compensatory mutations that restore efficient mating are also present in the same receptor sequence. Instead, other physiological mechanisms such as altered mating kinetics or germination timing may allow pre-zygotic isolation [34-36]. Thus, our evolution experiment shows that although receptors that generate both high discrimination and high efficiency exist, they may be inaccessible in nature due to the shape of the local adaptive landscape [8,37].

Author Contributions

D.W.R. conceived the study, designed the experiments, constructed strains, performed the experiments, analysed the results, and wrote the paper. J.A.D. conceived the study, designed and constructed plasmids, constructed strains, and performed the mutagenesis. E.M. performed the experiments. D.G. designed the experiments and wrote the paper.

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Figure Legends

Fig. 1. **Mating efficiency of *S. cerevisiae* with cells expressing conspecific and heterospecific pheromones.** Mating efficiency was calculated as the percentage of total MAT**a** (for α-pheromone efficiency) or MATα (for **a**-pheromone efficiency) alleles found in zygotes after 6h access to a 10× excess of mating partners (bars = mean ± SEM). The species from which each pheromone was predicted is shown in the centre column. Peptide sequences were mapped to a topology of the *Saccharomyces* complex [17]; branch lengths are arbitrary. Asterisks indicate the whole genome duplication event. Residues that differ from the principal *S. cerevisiae* α-pheromone (WHWLQLKPGQPMY) and **a**-pheromone (YIIKGLFWDPAC) sequences are shown in grey. All strains (with the exception of those producing GWMRLRIGQPL, FMLGSNYDPAC, and FMLGGSTSYYGC) were capable of mating, or improving mating, with tester strains indicating successful pheromone production and secretion (Fig. S2). "No pheromone" controls were identical to experimental strains but contained proprotein sequences lacking the mature-pheromone-encoding unit. Strains were generated as described in the Supplemental Experimental Procedures, Fig. S1, and Fig. S4. Mature pheromone predictions are listed in Table S2.

Fig. 2. **Responses to selection.** Mating efficiency is represented by vertical bars (left y-axis). Filled bars represent mating efficiency with conspecific-α-pheromone producers; open bars represent mating efficiency with heterospecific-α-pheromone producers. The height of stacked bars represents total mating efficiency. Mate discrimination is represented by circles (right y-axis). Results are shown for each pool of mutagenized receptors (L1, L2, L3, H1, and H2) both prior to selection (*pre-selection*) and after 5 cycles of evolution in either the *compatible-only*

treatment or the *mixed* treatment. Both conspecific-α-pheromone producers and heterospecific-α-pheromone producers were post-zygotically compatible with MAT**a** cells. **A.** Mating efficiency with an equal number of conspecific-α-pheromone producers. **B.** Mating efficiency with a 9× excess of heterospecific-α-pheromone producers. **C-E.** Mating efficiency and mate discrimination with a 1:9 mixture of conspecific-α-pheromone producers and heterospecific-α-pheromone producers in evolved lines (**C**), after transfer of evolved plasmid pools into the ancestral strain (**D**), after transfer of individual plasmids from evolved lines into ancestral strain (**E**); amino acid changes in evolved plasmids are indicated on the x-axis. **F.** Discrimination against other heterospecific-α-pheromones for three different plasmids. Colors correspond to panel E. Scer* represents the minor *S. cerevisiae* α-pheromone WHWLNLRPGQPMY. Statistical analysis of these responses is presented in Table S2. The evolution experiment was carried out as described in Fig. S3.

Fig. 3. **Effect of individual mutations on mating efficiency and mate discrimination. A.** Mating efficiency and mate discrimination of single-mutation-receptors with a 1:9 mixture of conspecific-α-pheromone producers and heterospecific-α-pheromone producers. Bars and circles as in Fig. 2. **B.** Inverse correlation between mating efficiency and mate discrimination for all single-mutation-receptors ($r = -0.93$, $n = 11$, $P < 0.0001$). Both efficiency and discrimination were normalized against the unmutated receptor (hatched lines). Each circle represents a single mutation (listed from lowest to highest discrimination): L8M, Q240K, T411N, A61V, I372V, N10Y, F377Y, M69T, T282I, T282P, F204Y. Mating efficiency was measured with a 1:9 mixture of conspecific-α-pheromone producers and heterospecific-α-pheromone producers.

