LETTERS

Incipient speciation by divergent adaptation and antagonistic epistasis in yeast

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Establishing the conditions that promote the evolution of reproductive isolation and speciation has long been a goal in evolutionary biology[1–3]. In ecological speciation, reproductive isolation between populations evolves as a by-product of divergent selection and the resulting environment-specific adaptations[4–6]. The leading genetic model of reproductive isolation predicts that hybrid inferiority is caused by antagonistic epistasis between incompatible alleles at interacting loci[1,7]. The fundamental link between divergent adaptation and reproductive isolation through genetic incompatibilities has been predicted[1,4,5], but has not been directly demonstrated experimentally. Here we empirically tested key predictions of speciation theory by evolving the initial stages of speciation in experimental populations of the yeast *Saccharomyces cerevisiae*. After replicate populations adapted to two divergent environments, we consistently observed the evolution of two forms of postzygotic isolation in hybrids: reduced rate of mitotic reproduction and reduced efficiency of meiotic reproduction. This divergent selection resulted in greater reproductive isolation than parallel selection, as predicted by the ecological speciation theory. Our experimental system allowed controlled comparison of the relative importance of ecological and genetic isolation, and we demonstrated that hybrid inferiority can be ecological and/or genetic in basis. Overall, our results show that adaptation to divergent environments promotes the evolution of reproductive isolation through antagonistic epistasis, providing evidence of a plausible common avenue to speciation and adaptive radiation in nature.

The development of reproductive isolation is a key aspect of speciation because it is important for both initial divergence and maintenance of distinct species. If two species are specialized to different environments, interspecific hybrids might have reduced fitness in both environments because they exhibit maladaptive intermediate phenotypes, underscoring the inherent link between ecological isolation and divergent adaptation[5,6,8,9]. The link between genetic isolation and divergent adaptation can be explained by Dobzhansky–Muller genic incompatibilities[1,7]. A progenitor population enters and adapts to divergent environments, and in each of these environments certain new alleles are favoured by selection. Hybridization creates novel combinations of alleles untested by selection[10,11], and negative interactions among such alleles can reduce hybrid fitness.

We investigated the relationship between adaptation to divergent environments and evolution of reproductive isolation using large replicate experimental populations of the yeast *Saccharomyces cerevisiae*. Twelve populations were initiated from a single diploid progenitor (P) and evolved for 500 generations by serial transfer in one of two sub-optimal liquid environments: high-salinity (S) or low-glucose minimal medium (M). Each of the 12 evolved populations displayed a significant increase in relative fitness (>1.0; one-tailed, $t > 3.15$, degrees of freedom d.f. $= 2$, $P < 0.05$ in each case), providing clear evidence for adaptation (Fig. 1). When competing in their

