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Molecular-Genetic Biodiversity in a Natural Population of the Yeast *Saccharomyces cerevisiae* From "Evolution Canyon": Microsatellite Polymorphism, Ploidy and Controversial Sexual Status

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ABSTRACT

The yeast *S. cerevisiae* is a central model organism in eukaryotic cell studies and a major component in many food and biotechnological industrial processes. However, the wide knowledge regarding genetics and molecular biology of *S. cerevisiae* is based on an extremely narrow range of strains. Studies of natural populations of *S. cerevisiae*, not associated with human activities or industrial fermentation environments, are very few. We isolated a panel of *S. cerevisiae* strains from a natural microsite, "Evolution Canyon" at Mount Carmel, Israel, and studied their genomic biodiversity. Analysis of 19 microsatellite loci revealed high allelic diversity and variation in ploidy level across the panel, from diploids to tetraploids, confirmed by flow cytometry. No significant differences were found in the level of microsatellite variation between strains derived from the major localities or microniches, whereas strains of different ploidy showed low similarity in allele content. Maximum genetic diversity was observed among diploids and minimum among triploids. Phylogenetic analysis revealed clonal, rather than sexual, structure of the triploid and tetraploid subpopulations. Viability tests in tetrad analysis also suggest that clonal reproduction may predominate in the polyploid subpopulations.

THE budding yeast *Saccharomyces cerevisiae* is one of the central model organisms of eukaryotic cell studies (Dickinson 2000). The wide knowledge about the genetic and molecular biology of the yeast *S. cerevisiae* has accumulated on an extremely narrow range of genotypes selected due to their specific technological features or suitability to laboratory conditions and, hence, hardly representing the species (Liu *et al.* 1996; Mortimer 2000).

The yeast *S. cerevisiae* is also a central component of many important industrial processes, including baking, brewing, distilling, and wine making. Once again, in this domain, most studies have included selected, commercially available yeast strains removed from the natural adaptation and evolution processes. Relatively limited genetic work has been done on commercial baking, wine, and brewing strains. In the last decade, some studies dealt with isolates from nature, wineries, and grapes (Sangorrin *et al.* 2001; van der Aa *et al.* 2001; Fay and Benavides 2005), as well as from contaminants of different lager breweries (van der Aa and Jespersen 1998; Jespersen *et al.* 2000). In fact, cells of *S. cerevisiae* are rarely isolated from natural grape surfaces except damaged grapes (Vaughan-Martini and Martini 1995; Martini *et al.* 1996; Mortimer and Polsinelli 1999), suggesting that insects such as bees or Drosophila are vectors for spreading of this microorganism (Stevic 1962; Snowdon and Cliver 1996; Mortimer and Polsinelli 1999).

Mortimer (2000) reported that attempts to find *S. cerevisiae* in regions remote from human activities have been unsuccessful. A model proposed by Naumov (1996) states that *S. cerevisiae* strains in European soil originate from human activity, *i.e.*, are found only in association with human civilization, especially in winery environments. On the other hand, *S. cerevisiae* was also found in exudates from North American oaks (Naumov *et al.* 1998). The foregoing evidence calls for extended population-genetic (Zeyl 2000) and molecular-genetic studies of yeast in nature (Liti and Louis 2005).

The yeast *S. cerevisiae* can exist in a vegetative mode in haploid, diploid, and higher ploidy states. Haploid cells of *S. cerevisiae* exhibit one of two phenotypes: mating types **a** or α. Correspondingly, diploid and polyploid cells can exhibit one of three mating phenotypes: **a**, α, or **a**/α. When cells of opposite mating types meet they participate in a mating process that results in cell and nuclear fusion to create an **a**/α-zygote. **a**/α-cells can either reproduce by mitosis or undergo meiosis and sporulation, with a further possibility of **a** × α gamete fusion.

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Mating type of haploid cells can be stable (heterothallic yeast) or unstable (homothallic yeast). In heterothallic yeasts, haploid culture derived from a single ascospore remains haploid until brought into contact with another haploid culture of the opposite mating type. In contrast, in homothallic culture, derived from a single ascospore, there is an alternation of haploid and diploid generations due to mating-type interconversion followed by mating (for review see Cox 1995). These aspects can be considered as regulation of the mode of reproduction in yeast, from full amphymixis (sex) to automixis. Zeyl and Bell (1997) show that sex increases mean fitness in an environment to which the populations were well adapted, but not in an environment to which new adaptation occurred, supporting the hypothesis that the advantage of sexuality lay in the removal of deleterious mutations.