Fig. 4. All mutated residues in sequenced plasmids isolated from evolved lines mapped to a topology plot of Ste2p. Mutations were pooled from 20 sequenced plasmids each from the *mixed* regime (yellow) and the *compatible-only* regime (blue). Mutations found in plasmids from both regimes are shown in green. The Ste2p topology is based on [26] and the plot was generated using Protter [27]. Residue numbers are shown in black. Residues mutated in more than one receptor are marked underneath with grey numbers (x2, x3, or x4). Truncations (and frameshifts) are indicated by diamonds. Amino acid changes observed in each receptor are listed in Table S3.

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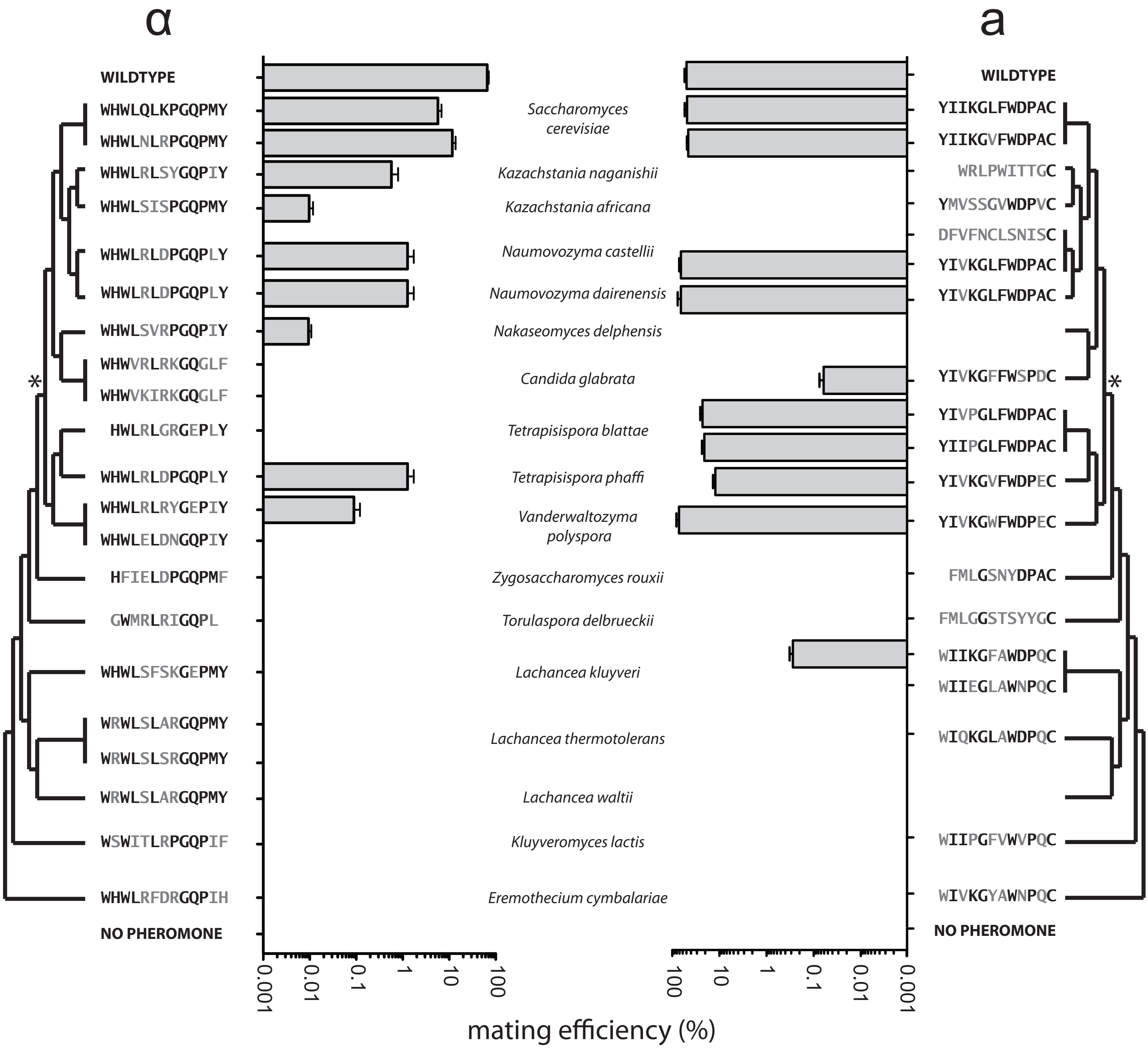
Figure 1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WHWLRFDRGQPIH
NO PHEROMONE
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Saccharomyces cerevisiae
Kazachstania naganishii
Kazachstania africana
Naumovozyma castellii