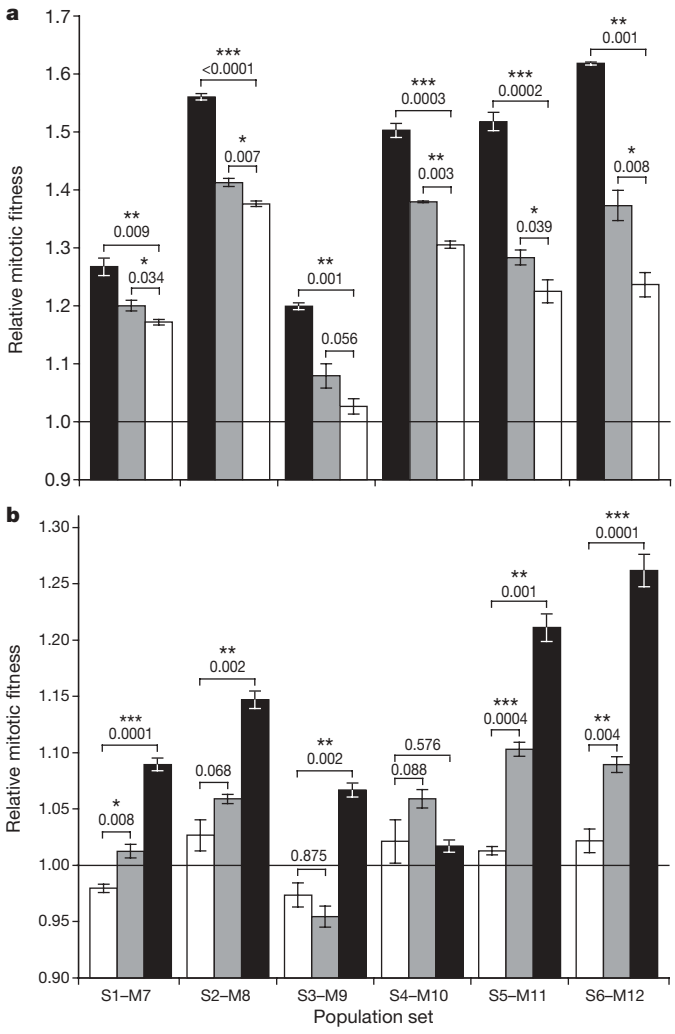

Figure 1 | Comparisons of mitotic fitness. a, Fitness assays in the high-salinity environment. Mean relative fitness estimates for populations evolved in high salinity (S, black), for hybrids between evolved populations and progenitors (S/P, grey), and for hybrids between populations evolved in high salinity and populations evolved in low glucose (S/M, white). **b**, Fitness assays in the low-glucose environment. Mean relative fitness estimates for populations evolved in low glucose (M, black), for hybrids between evolved populations and progenitors (M/P, grey), and for hybrids between populations evolved in high salinity and populations evolved in low glucose (S/M, white). Statistical significance (P values) of comparisons involving S/M hybrids is shown above the brackets. Error bars, ± 1 s.e.m. ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

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selective environment, evolved populations had an average fitness advantage of 28.8% over the progenitor. By contrast, when competing in the permissive or alternative selective environment, the mean relative fitness of evolved populations was not significantly increased (one-tailed, $t < 1.58$, d.f. $= 11$, $P > 0.42$ in each case). The fitness advantages gained during evolution represented environment-specific adaptations rather than an overall increase in general fitness (Fig. 2).

The hallmark of ecological speciation is environment-dependent (conditional) isolation, in which the fitness of hybrids is lower than that of pure populations in their selective environments. Each S population was paired with an M population, creating six independent comparisons. We tested for reproductive isolation by measuring fitness of the S/M hybrid diploids in all environments (permissive, high salinity and low glucose). In high salinity, S/M hybrid fitness was significantly lower than the S population fitness for each of the six population sets (Fig. 1). Similarly, in low glucose, S/M hybrid fitness was significantly lower than the M population fitness in five of six sets (Fig. 1). The same patterns of hybrid inferiority were observed when data were pooled ($P \leq 0.0001$ for each environment). Conversely, competition in the permissive environment revealed no significant difference between the mean fitness of S/M hybrids and that of pure populations (one-tailed, $t = 0.09$, d.f. $= 36$, $P = 0.53$), indicating that hybrid fitness reductions were dependent on the selective environments. As a control, we also constructed hybrids between populations that had evolved in the same environment. Overall, the fitness of hybrids between parallel-adapted populations was not significantly lower than that of pure populations (one-tailed, $P \geq 0.35$ for each environment). These patterns of conditional reproductive isolation can result only from environment-based divergent selection[12,13].

Our experimental yeast system provided the unique ability to construct hybrids between evolved populations (S or M) and the unevolved progenitor (P), resulting in 'progenitor hybrids' (S/P and M/P). Fitness gains of progenitor hybrids, which possessed only one evolved genome component, were only 0.51 ± 0.08 (mean $\pm$ standard error of mean, s.e.m.) of that of pure populations, which possessed two evolved genome components. Thus, reduced hybrid fitness was caused in part by incomplete dominance of the net effects of beneficial adaptive factors. These data provide a clear example of ecological isolation, because hybrid inferiority is determined by an interaction between genotype and environment[12,13].