Zeyl *et al.* (2003) tested rates of adaptation using asexual diploid and haploid yeast populations. They showed that diploidy may slow down adaptation, but by large populations only. In mutagenesis experiments (Mable and Otto 2001) haploids displayed a more pronounced decrease in apparent growth rate than diploids. Tetraploids did not show increased benefits of masking deleterious mutations compared with diploids. However, all treated tetraploid strains decreased in ploidy level whereas some of the treated haploid lines increased in ploidy level. These findings shed a new light on ploidy level as an adaptive evolutionary trait.

A panel of *S. cerevisiae* strains, among 25 different yeast species, belonging to 14 genera (Nagornaya *et al.* 2003), was isolated from a well-studied natural microsite called "Evolution Canyon," which is located in Lower Nahal Oren in Mount Carmel National Park, Haifa, Israel (32° 24′ N, 34° 58′ E) (Nevo 1995, 1997, 2001). The opposing slopes of this canyon, the "African" south-facing slope (SFS) and the "European" north-facing slope (NFS), are separated by only 100 m at the bottom and 400 m at the top. Despite sharing the same geology and macroclimate, the two slopes differ sharply in microclimatic conditions, because of the higher (up to 200–800%) solar radiation on the SFS as compared with the NFS (Pavlicek *et al.* 2003), causing also substantial interslope biotic contrasts. The SFS is warmer, drier, microclimatically more variable, and less predictable than the NFS. Parallel genotypic and phenotypic diversity patterns across phylogeny of bacteria, fungi, plants, and animals on the opposing slopes of Evolution Canyon suggest that natural selection plays a major role in biodiversity evolution (Nevo 1995; Andreyuk *et al.* 1999; Kalendar *et al.* 2000).

Higher genetic polymorphisms on the SFS are naturally selected as an adaptive strategy to cope with its higher ecological heterogeneity and stress (Lamb *et al.* 1998; Krugman *et al.* 2001; Nevo 2001; Saleem *et al.* 2001; Satish *et al.* 2001). Indeed, genetic diversity was higher on the more heterogeneous and stressful SFS in 11 of 14 model organisms tested at this site, including wild barley, fruit flies, beetles, and cyanobacteria (Gutterman and Nevo 1994; Rankevich *et al.* 1996; Lamb *et al.* 1998; Andreyuk *et al.* 1999; Krugman *et al.* 2001; Michalak *et al.* 2001; Nevo 2001; Saleem *et al.* 2001; Satish *et al.* 2001). This could highlight the importance of ecological stress in evolution and its remarkable effect on the genetic system (Grishkan *et al.* 2003). For instance, strong interslope differentiation for a complex of adaptive traits of Drosophila was found, including physiological and behavioral traits as well as different rates of mutation and recombination (Nevo *et al.* 1998; Korol *et al.* 2000; Iliadi *et al.* 2001; Michalak *et al.* 2001; Lupu *et al.* 2004; Singh *et al.* 2005; Zamorzaeva *et al.* 2005; Rashkovetsky *et al.* 2006). This remarkable differentiation has evolved in spite of the small interslope distance. Thus, the obtained evidence leads to a conclusion that strong microclimatic natural selection can override migration and random drift and generate slope-specific multitrait adaptive gene complexes that contribute to fitness at a microsite.

Here we use microsatellite loci to study *S. cerevisiae* biodiversity in isolates from Evolution Canyon. Microsatellites, or simple sequence repeats (SSRs), represent a class of DNA sequences consisting of tandemly organized, reiterated motifs. SSRs are abundant across eukaryotic genomes including yeasts that show high levels of polymorphism in motif copy number. SSR diversity may result from both external (ecological) and internal (genetic) effects (Li *et al.* 2002, 2004). In addition to their use as DNA markers, microsatellites have been described as molecular switches controlling gene expression in microbial and eukaryotic species (Trifonov 1989; Kashi *et al.* 1997; King *et al.* 1997; van Belkum *et al.* 1998; Li *et al.* 2004; Trifonov 2004). Functional significance of at least some SSRs has been demonstrated in critical experiments in many biological phenomena (reviews: Kashi *et al.* 1997; Kashi and Soller 1999; Li *et al.* 2002, 2004). SSRs might be a major source of genetic diversity and evolutionary adaptation to environmental stress. Indeed, evidence for microclimatic selection acting on SSR loci has been already reported (Li *et al.* 2000).

In this article we present results demonstrating high SSR polymorphism, ploidy variation, and clonal rather than sexual population structure, in the natural population of *S. cerevisiae* in Evolution Canyon.

