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Heterothallism in *Saccharomyces cerevisiae* isolates from nature: effect of *HO* locus on the mode of reproduction

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Abstract

Understanding the evolution of sex and recombination, key factors in the evolution of life, is a major challenge in biology. Studies of reproduction strategies of natural populations are important to complement the theoretical and experimental models. Fungi with both sexual and asexual life cycles are an interesting system for understanding the evolution of sex. In a study of natural populations of yeast *Saccharomyces cerevisiae*, we found that the isolates are heterothallic, meaning their mating type is stable, while the general belief is that natural *S. cerevisiae* strains are homothallic (can undergo mating-type switching). Mating-type switching is a gene-conversion process initiated by a site-specific endonuclease *HO*; this process can be followed by mother–daughter mating. Heterothallic yeast can mate with unrelated haploids (amphimixis), or undergo mating between spores from the same tetrad (intratetrad mating, or automixis), but cannot undergo mother–daughter mating as homothallic yeasts can. Sequence analysis of *HO* gene in a panel of natural *S. cerevisiae* isolates revealed multiple mutations. Good correspondence was found in the comparison of population structure characterized using 19 microsatellite markers spread over eight chromosomes and the *HO* sequence. Experiments that tested whether the mating-type switching pathway upstream and downstream of *HO* is functional, together with the detected *HO* mutations, strongly suggest that loss of function of *HO* is the cause of heterothallism. Furthermore, our results support the hypothesis that clonal reproduction and intratetrad mating may predominate in natural yeast populations, while mother–daughter mating might not be as significant as was considered.

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Y.K., T.K.E. and M.B. are interested in yeast biodiversity, microsatellite DNA and applied genomics. A.K. and Z.F. are interested in population genetics, evolution of sex and recombination, and genome mapping. A.M. is interested in chromosome transmission, experimental population genetics, laboratory evolution, and mating in fungi. A.V.S. is interested in the evolution and genetic architecture of complex traits and diseases. J.-Y.L. is interested in the mechanisms of speciation and S.-L.C. is interested in adaptation of organisms to novel environments.

Keywords

heterothallism; HO; reproduction; *Saccharomyces cerevisiae*

Introduction

Sexual reproduction is considered one of the key factors in evolution of life, and understanding the selection pressures responsible for the evolution of sex and recombination remains a major challenge in biology. Sexual reproduction is thought to be an 'expensive' process because offspring yield per sexual parent is less than that per clonal parent. For fungi, the actual cost of sex is considered much lower compared to animals and plants (e.g. Aanen & Hoekstra 2007). Due to meiotic recombination, sexual reproduction generates more variability than clonal reproduction that may serve as raw material for natural selection (Kondrashov 1993; Korol *et al.* 1994; Barton & Charlesworth 1998; Burt 2000; Rice 2002; Agrawal 2006). Sex facilitates combination of new favourable genes (Fisher–Muller hypothesis) and selection against deleterious mutations (Muller hypothesis) (Kondrashov 1993; Elliot 1994; Michod & Levin 1988; Goddard 2007). Thus, sexual reproduction can be considered in two perspectives: as a 'conservative' mechanism preserving the genome by facilitating purifying selection against harmful mutations, and as a factor of genome flexibility that increases the probability of survival in a competitive and / or changing environment and expedites the appearance of evolutionary innovations (Maynard Smith 1978; Elliot 1994; Korol *et al.* 1994; Otto & Gerstein 2006; Goddard 2007). Despite of the ever-increasing diversity of theoretical (mathematical) models aimed at explaining the evolution of sex, there are more models that are relevant to animals and plants than to the majority of eukaryotic organisms, including fungi (Birky 1999). Still, during the last two decades fungi have become one of the most popular taxa for laboratory testing of sexual causation (Birdsell & Wills 1996; Zeyl & Bell 1997; Greig *et al.* 1998; Goddard *et al.* 2005; Otto 2009). However, despite the large contribution of controlled laboratory experiments, the major problem remains enigmatic and needs elucidation from critical field observations.

Fungi can have both sexual and asexual life cycles. Sex in fungi is typically achieved by mating of individuals of different mating types that are controlled by the mating-type (*MAT*) locus. Fungi have evolved two major mating-type systems: bipolar or tetrapolar (Kronstad & Staben 1997; Fraser & Heitman 2003). Bipolar fungi have a single *MAT* locus that gives rise to two opposite mating types. Bipolar mating systems are common in ascomycetes, which includes for example budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) (Haber 1998a), pathogenic yeast *Candida albicans* (Hull & Johnson 1999) and *Cryptococcus neoformans* (Lengeler *et al.* 2002). On the other hand, species with the tetrapolar mating system have two unlinked *MAT* loci, which can generate a wide range of mating types (thousands in some species). Basidiomycetes fungi such as the mushroom fungi *Schizophyllum commune* and *Coprinus cinereus* have the tetrapolar mating system, which yields over 20 000 (Kothe 1999) and 50 000 (Kues 2000) mating types, respectively. Recently, it was shown that homothallic *Candida albicans* and *Cryptococcus neoformans* can go through sexual cycles without a mate of the opposite mating type (Lin *et al.* 2005). It is still unclear how common this is in the fungal kingdom.