Naumovozyma dairenensis
Nakaseomyces delphensis
Candida glabrata
Tetrapisispora blattae
Tetrapisispora phaffi
Vanderwaltozyma polyspora
Zygosaccharomyces rouxii
Torulaspora delbrueckii
Lachancea kluyveri
Lachancea thermotolerans
Lachancea waltii
Kluyveromyces lactis
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DFVFNCLSNISC
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WIIEGLAWNPQC
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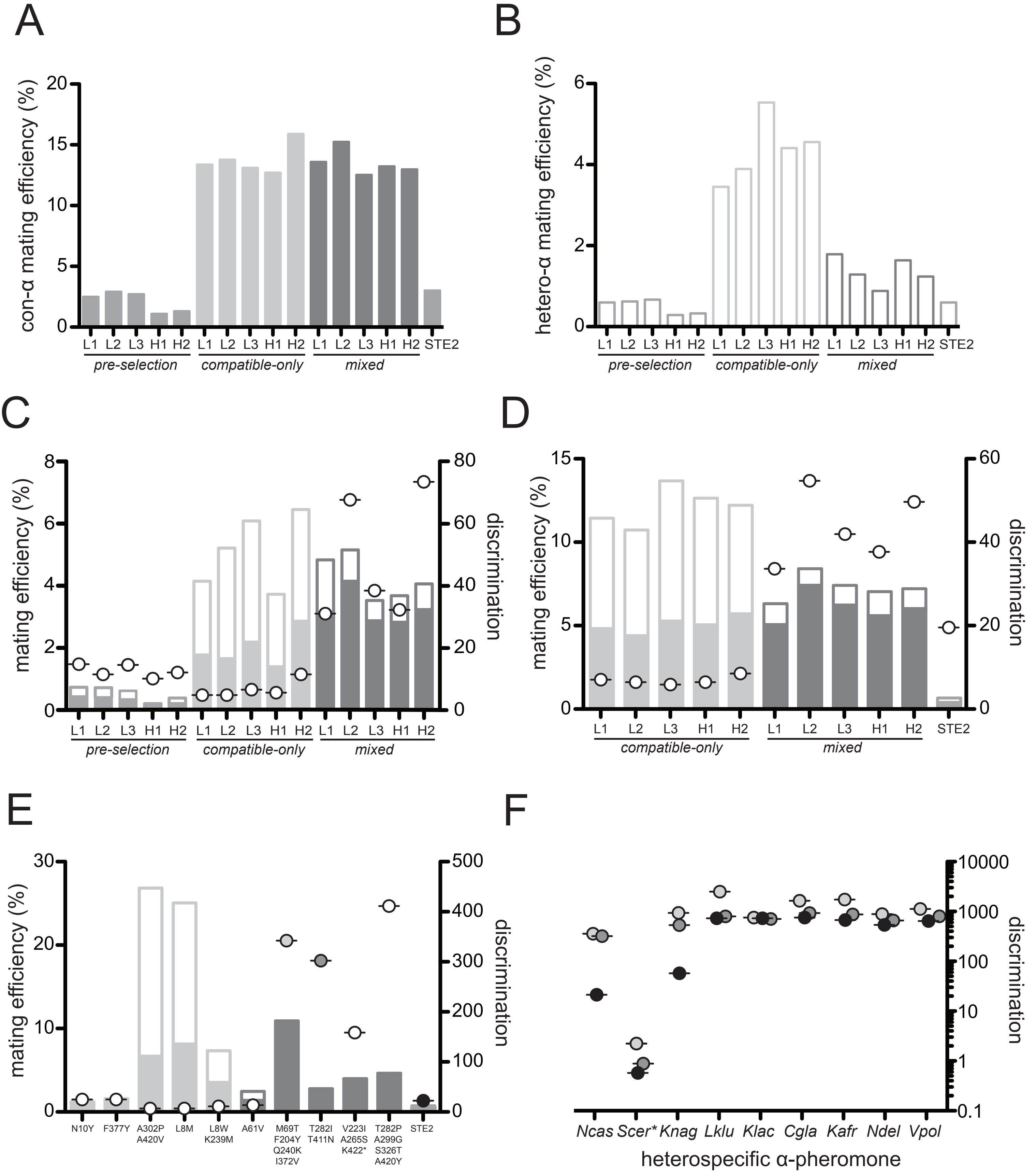
Figure 2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discrimination
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20
0
L1 L2 L3 H1 H2 L1 L2 L3 H1 H2 STE2
compatible-only
mixed
E
mating efficiency (%)
30
20
10
0
discrimination
500
400
300
200
100
0
N10Y
F377Y
A302P A420V
L8M
L8W K239M
A61V
M69T F204Y Q240K I372V
T282I T411N
V223I A265S K422*
T282P A299G S326T A420Y
STE2
F
discrimination
10000
1000
100
10
1
0.1
Ncas Scer* Knag Lklu Klac Cgla Kafr Ndel Vpol
heterospecific α-pheromone