MATERIALS AND METHODS

Strains: Sixty-eight *S. cerevisiae* yeast strains were isolated from Evolution Canyon at seven collection sites, at three altitudes on each slope (lower, middle, and upper) and at valley bottom (VB) (see supplemental figure at http://www.genetics.org/supplemental/) as described in Nagornaya *et al.* (2003). In short, soil and plant samples were collected in sunny and shady places (including adjacent sites), hereafter sunny, shady, and leaf, respectively. Isolation of *S. cerevisiae* was

TABLE 1

List of SSR loci selected for the analysis of genomic diversity within *S. cerevisiae* isolates from Evolution Canyon

Name of SSR locus	Chromosome no.	Coordinates (bp)	Core motif	No. of alleles found
ChII-ATP I	II	370450	$(AT)_{13}$	6
ChIV-1038.6	IV	1038641	$(TCA)_{10}$	3
ChVI-FAB1	VI	186212	$(ATA)_{19}$	6
ChIX-105.7	IX	105769	$(TAA)_{19}$	7
ChX-188.7	X	188731	$(TCT)_{6}$	4
ChX-403.7	X	403763	$(CAG)_{8}$	4
ChX-423.8	X	423865	$(AT)_{12}$	5
ChX-469.8	X	469823	$(TAA)_{16}$	6
ChX-518.90	X	518948	$(CA)_{20}$	9
ChX-GRR	X	593880	$(TTG)_{10}$	3
ChX-639.6	X	639684	$(AC)_{19}$	6
ChXI-126.1	XI	126106	$(GAA)_{10}$	9
ChXI-184.5	XI	184504	$(TAA)_{8}$	3
ChXI-576.1	XI	576129	$(TCG)_{13}$	7
ChXII-511.5	XII	511525	$(CAG)_{10}$	5
ChXII-823.4	XII	823442	$(GA)_{32}$	6
ChXIII-CMP II	XIII	159854	$(AT)_{12}$	5
ChXIII-ORF4	XIII	209872	$(TGA)_{11}$	4
ChXIII-388.6	XIII	388685	$(AT)_{14}$	4

The number of alleles found in SSR loci analyzed in the panel is shown. The name of the locus, its chromosomal location, the SSR motif, and the number of repeats according to the published genome are given.

conducted by dilution technique and the enrichment method (Beech and Davenport 1971). For identification, morphological, physiological, and biochemical criteria for Saccharomyces were employed (Kurtzman and Fell 1998). For reference, laboratory strains were used: Y422 (Sherman *et al.* 1993), Y102 and Y103 (*MAT***a** and *MAT*α testers, respectively) (all kindly provided by Y. Kassir), and S288C (Mortimer and Johnston 1986) (kindly provided by R. K. Mortimer).

Tetrad dissection: A micromanipulator equipped with a glass needle was used to isolate spores from asci (kindly made available by D. Kornitzer). The ascus walls were removed using 0.25 mg/ml Zymolase T100 (Sigma, St. Louis) in 1 M sorbitol.

Media: Yeast cells were usually grown on YPD complete medium (prepared as in Sherman 1991) at 30°. Sporulation was induced on sporulation medium (SPO) (described in Kassir and Simchen 1991). Prototrophy was tested on synthetic dextrose minimal medium (Kaiser *et al.* 1994).

Mating-type tests: Mating-type tests were performed as described in Sherman (1991). The tested strain was mixed, in parallel with the *MAT***a** and the *MAT*α tester strains on a YPD plate and incubated at 30° overnight. These cultures were then replica plated on a SPO plate, along with the original strain. Sporulation was tested under a microscope after 24 and 48 hr of incubation at room temperature.

DNA extraction: DNA was extracted by the yeast DNA miniprep method described in Kaiser *et al.* (1994).

Sequence polymorphism: *Microsatellite analysis:* Nineteen SSR (or microsatellite) loci were used to characterize the panel. The published genome of *S. cerevisiae* (http://www.yeastgenome.org/) was scanned by a computer program developed in our laboratory (ftp://ftp.technion.ac.il/pub/supported/biotech/) to detect SSR loci. Unique PCR primers were designed to amplify selected SSR loci according to the published genome of *S. cerevisiae*. In addition, some published microsatellite markers were used as well (Field and Wills 1998). The employed SSRs included di- and trinucleotide repeats dispersed over nine chromosomes (Table 1).