At the population level, a bipolar mating system promotes inbreeding and a tetrapolar mating system promotes outbreeding (Fraser & Heitman 2003). The frequency of outcrossing depends on the number of *MAT* alleles in the population (Hsueh & Heitman 2008). Notably, some fungi, such as the budding yeast *S. cerevisiae* and some filamentous ascomycetes are able to switch their mating types (Russell *et al.* 1986; Perkins 1987; Haber 1998b). Why have fungi evolved such diverse modes of sexual reproduction? Recent studies

have shown that in some pathogenic fungi, sexual reproduction is correlated with their virulence (Heitman 2006; Hsueh & Heitman 2008) or involved in host niche adaptation (Bennett & Johnson 2005).

Despite great variation in the details of sexual reproduction, sex in eukaryotic species, including gamete fusion in animals, fusion between pollen and female gametophytes in plants, and mating between different mating types in fungi, share some general cellular processes. The central role in the reproduction mode is played by genetically controlled recognition of the fusion partner (Clark *et al.* 2006; Chen *et al.* 2007). Studies among different taxa of animals, plants, and fungi have led to the conclusion that reproductive genes evolve more rapidly than other genes (Wik *et al.* 2008). Moreover, the structure of fungal mating-type loci displays features in common with sex chromosomes of animal and plants. Similar mechanisms and genomic elements are thought to drive the evolution of sex determining regions in all three eucaryotic kingdoms. These include the critical role of repeated DNA and recombination in shaping the sex chromosomes and *MAT* loci (e.g. Fraser *et al.* 2004).

In the sexual cycle of budding yeast *S. cerevisiae*, mating is achieved by fusing cells of two different haploid types, **a** and **α**, to form diploid **a / α** cells. When under stress conditions, diploid cells undergo meiosis to produce four haploid spores inside asci. The spores are more resistant to stresses than vegetative cells and germinate when conditions have improved. After germination, haploid cells have three alternative ways to return to a diploid state, which is necessary for meiosis: amphimixis, automixis and haplo-selfing. Amphimixis is the mating of unrelated haploids from different tetrads and automixis is mating between spores from same tetrad. Haplo-selfing, which involves mating-type switching, is a mother-daughter mating behaviour in homothallic strains (Knop 2006).

The *MAT* locus is responsible for the mating type of haploid yeast cells. The two variants of the *MAT* locus, *MATa* and *MATα*, differ in sequence, size and gene content. In homothallic strains, a haploid cell can switch its *MAT* genotype (from *MATa* to *MATα* and vice versa) by a gene-conversion process that replaces the current *MAT* allele with a copy from one of two silent loci, *HML* or *HMRa*. The HO endonuclease initiates this gene-conversion process by generating a site-specific double-stranded DNA break during cell division (Russell *et al.* 1986). Most natural isolates of *S. cerevisiae* tested so far are able to undergo mating-type switching and therefore are considered homothallic (Mortimer 2000; Butler *et al.* 2004), whereas strains that cannot undergo switching for any reason are considered heterothallic. As heterothallic yeast cells are unable to do haplo-selfing, heterothallic populations may have different outcrossing rates and inbreeding levels from those of homothallic populations.

In the last decade, surprisingly, additional roles for the mating-type loci were revealed. Verna & Ballester (1999) showed that the mating-type genes of *S. cerevisiae* take part in maintaining cell wall integrity and heat shock response. Another study showed that simultaneous expression of both mating-type alleles in haploid yeasts suppresses mutations in the microtubule motor genes (Steinberg-Neifach & Eshel 2000). The authors suggest that properties of microtubules in diploid cells are modified in a pathway controlled by the mating-type genes.

The *HO* gene is expressed only in cells that have budded once and carry only one type of *MAT* allele (hence, are phenotypically either *MATa* or *MATα*, but not *MATa / α*). This means that during vegetative growth only mother cells switch mating type (Herskowitz 1988). Recently, it was shown that the *HO* endonuclease is a relatively new evolutionary addition to a pre-existing cassette-based switching system. The authors claim that the mating

system of hemiascomycete yeasts has evolved from an obligate heterothallic system to HO-catalyzed homothallic switching via a series of steps (Butler *et al.* 2004).