Progenitor hybrids and S/M hybrids should have equal fitness if fitness is determined solely by adaptive mutations. In high salinity, S/M hybrids were less fit than S/P hybrids in all six population sets, and significantly so for five sets (Fig. 1a). In low glucose, S/M hybrids were less fit than M/P hybrids in five sets, and significantly so for three sets (Fig. 1b). The inferiority of S/M hybrids compared to progenitor hybrids can be explained by two factors: first, antagonistic epistasis[10,14] between S and M genome components, and, second, detrimental pleiotropic effects of adaptation on fitness in the alternative environment. The percentage of total reduction in S/M hybrid fitness caused by antagonistic epistasis and pleiotropy together averaged $27.6 \pm 3.5\%$. In general, the detrimental pleiotropic effects of adaptation were relatively minor (Fig. 2). Mean fitness for the 12 pure populations in the alternative environment was 0.98 ± 0.01, suggesting that antagonistic epistasis was responsible for a considerable proportion of S/M hybrid fitness deficits. For the five comparisons in which detrimental effects of pleiotropy were absent (fitness of pure population in alternative environment ≥ 1.0; S1–M7 in high salinity, and S1–M7, S2–M8, S4–M10 and S6–M12 in low glucose (see Methods)), S/M hybrids were significantly less fit than progenitor hybrids (paired-sample t-test, $P < 0.0001$; see Fig. 1 for individual comparisons). In these cases, S/M hybrid inferiority was caused solely by genetic isolation by means of antagonistic epistasis.

In addition to mitotic reproduction, S/M hybrids were severely inferior to pure populations in their ability to reproduce meiotically (sexually). In an environment permissive to sexual reproduction, mean meiotic efficiency of pure populations was $63.6 \pm 1.2\%$, whereas S/M hybrids had a mean of only $34.4 \pm 1.7\%$. For all six independent comparisons, meiotic efficiency of S/M hybrids was significantly lower than that of both pure populations (Fig. 3). The mean meiotic efficiency of hybrids between parallel-adapted populations (45.84 ± 1.58) was significantly greater than the S/M hybrid mean (one-tailed, $t = 4.97$, d.f. $= 58$, $P < 0.0001$), but was still less than that of the pure populations (one-tailed, $t = 8.89$, d.f. $= 64$, $P < 0.0001$). Selection in divergent environments resulted in greater reproductive isolation than parallel selection in the same environment. These unconditional reductions in hybrid meiotic efficiency represent another clear example of genetic isolation by means of antagonistic epistasis between divergently adapted genomic components.

We are able to reject the two alternative explanations for reduced meiotic reproduction of hybrids: chromosomal rearrangements[15–17] and the anti-recombination activity of the mismatch repair system[18,19]. Both mechanisms have extremely detrimental effects on progeny viability resulting from abnormal disjunction, aneuploidy and deficiency[16–19]. In contrast, meiotic progeny from S/M hybrids all had high viabilities, ranging from 97.7–100% (mean $= 99.4\% \pm 0.4$), with normal mendelian segregation of markers. This observation

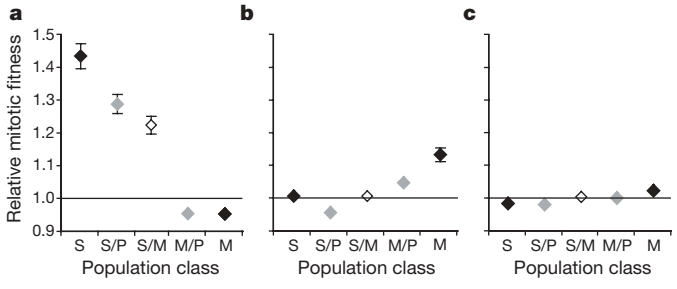

Figure 2 | General patterns of mitotic fitness across population classes. Mean relative fitness estimates for pure populations (S and M, black filled), progenitor hybrids (S/P and M/P, grey filled), and S/M hybrids (S/M, white filled) assayed in: **a**, the high-salinity environment, **b**, the low-glucose environment, and **c**, the permissive environment. Data were pooled for all six populations sets; error bars, ± 1 s.e.m. ($n = 18$), if greater than the symbol size.