Size of PCR products was determined using an automated sequencer, ALFexpress (Pharmacia, Uppsala, Sweden). Sequencing gel-running conditions were: 1800 V, 50 mA, and 50 W at 50° for 5 hr. Product sizes were calculated by the ALFwin fragment analysis software using an ALF size marker (50–500 bp) as an external marker. For validation of SSR marker analysis, direct sequencing was conducted for some of the amplified fragments.

Taxonomic analysis: To validate the allocation of the natural isolates to *S. cerevisiae*, sequence analyses of two loci were performed in a part of our isolates (see Kurtzman and Robnett 2003):

1. ITS1-5.8S-ITS2 rDNA: primers (for amplification and sequencing) were TCCTCCGCTTATTGATAT(f) and GGAAGTAAAAGTCGTAACAAGG(r). The annealing temperature for PCR was 50°.
2. Translation EF-1 αA gene: primers (for amplification and sequencing) were TTCTTCGACTATGCTGGAGG(f), and TAAGGTTACCAAGGCTGCTC(r). The annealing temperature for PCR was 58°.

All sequences were aligned with reference sequences of all Saccharomyces *sensu stricto* species using ClustalW. GenBank accession numbers for the reference sequences are AY046146–AY046152 and AY130303–AY130313 for the ITS1-5.8S-ITS2 locus and AF402004–AF402009, AF402010–AF402016, AY130808, and AY130810–AY130813 for the translation EF-1 αA locus. Phylogenetic analysis was conducted for the ITS1-5.8S-ITS2 locus using the Fitch–Margoliash algorithm (Fitch and Margoliash 1967) in the PHYLIP software package. The minimum evolution distance matrix method was used to obtain the best tree (not shown).

Mating-type and HO analysis: Mating type was tested by PCR as described in Bradbury *et al.* (2006) with minor changes: each PCR reaction included only two primers, flanking either the **a**- or the α-mating type. Amplification products were loaded on a 1.5% agarose gel to examine which primer set produced amplification. The HO gene encodes an endonuclease

responsible for initiating mating-type switching, a gene-conversion process where *MAT***a** cells change to *MAT*α cells or vice versa through the generation of a double-strand DNA break (Russell *et al.* 1986). This locus was amplified using the primers TTGAGAAAGGCTGAAGTTGG(f) and TGTTGAAG CATGATGAAGCG(r). The annealing temperature for PCR was 56°. Primers for sequencing were: ACACTCTGGTCCTT TAAC, GACATTGGACTTTTCTTCC, CTGGCTCTTTTGTTG TAC, and TCACCTTCAAAAGCTCTG, along with the forward PCR primer. The resulting sequences were aligned with the wild-type sequence, GenBank accession no. M14678.

Assessment of ploidy level: The results of SSR analysis indicated that the collected strains varied in ploidy level, from haploid up to tetraploid. Independent scoring of the ploidy level was conducted using flow cytometry analysis (FACS). Protocol was adopted for this scoring from Foiani *et al.* (1994), using a Becton–Dickinson FACScan analyzer. In particular, yeast cultures were grown to logarithmic phase, fixated in ethanol, treated by pepsin and ribonuclease A, and stained with propidium iodide. Each tested strain was scored a few times, in each experiment with two replicates. Each experiment included at least two reference strains, haploid and diploid. The ploidy level was scored on the basis of the fluorescence intensity compared to the haploid and diploid reference stains.

Statistical analysis: Cluster analysis and phylogenetic reconstructions based on SSR polymorphism were made using the Fitch–Margoliash algorithm (Fitch and Margoliash 1967). Bootstrap consensus trees were constructed using the PHYLIP software package. The minimum evolution distance matrix method was used to obtain the best tree. We used normalized Euclidian distance $d(i, j) = \sqrt{\sum_l \left(d_l(i, j)/\max_{p,q} d_l(p, q)\right)^2}$ between strains, where d_l is the contribution of the lth locus. Distance (d_l) between two tetraploid strains was calculated in the coordinate system of *ordered allele sizes*: $d_l(i, j) = \sqrt{\sum_{a=1}^{4} \left(\mathrm{Len}(i_{(a)}^{(l)}) - \mathrm{Len}(j_{(a)}^{(l)})\right)^2}$, where $\mathrm{Len}(p_{(a)}^{(l)})$ is the length of the ath allele at locus l of strain p. Our way of genetic distance calculation takes into account the size of alleles, but is different from the standard one (reviewed in Takezaki and Nei 1996). In contrast to standard models, we suppose that population is asexual and mutations are rare and cause only small changes in allele length. Actually, we consider mutation process like a four-dimensional Brownian motion in the space of allele lengths transforming one tetraploid genotype to another. According to our definition, genetic distance between two tetraploid genotypes is equal to the length of the shortest (and most probable) mutation way. This value is proportional to mean time required to reach the observed level of genetic difference between the compared genotypes (Feller 1957).