HO is a homing endonuclease (rare cutting enzyme) that belongs to the LAGIDADG family (Belfort & Roberts 1997) and consists of 586 amino acids, in which the carboxyl-terminal 100 amino acids contain five zinc fingers (Russell *et al.* 1986; Bakhrat *et al.* 2004). These zinc fingers are proven to be crucial for the binding of the HO protein to DNA and, therefore, for its activity (Jin *et al.* 1997). The *HO* gene seems to have been gained by yeasts before the whole genome duplication that occurred in the shared ancestor of several yeast species. Apart from *S. cerevisiae* and its close relatives, the *HO* gene is present only in *Candida glabrata*, *Saccharomyces castellii* and *Zygosaccharomyces*. Some other yeast species, which do not have an orthologue of *HO*, can switch mating types by different mechanisms, while others are strictly heterothallic (Haber & Wolfe 2005).

Most natural isolates of *S. cerevisiae* are considered to be diploid and phenotypically homothallic (Mortimer 2000; Butler *et al.* 2004). If this is indeed the case, then the HO endonuclease enzyme should be predominantly functional in *S. cerevisiae* strains in nature. We studied the genetic diversity of *S. cerevisiae* in a natural population isolated from a well-studied natural site, called 'Evolution Canyon' on Mount Carmel, Israel, where dozens of other species were analysed at the phenotypic, genetic and molecular-genetic levels (Saleem *et al.* 2001; Zamorzaeva *et al.* 2005; Nevo 2009). The yeast strains were isolated from Evolution Canyon at seven collection sites, from soil and plant samples collected in sunny and shady places (Katz Ezov *et al.* 2006). Inspection of SSR (short sequence repeats, microsatellites) polymorphism, tetrad analysis, and flow cytometry showed that the isolates differ in ploidy level; diploids, triploids and tetraploids were found in the collected material. An SSR-based phylogenetic analysis revealed a clonal, rather than a sexual, structure of the triploid and tetraploid, but not diploid subpopulations. Maximum genetic diversity was observed among diploids and minimum diversity among triploids (Katz Ezov *et al.* 2006). An interesting result was revealed in tetrad analysis: haploids derived from 21 diploids as well as some of the diploids derived from tetraploids displayed stability of mating type (being therefore heterothallic). Based on this finding, we decided to investigate the molecular basis of heterothallism in these natural populations.

Clearly, one of the most relevant candidates for molecular testing is the *HO* gene. Hence, we sequenced the *HO* gene in multiple isolates from the study site and compared the sequences to the standard homothallic *HO* sequence. Sequence analysis was conducted on 26 representative strains and offspring, chosen based on the observed population structure, with emphasis on diploids, which showed the highest variability (Katz Ezov *et al.* 2006). Several point mutations, including insertions, deletions and single-nucleotide polymorphisms (SNPs) were found whose nature suggests that HO is not functional in these strains. We also performed functional assays to test whether the mating-type switching genes upstream or downstream to HO in the mating-type switching pathway are intact. The experimental results further support the view that loss of mating-type switching ability in the natural isolates is most likely due to loss of function of HO.

Materials and methods

Strains

Sixty-eight *Saccharomyces cerevisiae* yeast strains were isolated from 'Evolution Canyon' on Mount Carmel, Haifa, Israel (Nagornaya *et al.* 2003). Based on our previous results on the structure of this population (Katz Ezov *et al.* 2006), 26 representative strains were chosen for the *HO* analysis (see Table 1). In addition, *S. cerevisiae* strain M12 was used.

Tetrad dissection

A micromanipulator equipped with a glass needle was used to isolate spores from asci. Asci were digested using 0.25 mg/mL Zymolyase T100 (Sigma) in 1 M sorbitol.

Media

Yeast cells were grown on Yeast-extract Peptone Dextrose (YPD) complete medium at 30 °C (Sherman 1991) unless stated differently. Sporulation was induced on sporulation medium (SPO) (Kassir & Simchen 1991). Yeast-Extract Peptone medium (YEP) was used for the mating-type switching assay (Kassir & Simchen 1991).

Mating-type tests

Mating-type tests were performed as described in (Sherman 1991). The diploid strains were induced to sporulate and tetrads were dissected. The tested haploid segregants were mixed in parallel with the *MATa* and the *MATα* tester strains on the YPD plate and incubated at 30 °C overnight. These cultures were then replicaplated on a SPO plate, along with the original strain. Sporulation was tested under a microscope after 24 and 48 h of incubation at room temperature.

DNA extraction

DNA was extracted using the yeast DNA miniprep method as described (Kaiser *et al.* 1994).