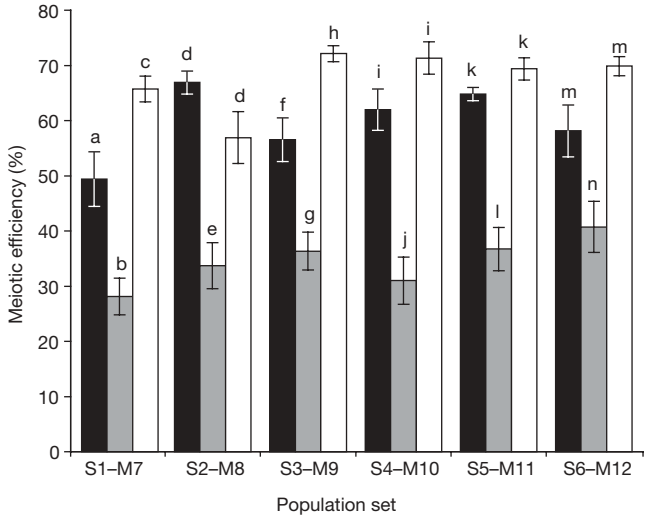

Figure 3 | Comparisons of meiotic efficiency. Mean meiotic efficiency values for pure populations (S, black; M, white) and for S/M hybrids (S/M, grey) from six population sets. Error bars, ± 1 s.e.m. ($n = 5$). For all six sets, the meiotic efficiency of hybrids was significantly less than that of both pure populations. Means that are not connected by the same letter are significantly different from each other, as determined by analysis of variance (all $F \geq 13.94$, $P \leq 0.0007$) and post-hoc Tukey tests ($P = 0.05$) performed independently for each population set.

alone excludes both alternative mechanisms (see Supplementary Information 1 for additional evidence).

To investigate whether mitotic fitness and meiotic efficiency of hybrids were affected by interactions between the same genetic factors, we compared these 2 phenotypes across 12 series of 6 hybrid diploids that had variable numbers of S-adapted and M-adapted factors (see Supplementary Methods and Supplementary Information 2). The S haploid component was fixed within each series, so differences in phenotype are caused by the variable number of M-adapted factors. A positive correlation between meiotic efficiency and mitotic fitness (in high salinity), which was predicted if the two phenotypes were affected by the same genetic interactions, was displayed by 5 of the 12 hybrid series, only one of which was significant ($R^2 = 0.78$, $P < 0.02$). Reductions in the two phenotypes were not inextricably linked, which suggests that a complex array of multiple independent interactions cumulatively resulted in concomitant negative effects on both phenotypes in S/M hybrids (Figs 1–3).

Microarray experiments were performed to compare genome-wide gene expression during sporulation[20] between non-hybrid and hybrid states of S and M genome components (see Supplementary Methods and Supplementary Information 3). Reference RNA was an equal mixture of RNA from two pure populations, and test RNA was from S/M hybrids. For the two population sets examined (S2–M8 and S6–M12), a consensus set of four genes displayed expression that was significantly altered in S/M hybrids: two genes were consistently underexpressed and two genes were overexpressed. Both underexpressed genes (*RCR1* and YLR194C) are involved in cell wall synthesis and organization; their mis-expression might affect both mitotic fitness and meiotic efficiency. An additional 31 genes that had altered expression were specific to one of the two population sets examined. Two sporulation genes (*SPS100* and *PFS1*) were underexpressed, providing evidence for disruption of the sporulation transcriptional cascade in hybrids and an entry point for future investigation of the underlying mechanism(s) of antagonistic epistasis.

Previous studies found no evidence for recessive[21] or dominant[22] incompatibilities causing yeast hybrid sterility. These studies differed from ours in one fundamental way: we examined *de novo* genetic isolation between recently diverged populations rather than pre-established isolation between species that diverged ~5 million years ago. The history of established species may comprise multiple episodes of adaptation, with successive gains or losses of mechanisms of isolation. The initial mechanisms of isolation are obscure.