Distance between a tetraploid strain and a strain with another ploidy level was calculated by "redefining" the non-tetra-strain as a virtual "tetraploid." This was done (separately for each locus) using the following rules, which obey the *triangle inequality* for the defined distance measure: haploids, tetraploids were constructed by taking four times the alleles of the haploid at every locus; diploids, tetraploids were constructed by taking two times the alleles of the diploid at every locus; triploids, tetraploids were constructed using the three alleles of the triploid and an addition of a fourth allele, with a length equal to that of the second-in-size allele of the triploid. The proposed distance definition has close physical sense in the case of strains with the same ploidy level. Also, it obeys the triangle inequality. By creating a virtual tetraploid from the triploid via a duplicating median allele, we get a genotype that can be produced from this triploid without additional mutations (mutations are considered as small and rare).

In the case of disconcordance between the ploidy and the number of observed alleles maximum-likelihood estimations were used. A χ^2-test was employed to assess the significance of population differentiation into ecological groups (*e.g.*, between SFS and NFS). Phylogenetic trees were drawn using the program TreeView (Page 1996). Bootstrap support for phylogenetic trees was determined from 1000 replications.

Expected homozygosity h_e was calculated as the sum of squares of allele frequencies. Allele diversity H_e and allele combination diversity D were calculated as $1 - \sum p_i^2$, where p_i is the frequency of allele i or frequency of genotype i, correspondingly (Weir 1990). This characteristic of allele diversity (and allele-combination diversity) is close to zero in the case when almost all alleles (or allele combinations) are the same at the considered locus across strains and is close to 1 when all of them are different. The value $H_e = 1 - h_e$ can characterize the mean expected heterozygote proportion in the *diploid case under panmixia*.

Segregation analysis: To test whether segregations of SSR loci in considered tetraploid yeasts are random we compared observed results of segregations with expected ones. Denote by $k = k(l)$ the number of different possible sets of four diploid genotypes at locus l that can be obtained in segregation of the tetraploid strain. If $k = 1$ (for example, for tetraploid genotype AAAa) then locus l is not informative for segregation analysis. If $k > 1$ then denote by $p_1, \ldots, p_k$ the probabilities to obtain a corresponding set of diploid genotypes. Denote by $m = m(l)$ the number of different possible sets of diploid genotypes at locus l, which can be obtained in segregation of a tetraploid strain when probably not all of four diploid genotypes are observed. Denote by p_{ij} ($i = 1, \ldots, k$; $j = 1, \ldots, m$) the probability to obtain a set of genotypes j in the case of segregation producing a set of four diploid genotypes i ($\sum_j p_{ij} = 1$).

If segregations are random and all diploid products have the same probability to be observed, then values p_i and p_{ij} ($i = 1, \ldots, k$; $j = 1, \ldots, m$) can be calculated using combinatorial formulas. Let N_j ($j = 1, \ldots, m$) be the number of segregation cases where a set of diploid genotypes j was observed ($N = \sum_j N_j$ is the total number of successful segregations). The number of cases where segregation produces a set of four diploid products i can be estimated by $N_i = \sum_{j=1}^{m} N_j (p_i p_{ij} / \sum p_q p_{qj})$ ($i = 1, \ldots, k$). If segregation is random, then we expect that the value $\chi^2_{k-1} = \sum_{i=1}^{k} ((N_i - N p_i)^2 / N p_i)$ will have standard χ^2-distribution with d.f. $= k - 1$.

RESULTS

All 68 isolates were found to be prototrophic and sporulated on SPO media. After tetrad dissection of representative strains the spores either were successfully mated with haploid testers *MAT***a** or *MAT*α or underwent additional sporulation that showed the polyploid nature of the parental strain. A very low sporulation rate was obtained in the second sporulation test (see below).

Microsatellite variation: Nineteen primer pairs designed for SSR analysis on the basis of the *S. cerevisiae* genome sequence proved efficient in amplifying fragments in the expected length range (see Table 2). To validate the relevance of these PCR products, parts of the amplimeres were sequenced. The resulting sequences were in agreement with the published genome and the predicted number of repeats from sizing analysis (data not shown). The scored microsatellite loci showed high polymorphism of allele size, with 2–9 alleles per locus and an average of 5.1 alleles per locus (Table 1).