Sequence analysis

The *HO* locus was amplified using the primers 5'-TTGAGAAAGGCTGAAGTTGG-3' (f) and 5'-TGTTGAAGCATGATGAAGCG-3' (r). Polymerase chain reaction (PCR) was performed using Super Nova *Taq* DNA polymerase (Madi Ltd), with annealing temperature 56 °C. The PCR was conducted using PTC-100 PCR machine (MJ research, Inc). Primers for sequencing both strands were: 5'-ACACTCTGGTCCTTTAAC-3', 5'-GACATTGGACTTTTCTTCC-3', 5'-CTGGCTCTTTTGTGTAC-3', 5'-TCACCTTCAAAAGCTCTG-3', 5'-GAAATC TGCAGCAATGTC-3', 5'-CAGGAAGACAAGGAATTTG-3', 5'-TGGGCTTACGATAGAAGG-3' and 5'-ACTAGAATGCTGGAGTAG-3' along with the forward and reverse PCR primers. Sequence was analysed from the ATG to the stop codon, and the resulting sequences were aligned with the wild-type sequence, GenBank Accession no. M14678 (Russell *et al.* 1986). The GenBank Accession nos for the *HO* gene sequenced in the following natural strains in this study are: strain #3 GQ923584, #7 GQ923585, #10 GQ923598, #13 GQ923586, #20 GQ923587, #33 GQ923599, #34 GQ923600, #35 GQ923601, #36 GQ923602, #39 GQ923603, #40 GQ923588, #45 GQ923604, #57 GQ923605, #58 GQ923606, #59 GQ923607, #60 GQ923608, #63 GQ923609, #66 GQ923589, #1-301 GQ923594, #1-302 GQ923595, #1-303 GQ923596, #1-304 GQ923597, #9-531 GQ923590, #9-532 GQ923591, #9-533 GQ923592, #9-534 GQ923593.

Phylogenetic analysis

Cluster analysis and phylogenetic reconstructions based on *HO* sequences were conducted using the Fitch–Margoliash algorithm (Fitch & Margoliash 1967). We used Euclidian

distance between strains *i* and *j* (possibly with different ploidy): $d^2(i, j) = \sum_{k=1}^N d_k^2(i, j)$, where *N* is the length of consensus *HO* sequence (in bp), and $d_k(i, j)$ is distance between

strains at single position *k* defined as $d_k^2(i, j) = \sum_{m=1}^5 w_m^2 d_{k,m}^2(i, j)$. Here w_m is the weight coefficient, and $d_{k,m}^2(i, j)$ is calculated by: $d_{k,m}^2(i, j) = 0.25(p_m(i_k) - p_m(j_k))^2 / ((p_m(i_k) + p_m(j_k))/2)$ [proportionally to corresponding term of chi-squared distance of distributions $p(i_k)$

and $p(j_k)$. Values $p_m(l)$ ($l = i$ or j) correspond to proportion of A, C, G, T and gap ‘-’ ($m = 1, 2, 3, 4$ and 5 respectively) at position k of strain l . For example, $p_1(A) = 1$, $p_m(A) = 0$ for $m = 2, \dots, 5$; $p_1(R) = p_3(R) = 0.5$, $p_2(R) = p_4(R) = p_5(R) = 0$. We used equal weight coefficients $w_m = 1$ for all letters ($m = 1, 2, 3, 4$) and twofold weight for a frameshift mutation, i.e. $w_5 = 2$. Cluster analysis and phylogenetic reconstructions based on SSR polymorphism were performed as described in Katz Ezov *et al.* (2006).

Mating-type switching assay

The haploid cells of representative strains (cluster I: #9, #10, #35, #36, #39, #57; cluster II: #13, #33, #34, #45, #59, #60, #63) were transformed with a CEN plasmid carrying a functional *HO* open reading frame (ORF), fused with the *GAL1* promoter (pGAL1-HO) and a dominant drug resistant gene to hygromycin. Plasmid transformations were carried out by the lithium acetate protocol (Ito *et al.* 1983). The transformants were inoculated in YEP with 2% raffinose and grown overnight. Cells were then diluted in YEP with 2% galactose and grown for two more hours. After galactose induction, at least 200 cells were plated on YPD plates to form single colonies. Mating types of each single colony were tested by halo assays as previously described method (Konopka *et al.* 1988).

Results

Studying a panel of 68 *Saccharomyces cerevisiae* natural isolates and their offspring revealed mating-type stability in offspring of 17 diploid strains, and two tetraploid strains (spores of triploid were inviable, hence, homothallism could not be tested phenotypically). These results were unexpected as natural *S. cerevisiae* strains are generally considered to be homothallic (Mortimer 2000), although few mutations in the *HO* gene have been reported earlier (Meiron *et al.* 1995). As we were working on newly isolated yeasts from a natural population, which appear not to be directly associated with any human activity, we were interested in characterizing and understanding the molecular basis of the observed heterothallism.

Sequencing and phylogenetic analysis

Based on our previous genomic diversity study (Katz Ezov *et al.* 2006), we selected a panel of strains, chosen to maximize both genetic diversity and naturally occurring heterothallism. In choosing the strains, we considered the population structure, with emphasis on diploids that harbour substantial diversity. This panel contains 16 diploid isolates, 2 triploids, 4 diploid offspring of a tetraploid isolate (a whole tetrad) and 4 haploid off-spring of a diploid isolate (a whole tetrad) (see Table 1). The ORF of the *HO* gene was sequenced in each strain, and all sequences were aligned, revealing a substantial amount of point mutations including SNPs, indels and nonsense mutations. All of the nucleotide mutations found are summarized in Table 2 and the amino acid mutations are summarized in Table 3.