Unlike natural species, our experimental populations have an evolutionary history that is known with certainty. We can therefore conclude that divergent adaptation caused the reproductive isolation observed in this investigation. Experimental evolution of reproductive isolation has been studied in a few eukaryotes (mainly *Drosophila*) with mixed results[23–27]. Previous research has focused mostly on prezygotic isolation, and we are aware of only a single study that reported successful evolution of postzygotic isolation by means of divergent selection[28]. We present the most striking example of experimental evolution of postzygotic isolation observed in any organism, and the first for the fungal kingdom[29].

Although the isolation that evolved *de novo* in our short-term experiment is partial, it represents incipient speciation. Given more time, complete reproductive isolation is likely to evolve. Effects of reproductive isolation are compounded over successive steps of the life cycle; pure populations have a 2.14-fold advantage (range, 2.00–2.31) over S/M hybrids when mitotic and meiotic reproduction are combined. Furthermore, these effects are multiplied over successive generations, allowing minor hybrid inferiority to have major detrimental consequences to overall gene flow among natural populations. Therefore, the most significant point in the speciation process is the initial development of reproductive isolation, regardless of its intensity, as studied here.

Our results validate the theoretical link between divergent adaptation and reproductive isolation. We show that specialization to alternative environments provides the phenotypic variation on which ecological mechanisms of reproductive isolation can operate. We also show that the processes of mutation and selection during divergent adaptation can facilitate the accumulation of antagonistically interacting genetic factors. Overall, our experiments support the theory that divergent adaptation promotes the evolution of reproductive isolation, caused by intrinsic genetic incompatibilities and/or extrinsic environmental factors, and is a major driving force for speciation.

METHODS SUMMARY

In the diploid progenitor strains, the entire *URA3* open reading frame was replaced with one of two antibiotic resistance cassettes (geneticin or nourseothricin) flanked by one of five DNA barcodes. Each of the 12 experimental populations was propagated in 10 ml of shaking liquid medium, with daily serial transfers (1/100 dilution) for a total of 500 generations. Six populations evolved in rich high-salinity medium (S1–S6), and six populations evolved in low-glucose minimal medium (M7–M12). Six independent population sets were designated by pairing S and M populations with alternative antibiotic resistance. To create hybrid populations, pure populations were sporulated, mated with each other, and then grown in medium containing both antibiotics to select for hybrids. The same procedure was used to create hybrids between pure populations and progenitors.

Mitotic fitness of populations was determined by direct competition against an alternatively tagged progenitor and by measurement of change in proportions of strains in mixed culture over time[30]. Genomic DNA was extracted from mixed cultures at the start and finish of a 24 h competition period. A segment of the tag region was amplified using polymerase chain reaction and transferred to membranes, which were probed with oligonucleotides complementary to the barcode tags and an invariant region shared by all competitors. The number of cell doublings was calculated from tag signal ratios and optical densities of cultures. Relative fitness was defined as the ratio of cell doublings by the assay population to cell doublings by the progenitor.

The meiotic efficiency of populations was defined as the percentage of diploid cells that underwent meiosis. After 72 h of sporulation, meiotic efficiency was determined by direct counting under ×400 magnification.

Microarray protocols were performed as described in ref. 14 except that cell populations were incubated to mid-log growth phase then transferred to sporulation medium for 12 h before RNA extraction.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions The research was conceived and planned by all authors. C.S. and J.R.D. performed the experiments, and J.R.D. analysed the data. J.R.D, J.B.A. and L.M.K. contributed to the writing of the manuscript, which was coordinated by J.R.D.

Author Information The full microarray data set has been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession series GSE6870. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to J.R.D. (jdettman@utm.utoronto.ca).

METHODS

Additional details are given in Supplementary Methods.

Strains. Progenitor strains were derived from diploid strains constructed in ref. 30. The entire *URA3* open reading frame was replaced with a geneticin (G418) antibiotic resistance cassette flanked by two DNA barcodes. The G418 cassette was replaced with a nourseothricin (NAT) resistance cassette in each strain to give ten different combinations of two antibiotic resistance cassettes and five barcode tags.