TABLE 2

PCR primers for amplification of the selected SSR loci

Locus	Forward	Reverse	Temperature
ChII-ATP I	tgccatccgtgtacgctagg	gcgaacagagccgtttaccg	57°
Ch IV-1038.6	aattgctgtcattggatctat	attattcctacgtatgaagtg	52°
ChVI-FAB1	ctacaattccaaaggtccttcgc	cgtgccattgtcgtttgaggg	53°
ChIX-105.7	gcacttgctgaacataagc	aggtagtttaggaagtgaggc	51°
ChX-188.7	cagaggaggaccaccagtttg	aaaggaaccacagcagcagg	53°
ChX-403.7	cacaaataggttagagacacag	ctaaatcgtcctcccattg	55°
ChX-423.8	gctggctctatatctcctctcg	actgtgtggcgggtaatgc	55°
ChX-469.8	caatgctaaaggacaccaag	ggcgaagagaagaagcatctg	55°
ChX-518.90	cgccgatattagacgtgtg	gggctttcactccactttac	53°
ChX-GRR	cgttgcatccctaacctcactt	gctgcacccacctgatatacatcc	53°
ChX-639.6	gtagcataacagcagcgtag	cttcaaactcagtagtcgtcc	53°
ChXI-126.1	tgaatctggcgacgatag	acttttggccaatttctcaagat	55°
ChXI-184.5	aagcgtcctaacatactatccacc	atttcaattggctatatatcctta	52°
ChXI-576.1	agatacagaagataagaacgaaaa	ttattgatgcttatctattatacc	55°
ChXII-511.5	cttaaacaacagctcccaaa	atgaatcagcgcatcagaaat	53°
ChXII-823.4	ctggaatgaaattaaacaaaagc	tcttccttttctactatcttctc	51°
ChXIII-CMP II	cggactctcgtcctactattg	ggggacaatgttggcgctag	60°
ChXIII-ORF4	Gctcgcagggagaaatctgcttcc	cttcatcggtatccgttccactagg	53°
ChXIII-388.6	Atgcactcaaacagtcgatcctt	cctatccatcgcttatagaacaa	57°

No significant differences were found in the level of SSR variation in any locus between the three major localities (NFS, SFS, and VB): the maximum of $\chi^2_{\text{d.f.}=2}$ among the loci in the likelihood test for microsite heterogeneity was 7.3 (found for locus X-188.70), $P = 0.05$. Likewise, between the three microniches (habitats) tested (sunny, shady, and leaf), the maximum of $\chi^2_{\text{d.f.}=2}$ among the loci in the likelihood test was 8.9 (found for locus XIII-388.60), $P = 0.03$. Keeping in mind the multiple-comparison nature of our analysis, these values cannot be considered as significant because the probability to reach a minimal *P*-value = 0.03 in 19 tests when H_0 (no real difference between the locations) is true is ~0.6 (Benjamini and Hochberg 1995).

Ploidy variation: High heterozygosity at the scored SSR loci indicated a variation in ploidy level, with one to four alleles per locus per strain. Flow cytometry analysis (FACS) was conducted for a sample of strains, representing the four putative levels of ploidy revealed from SSR analysis, and for a part of their progeny obtained by tetrad dissection. Our diploid isolates showed the same level of fluorescence per cell as the laboratory diploid control strain, whereas presumably tetraploid strains (according to SSR analysis) showed a doubled level of fluorescence per cell. High consistence was found between these sources of ploidy assessment (see Figure 1).

Consequently, the numbers of di-, tri-, and tetraploid strains in our panel were found to be 21, 7, and 40 (31, 10, and 59%), correspondingly. No significant differences were found between the three microsite subpopulations (NFS, SFS, and VB) or the three microniches (sunny, shady, and leaf) with respect to variation in ploidy level ($\chi^2_4 = 5.4$ and $\chi^2_4 = 1.5$, correspondingly, $P > 0.1$).

At all three ploidy levels (di-, tri-, and tetraploid) the sporulation test was positive for all strains. As expected, our trials for tetrad analysis of triploid strains gave an extremely low level of viable spores. An interesting result was obtained by tetrad analysis of tetraploid strains: they showed high sporulation level and high viability of spores. Unlike the tetraploids themselves, a part of the offspring derived from the tetraploids displayed low