Phylogenetic analysis revealed two major clusters (Fig. 1). The first cluster (cluster I) contains seven identical sequences, from the four tetrad offspring of diploid strain #9 alongside with diploid isolates #36, #40 and #58, that are homozygous for *ho*. All these sequences have a nonsense mutation (SNP at position 238: CAA → TAA, i.e. Gln → STOP), resulting in an 80 amino acid protein that has the same amino acid sequence as the wild type to that point. Downstream of the nonsense mutation we observed an accumulation of seven point mutations one of which is a frameshift. This cluster also contains strains #39, #35, #10 and #57, which have the nonsense and frameshift mutations.

The second major cluster (cluster II), that includes the wild-type *HO* sequence (from GenBank), is divided into three subclusters, a, b and c. Subcluster a contains only strain #63, a diploid isolate with three missense SNPs in *HO*; two of them are unique.

The next group, subcluster b, contains five identical sequences (strains #13, #34, #45, #59 and #60, all from diploid isolates) with four nonsilent SNPs. Some of the mutations are shared with other strains, including a SNP at position 1424 (CAT → CTT, His → Leu), which was shown in one study to cause loss of function of the HO endonuclease and is thought to prevent the binding of the HO protein to DNA (Meiron *et al.* 1995). In another study, the same mutation was found to reduce, but not abolish, the ability to switch mating type (Ekino *et al.* 1999). This mutation was found in all strains sequenced in our study except #63.

The third group, subcluster c, contains the GenBank *HO* sequence along with three almost identical sequences of strains that share four mutations: #3 (diploid), #1-303 and #1-302 (both diploid progeny of tetraploid strain #1). It also contains four identical sequences in strains: #7 (diploid), #20 (triploid), #1-301, and #1-304 (both diploid progeny of tetraploid strain #1), and strain #66 (triploid), which contains the same mutations as these four strains plus two unique mutations. These sequences share four missense mutations.

In addition to the mutation at position 1424, Meiron *et al.* (1995) identified three more point mutations in the *ho* gene in strain S288c, which is a derivative of a yeast strain isolated from a rotten fig in 1938 near Merced, California (Mortimer & Johnston 1986). Interestingly, we found all of these mutations in the sequences of our natural isolates:

- Position 565 (ACA → GCA, Thr → Ala); found in strains #7, #13, #20, #33, #34, #40, #45, #59, #60, #66, #1-301 and #1-304, while #1-302 and #1-303 were heterozygous for this mutation. In addition, an insertion at this codon (ACA → AGCA) was found in #3.
- Position 667 (GGT → AGT, Gly → Ser); found in #13, #33, #34, #45, #59 and #60, which are all diploids. Another mutation (GGT → GAT, Gly → Asp) was found at this codon in #7, #20, #66, #1-301 and #1-304, whereas #3, #1-302 and #1-303 were heterozygous (Gly / Asp). The Gly → Ser mutation was shown to be enough to cause loss of mating-type switching (Ekino *et al.* 1999).
- Position 1214 (TTA → TCA, Leu → Ser); found in #13, #33, #34, #45, #59 and #60, which are all diploids.

The probability of coincidental occurrence of the same four point mutations in our tested strains (out of the detected mutations, in a 1761-bp-long ORF) and in unrelated yeast strains from another continent is $\sim 10^{-7}$. This result calls for further study, though we wonder if the most likely possibility is that the Merced strain was taken from Europe to California by humans, in which case it can represent a sample from a European gene pool with some wide geographic distribution of individual mutations.

Testing upstream and downstream genes to *HO*

In addition to mutations in the *HO* gene, the heterothallism in the isolates might be caused by mutations in the upstream or downstream genes of the mating-type switching pathway. To further investigate these possibilities, we conducted the following experiments. In the first experiment, a wild-type *HO* ORF fused with the *GAL1* promoter was transformed into haploid isolates from the two major clusters: (#9, #10, #35, #36, #39, #57, #59) vs. (#13, #33, #34, #45, #59, #60, #63). The transformants were then grown in medium containing galactose to induce the *HO* expression. All the transformants were able to switch their

mating types after galactose induction, indicating that the downstream genes of the HO pathway were still functional.