Evolution regimen. Six populations, designated S1 to S6, were evolved in rich high-salinity medium (environment S, yeast peptone dextrose (YPD: 1% yeast extract, 2% peptone, 2% dextrose) plus 1.0 M NaCl). The other six populations, designated M7 to M12, were evolved in low-glucose minimal medium (environment M, 0.67% yeast nitrogen base without amino acids, 0.25% dextrose, 0.02 mg ml^{-1} uracil). Populations were propagated in 10 ml of liquid medium at 30 °C on a rotary shaker at 250 r.p.m. After each 24 h of growth, which represented an average of 6.64 generations, 100 µl of culture was transferred to 9.9 ml of fresh medium. This cycle was repeated until populations had evolved for a total of 500 generations. Every 100 generations, 8 populations (S1 to S4, M7 to M10) were sporulated, diploidized by intra-population mating, and then used to re-initiate the evolution regimen. Every 100 generations, aliquots of all populations were archived at –80 °C. No cross-contamination was detected, and each tag and resistance marker appeared where expected without exception.

Population sets and construction of hybrids. Population sets were designated by pairing populations that had alternative antibiotic resistance cassettes. To create hybrid populations, the two pure populations were sporulated, mated with each other, and then grown in liquid YPD with both NAT and G418 antibiotics. The same procedure was used to create hybrids between pure populations and alternatively tagged progenitors. All sporulations and matings were performed *en masse*, so assay results represent population averages rather than specific values for single genotypes.

Mitotic fitness. Mitotic fitness of populations was determined by direct competition against an alternatively tagged progenitor and by measurement of change in proportions of strains in mixed culture over time[30]. The two competitors were mixed in equal proportions and competition was initiated (time 0) by inoculating 10 µl into 9.99 ml of competition medium. Competitions were run for 24 h (time 1) under conditions identical to both selective environments (high salinity or low glucose) as well as in a rich permissive environment (YPD). Genomic DNA was extracted from mixed cultures at time 0 and time 1, and a segment of the *URA3* replacement region was amplified by polymerase chain reaction. Amplicons were transferred to membranes, which were probed in succession using oligonucleotides complementary to the barcode tags and an invariant region shared by all competitors. The number of cell doublings was calculated as $\log_2([R_f \times D_f] / [R_i \times D_i])$, where R_f is the ratio of tag signal to invariant probe signal at time 1 and R_i is the corresponding ratio at time 0. D_f and D_i are optical densities of cultures at time 1 and time 0, respectively. Relative fitness was defined as the ratio of cell doublings by the assay population to cell doublings by the progenitor. To estimate the mean dominance of the net effects of all adaptive factors present in the adapted genome, we compared fitness of pure populations to that of progenitor hybrids. Here, $w_{X(Y)}$ equals the relative fitness of population X in the Y environment. The degree of dominance for S-adapted factors was calculated as $(w_{S/P(S)} - 1) / (w_{S(S)} - 1)$, and for M-adapted factors as $(w_{M/P(M)} - 1) / (w_{M(M)} - 1)$. The combined effects of antagonistic epistasis and pleiotropy were estimated by the fitness difference between S/M hybrids and progenitor hybrids. By controlling one haploid component in the diploid, we could determine the effects of the other classes of haploid components, and associated interactions. The proportion of total reduction in S/M hybrid fitness attributable to antagonistic epistasis and pleiotropy together was calculated by $(w_{S/P(S)} - w_{S/M(S)}) / (w_{S(S)} - w_{S/M(S)})$ and $(w_{M/P(M)} - w_{S/M(M)}) / (w_{M(M)} - w_{S/M(M)})$.

Meiotic efficiency. After 72 h of sporulation, the percentage of diploid cells that underwent, or were undergoing, meiosis was determined by direct counting under ×400 magnification. The presence of dyads, triads and tetrads constituted evidence for meiosis. An average of >100 cells were examined per meiotic efficiency replicate.

Microarrays. Protocols were performed as described[14] with the exception that cell populations were incubated to mid-log growth phase and then transferred to the sporulation medium for 12 h before RNA extraction. For each hybrid versus non-hybrid comparison, four independently replicated microarray hybridizations, with balanced dye swapping, were performed. Gene lists were made from genes that were differentially expressed by ≥1.5-fold in S/M hybrid populations relative to the reference.