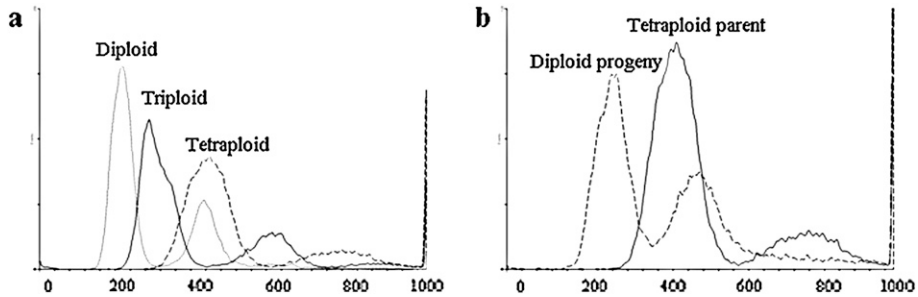

Figure 1.—Analysis of DNA content by flow cytometry. Yeast cells were stained with propidium iodide for cell cycle analysis. Cell counts and relative DNA content are shown. (a) Diploid, triploid, and tetraploid natural isolates. (b) A tetraploid natural isolate and one of its diploid offspring.

TABLE 3

Variation in allele diversity across ploidy levels and in entire population of *S. cerevisiae* isolates from Evolution Canyon

	Allele diversity (H_e)				*P*-value (for test of allele diversity equivalence)		
Locus	Diploids	Triploids	Tetraploids	Entire population	Di-triploids	Di-tetraploids	Tri-tetraploids
ChII-ATP I	0.62	0.00	0.38	0.44	<0.001	<0.001	<0.001
Ch IV-1038.6	0.61	0.09	0.37	0.57	<0.001	<0.001	<0.001
ChVI-FAB1	0.67	0.67	0.63	0.76	NS	0.01	NS
ChIX-105.7	0.73	0.67	0.70	0.75	0.01	0.02	NS
ChX-188.7	0.49	0.09	0.38	0.55	<0.001	<0.001	<0.001
ChX-403.7	0.39	0.44	0.38	0.47	0.02	NS	0.02
ChX-423.8	0.69	0.44	0.63	0.67	<0.001	0.002	<0.001
ChX-469.8	0.69	0.67	0.63	0.69	NS	0.002	NS
ChX-518.90	0.80	0.67	0.75	0.82	<0.001	0.005	0.005
ChX-GRR	0.41	0.44	0.50	0.50	NS	<0.001	0.01
ChX-639.6	0.74	0.44	0.50	0.60	<0.001	<0.001	0.01
ChXI-126.1	0.75	0.44	0.65	0.75	<0.001	<0.001	<0.001
ChXI-184.5	0.56	0.00	0.38	0.46	<0.001	<0.001	<0.001
ChXI-576.1	0.83	0.00	0.38	0.52	<0.001	<0.001	<0.001
ChXII-511.5	0.52	0.00	0.38	0.47	<0.001	<0.001	<0.001
ChXII-823.4	0.77	0.44	0.39	0.57	<0.001	<0.001	0.02
ChXIII-CMP II	0.70	0.00	0.50	0.61	<0.001	<0.001	<0.001
ChXIII-ORF4	0.60	0.44	0.62	0.67	<0.001	NS	<0.001
ChXIII-388.6	0.35	0.00	0.02	0.09	<0.001	<0.001	NS

NS, not significant. Allele diversity H_e was calculated as $1 - \sum p_i^2$, where p_i is the frequency of allele i (as described in *Statistical analysis* in MATERIALS AND METHODS). Zero allele diversity means monomorphism at the locus. Results on significance of differences in allele diversity between different ploidy levels are presented in the right side. *P*-values are calculated using a maximum-likelihood test (see text).

sporulation level (<1%), with no viable spores, and therefore were considered **a**/α-diploid. Other diploid offspring, derived from meiosis of tetraploid strains, did not sporulate at all. These strains were successfully mated with an α-tester strain and the resulting triploids were able to undergo sporulation. The mating type deduced from these tests was confirmed by PCR. Almost all haploids derived from diploids as well as diploids derived from tetraploids displayed stability of mating type (being therefore heterothallic). It is noteworthy that homothallism is believed to be characteristic of most yeast in nature (*e.g.*, MORTIMER 2000). In light of the foregoing results obtained in our mating experiments, it was desirable to check the sequence of the *HO* locus that controls mating-type switch (RUSSELL *et al.* 1986). Such tests were conducted on a few haploids derived from diploids. Sequence analysis followed by alignment to the standard *HO* sequence revealed several point mutations, including deletions and SNPs. The first substitution, causing a nonsense mutation, appeared at position 235 (after the ATG codon). This mutation causes the formation of a short mutant polypeptide of only 79 amino acids, while the wild-type protein is 586 amino acids long (RUSSELL *et al.* 1986).