The second experiment was designed to test whether the genes that induce *HO* expression were intact. We selected three representative strains (#9, #60 and #63) and mated the haploids with a wild-type homothallic yeast strain M12. The hybrid diploids were then induced to sporulate and 10 tetrads were dissected from each diploid to assay the mating-type switching ability of their progeny. All the spores carrying the wild-type allele of *HO* (detected through *HO* sequencing) were able to switch their mating types. The most likely explanation for this result is that the upstream genes of the HO pathway in the isolates are functional. In this experiment, we also observed that all the spores carrying the *HO* allele of the natural isolates could not switch their mating types. The results of these two experiments suggest that all the *HO* alleles of the natural isolates, including the *HO* allele of isolate #63 that only contains uncharacterized mutations, have lost their function.

Concordance between *HO* and SSR-based phylogenetic structure of the inspected population

In our previous work (Katz Ezov *et al.* 2006), we employed SSR markers to evaluate the genomic biodiversity of the collected natural isolates. Using these previously obtained results, we constructed an SSR-based phylogenetic tree, containing only isolates selected for this study, in order to assess the similarity in variation of the two polymorphic systems, SSR alleles across eight chromosomes and *ho* sequences (see Figs 1 and 2, respectively). SSR loci and *HO*, which is part of the mating system, are both fast evolving components of the genome, and thus, their variation may reflect very recent changes in the population structure. Comparison of the trees indeed demonstrates good correspondence. To score the similarity between SSR and *HO* trees, we needed to have the same set of elements. Thus, averaged data for offspring of strains #1 and #9 were used. Spearman rank correlation of the pairwise distances (distance matrices) for all 20 strains based on SSR variation and *HO* sequence was highly significant ($R = 0.73$, $P < 10^{-6}$).

Discussion

Role of HO endonuclease in heterothallism in the wild

The mutations found in the *HO* sequences of the heterothallic natural *Saccharomyces cerevisiae* isolates from Mount Carmel are strong indicators of full or partial HO loss of function. Indeed, all strains from cluster I shared a nonsense mutation, resulting in an 80 amino acid protein, which is <15% of the total enzyme length. Likewise, strains from subcluster II-b and subcluster IIc all have a missense mutation at nucleotide position 1424 (also found in cluster I), which was previously shown to inactivate the HO endonuclease (Meiron *et al.* 1995). Strains from subcluster II-b have another missense mutation at position 667 also shown to cause heterothallism (Ekino *et al.* 1999). Strain #63, which constitutes cluster II-a, does not share any mutations with the other strains, but has a unique missense mutation at amino acid 70, which is at a highly conserved residue from *S. cerevisiae* to *Saccharomyces castelli* and *Saccharomyces kudriavzevii*. *In vivo* assays that tested the functionality of the mating-type switching pathway upstream and downstream to HO further support the conclusion that loss of function of HO is the most probable explanation for heterothallism in the natural isolates.

Selection of heterothallism in nature and intratetrad mating

Why did our wild isolates lose their ability to switch mating type and thus their ability to undergo mother–daughter mating? The most parsimonious explanation is that the population was founded from a small founder population that was heterothallic (carrying the mutation

at nucleotide 1424), and is sufficiently isolated, so that it has not been invaded by other heterothallic mutations, aside for strain #63. This assumption is supported by the mainly clonal nature of the population (Katz Ezov *et al.* 2006). These strains then accumulated additional mutations that were probably phenotypically silent. In this case, accumulation of other mutations at the already nonfunctional HO was not under selection. Support of this possibility is given by Wik *et al.* (2008) who found that homothallic *Neurospora* shows rapid evolution in *MAT* genes compared to heterothallic *Neurospora*. Other possibilities that require more assumptions are that the environmental conditions in Evolution Canyon on Mount Carmel lead to selection against homothallism (e.g. to increase allelic diversity), or to lack of selection to maintain homothallism (e.g. because viable mating partners are typically available).

The lack of a functional HO endonuclease in the tested natural yeast isolates means that they are able to undergo vegetative growth, amphimixis (mating of unrelated haploids) or automixis (intratetrad mating, i.e. between spores from the same tetrad), but not haplo-selfing (mother–daughter mating that requires mating-type switching). Lack of nutrients and different stress conditions require sporulation for survival, promoting selection for the ability to return to a diploid state.

We speculate that in nature most mating occurs between spores from the same ascus (automixis) for two reasons. First, for a heterozygous diploid cell, such an intratetrad mating event leads to an excess of heterozygote products ($2/3$ compared to $1/2$ proportion expected under panmixia), balancing thereby some of the costs and benefits of sex and meiosis (Zakharov 2005; Knop 2006). As such, intratetrad mating can be seen as an advantageous alternative to haplo-selfing, which allows formation of a diploid while keeping allelic variation (Knop 2006). Second, intratetrad mating was found to be more prevalent than haplo-selfing even in a homothallic yeast cell (Taxis *et al.* 2005). Taxis and colleagues used a homothallic *S. cerevisiae* strain to analyse populations of asci after growth on rich medium for 24 h for the formation of diploid cells. They found that all cells were diploid, and 75–80% of all mating events occurred in the absence of any haploid mitotic division. Hence, mating occurred between spores in the same asci, despite the ability to form a diploid through haplo-selfing. Based on these results, the authors concluded that mating-type switching may not be that common in nature. Moreover, a high frequency of intratetrad mating was recently found in the wild yeast *Saccharomyces paradoxus* as well (Tsai *et al.* 2008), and seems to be the predominant mode of reproduction in the fungus *Microbotryum violaceum* (Giraud *et al.* 2008). These observations support the ongoing hypothesis that intratetrad mating plays a key role in the processes of yeast reproduction in nature, while haplo-selfing might not be as significant as considered earlier.