Figure 3

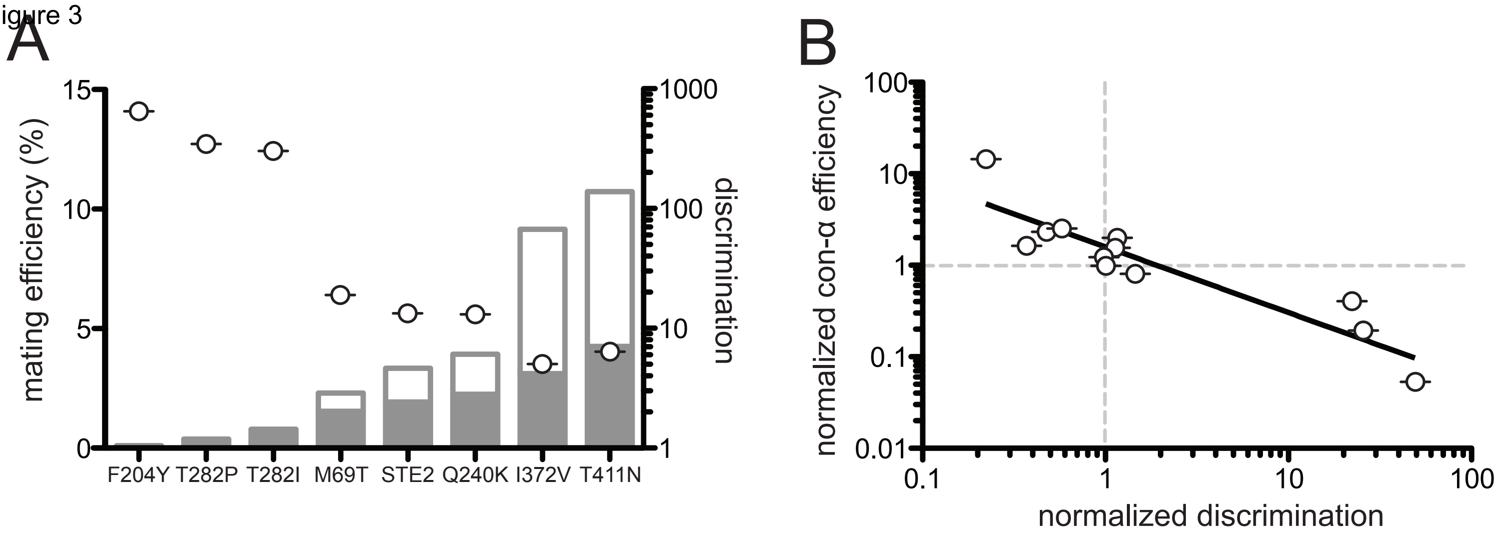
A
mating efficiency (%)
15
10
5
0
discrimination
1000
100
10
1
F204Y
T282P
T282I
M69T
STE2
Q240K
I372V
T411N
B
normalized con-α efficiency
100
10
1
0.1
0.01
0.1
1
10
100
normalized discrimination

Figure 4