One other line of support for intratetrad mating lies in the following observation. Kurtzman & Fell (1998) reported that the ascus of *S. cerevisiae* is ‘persisting’ in terms of readiness of releasing the spores (meaning that it is highly resistant to the environment). It is interesting that yeast species that have a preference for a diploid life cycle, like *S. cerevisiae*, were categorized in that study as having persisting asci.

Heterothallism and the population characteristics

In our previous work (Katz Ezov *et al.* 2006), we found that in collected diploid isolates, the majority of chromosomes displayed an excess of homozygotes relative to the Hardy–Weinberg (HW) expectation (an exception was chromosome X with only one marker of seven that showed deviation from HW proportions in the opposite direction). Mitotic recombination between the markers and the centromere could contribute to homozygosity. However, the fact that significant paucity of heterozygotes compared to HW expectation was found for markers located both distant and close to the centromere indicates that mitotic

recombination alone cannot be the only cause, and selfing, e.g. in the form of intratetrad mating, should be a playing factor here.

When comparing the population structure estimated based on SSR markers and the structure based on the *HO* sequence, the clustering shows high similarity. Such concordance between a single locus and 19 SSR markers that are spread over eight different chromosomes supports the hypothesis that clonal reproduction and selfing (in the form of intratetrad mating) predominate in the tested population. Thus, our new results on *ho* heterothallic mutations support the previous evidence on the structure of the inspected natural population (Katz Ezov *et al.* 2006), and favour the hypothesis that clonal mitotic reproduction and intratetrad mating is an important mode of reproduction in this *S. cerevisiae* natural population.

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Fig. 1. Phylogenetic tree of 26 yeast isolates and offspring from 'Evolution Canyon' based on the *HO* locus sequence using Fitch–Margolis algorithm. The numbering of each cluster is given on the right.

Fig. 2. Phylogenetic tree based on SSR analysis of the tested strains. The numbering of each cluster is given respectively to the *HO* sequence based tree.

Table 1Natural *Saccharomyces cerevisiae* isolates and offspring used in the study

Ploidy	Strains
1n	#9-531 [*] , #9-532 [*] , #9-533 [*] , #9-534 [*]
2n	#3, #7, #10, #13, #33, #34, #35, #36, #39, #40, #45, #57, #58, #59, #60, #63, #1-301 [†] , #1-302 [†] , #1-303 [†] , #1-304 [†]
3n	#20, #66

^{*} Progeny of a diploid (#9), a whole tetrad.

[†] Progeny of a tetraploid (#1), a whole tetrad.

Table 2

Summary of mutations found in the *HO* nucleotide sequence in comparison to *HO* and *ho* sequences from GenBank

Strain / position	209	238	279	369	413	417	485	486	492	506	527	565	667	668	789	836	1009	1184	1214	1377	1424	1635	1710	1740	1741	1759
<i>HO</i> GenBank	G	C	C	G	C	T	T	T	T	T	T	A	G	G	A	A	G	A	T	C	A	C	C	C	G	T
<i>ho</i> S288c	G	C	C	G	C	T	T	T	T	T	T	G	A	G	A	A	G	A	C	C	T	C	C	C	G	T
#3	G	C	C	G	C	T	T	T	T	T	T	R	G	R	A	A	R	R	T	C	T	C	C	C	G	T
#7	G	C	C	G	C	T	T	T	T	T	T	G	G	A	A	A	G	G	T	C	T	C	C	C	G	T
#10	G	T	A	A	—	G	T	G	G	C	C	A	G	G	G	A	A	A	T	T	T	T	T	T	G	T
#13	G	C	C	G	C	T	T	T	T	T	T	G	A	G	A	A	G	A	C	C	T	T	T	T	G	T
#20	G	C	C	G	C	T	T	T	T	T	T	G	G	A	A	A	G	G	T	C	T	C	C	C	G	T
#33	G	C	C	G	C	T	T	T	T	T	G	G	A	G	A	A	G	A	C	C	T	T	T	T	G	T
#34	G	C	C	G	C	T	T	T	T	T	T	G	A	G	A	A	G	A	C	C	T	T	T	T	G	T
#35	G	T	A	A	—	T	T	G	T	T	C	A	G	G	G	A	A	A	T	T	T	T	T	T	G	T
#36	G	T	A	A	—	T	T	T	T	T	T	A	G	G	G	A	A	A	T	T	T	T	T	T	G	T
#39	G	T	A	A	—	C	T	T	C	T	T	A	G	G	G	A	A	A	T	T	T	T	T	T	G	T
#40	G	T	A	A	—	T	T	T	T	T	T	A	G	G	G	A	A	A	T	T	T	T	T	T	G	T
#45	G	C	C	G	C	T	T	T	T	T	T	G	A	G	A	A	G	A	C	C	T	T	T	T	G	T
#57	G	T	A	A	—	C	C	C	C	C	C	A	G	G	G	A	A	A	T	T	T	T	T	T	G	T
#58	G	T	A	A	—	T	T	T	T	T	T	A	G	G	G	A	A	A	T	T	T	T	T	T	G	T
#59	G	C	C	G	C	T	T	T	T	T	T	G	A	G	A	A	G	A	C	C	T	T	T	T	G	T
#60	G	C	C	G	C	T	T	T	T	T	T	G	A	G	A	A	G	A	C	C	T	T	T	T	G	T
#63	A	C	C	A	C	T	T	T	T	T	T	A	G	G	G	C	A	A	T	C	A	C	C	C	G	T
#66	G	C	C	G	C	T	T	T	T	T	T	G	G	A	A	A	G	G	T	C	T	C	C	C	K	Y
#1-301	G	C	C	G	C	T	T	T	T	T	T	G	G	A	A	A	G	G	T	C	T	C	C	C	C	T
#1-302	G	C	C	G	C	T	T	T	T	T	T	R	G	R	R	A	R	R	T	C	T	C	C	C	G	T
#1-303	G	C	C	G	C	T	T	T	T	T	T	R	G	R	R	A	R	R	T	C	T	C	C	C	G	T
#1-304	G	C	C	G	C	T	T	T	T	T	T	G	G	A	A	A	G	G	T	C	T	C	C	C	G	T
#9-531	G	T	A	A	—	T	T	T	T	T	T	A	G	G	G	A	A	A	T	T	T	T	T	T	G	T
#9-532	G	T	A	A	—	T	T	T	T	T	T	A	G	G	G	A	A	A	T	T	T	T	T	T	G	T
#9-533	G	T	A	A	—	T	T	T	T	T	T	A	G	G	G	A	A	A	T	T	T	T	T	T	G	T
#9-534	G	T	A	A	—	T	T	T	T	T	T	A	G	G	G	A	A	A	T	T	T	T	T	T	G	T

Table 3

Summary of mutations found in the *HO* amino acid sequence in comparison to *HO* and *ho* sequences from Gen-Bank

Strain / position	70	80	138	189	223	337	395	405	475	580	587
<i>HO</i> GenBank	R	Q	T	T	G	V	Y	L	H	A	*
<i>ho</i> S288c	R	Q	T	T	S	V	Y	S	L	A	*
#3	R	Q	T	S	G/D	V/M	Y/C	L	L	A	*
#7	R	Q	T	T	D	V	C	L	L	A	*
#10	R	*	K	T	G	M	Y	L	L	A	*
#13	R	Q	T	T	S	V	Y	S	L	A	*
#20	R	Q	T	T	D	V	C	L	L	A	*
#33	R	Q	T	T	S	V	Y	S	L	A	*
#34	R	Q	T	T	S	V	Y	S	L	A	*
#35	R	*	K	T	G	M	Y	L	L	A	*
#36	R	*	K	T	G	M	Y	L	L	A	*
#39	R	*	K	T	G	M	Y	L	L	A	*
#40	R	*	K	T	G	M	Y	L	L	A	*
#45	R	Q	T	T	S	V	Y	S	L	A	*
#57	R	*	K	T	G	M	Y	L	L	A	*
#58	R	*	K	T	G	M	Y	L	L	A	*
#59	R	Q	T	T	S	V	Y	S	L	A	*
#60	R	Q	T	T	S	V	Y	S	L	A	*
#63	H	Q	T	T	G	M	Y	L	H	A	*
#66	R	Q	T	T	D	V	C	L	L	A/S	*/Q
#1-301	R	Q	T	T	D	V	C	L	L	A	*
#1-302	R	Q	T	T	G/D	V/M	Y/C	L	L	A	*
#1-303	R	Q	T	T	G/D	V/M	Y/C	L	L	A	*
#1-304	R	Q	T	T	D	V	C	L	L	A	*
#9-531	R	*	K	T	G	M	Y	L	L	A	*
#9-532	R	*	K	T	G	M	Y	L	L	A	*
#9-533	R	*	K	T	G	M	Y	L	L	A	*
#9-534	R	*	K	T	G	M	Y	L	L	A	*

* Stop codon.

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