SSR allele diversity varied across ploidy levels (Table 3). Two tests shown below were conducted to estimate the significance of this variation.

Likelihood test for equivalence of allele diversity for different ploidy levels: This test employs maximum-likelihood estimation of allele frequencies, under two conditions for allele diversity equivalence. We compared likelihood function for observed data under hypotheses H_1 and H_0: "allele distribution was different (H_1) *vs.* the same (H_0) for different ploidy levels." Supposing multinomial distribution of the allele numbers in the sampled strains, we calculated log-likelihood as

$$\ln L(n_1^{\mathrm{I}}, \ldots, n_{k_{\mathrm{I}}}^{\mathrm{I}}; n_1^{\mathrm{II}}, \ldots, n_{k_{\mathrm{II}}}^{\mathrm{II}} \mid p_1^{\mathrm{I}}, \ldots, p_{k_{\mathrm{I}}}^{\mathrm{I}}; p_1^{\mathrm{II}}, \ldots, p_{k_{\mathrm{II}}}^{\mathrm{II}}) \propto \sum n_i^{\mathrm{I}} \ln p_i^{\mathrm{I}} + \sum n_j^{\mathrm{II}} \ln p_j^{\mathrm{II}}.$$

Here $n_1^{\mathrm{I}}, \ldots, n_{k_{\mathrm{I}}}^{\mathrm{I}}$ and $n_1^{\mathrm{II}}, \ldots, n_{k_{\mathrm{II}}}^{\mathrm{II}}$ are the observed numbers of alleles for two ploidy levels I and II; $p_1^{\mathrm{I}}, \ldots, p_{k_{\mathrm{I}}}^{\mathrm{I}}$ and $p_1^{\mathrm{II}}, \ldots, p_{k_{\mathrm{II}}}^{\mathrm{II}}$ are expected allele frequencies (they are positive and fit the trivial condition $\sum p_i^{\mathrm{I}} = \sum p_j^{\mathrm{II}} = 1$). The symbol "$\propto$" denotes that, in the equality, the term independent of $p_1^{\mathrm{I}}, \ldots, p_{k_{\mathrm{I}}}^{\mathrm{I}}$ and $p_1^{\mathrm{II}}, \ldots, p_{k_{\mathrm{II}}}^{\mathrm{II}}$ is omitted. For the compared hypotheses H_1 and H_0,

$$L_1 = \max_{p_1^{\mathrm{I}}, \ldots, p_{k_{\mathrm{I}}}^{\mathrm{I}}; p_1^{\mathrm{II}}, \ldots, p_{k_{\mathrm{II}}}^{\mathrm{II}}} L(n_1^{\mathrm{I}}, \ldots, n_{k_{\mathrm{I}}}^{\mathrm{I}}; n_1^{\mathrm{II}}, \ldots, n_{k_{\mathrm{II}}}^{\mathrm{II}} \mid p_1^{\mathrm{I}}, \ldots, p_{k_{\mathrm{I}}}^{\mathrm{I}}; p_1^{\mathrm{II}}, \ldots, p_{k_{\mathrm{II}}}^{\mathrm{II}})$$

and

$$L_0 = \max_{\substack{p_1^{\mathrm{I}}, \ldots, p_{k_{\mathrm{I}}}^{\mathrm{I}}; p_1^{\mathrm{II}}, \ldots, p_{k_{\mathrm{II}}}^{\mathrm{II}} \\ 1 - \sum (p_i^{\mathrm{I}})^2 = 1 - \sum (p_j^{\mathrm{II}})^2}} L(n_1^{\mathrm{I}}, \ldots, n_{k_{\mathrm{I}}}^{\mathrm{I}}; n_1^{\mathrm{II}}, \ldots, n_{k_{\mathrm{II}}}^{\mathrm{II}} \mid p_1^{\mathrm{I}}, \ldots, p_{k_{\mathrm{I}}}^{\mathrm{I}}; p_1^{\mathrm{II}}, \ldots, p_{k_{\mathrm{II}}}^{\mathrm{II}}).$$

TABLE 4

Allele combination diversity for the three ploidy levels of *S. cerevisiae* isolates from Evolution Canyon

	Allele combination diversity (*D*)					
	Observed			Expected[a]		
Locus	Diploids	Triploids	Tetraploids	Diploids	Triploids	Tetraploids
ChII-ATP I	0.66	0.00	0.05	0.78	0.00	0.68
Ch IV-1038.6	0.74	0.24	0.05	0.80	0.24	0.67
ChVI-FAB1	0.72	0.00	0.14	0.85	0.87	0.89
ChIX-105.7	0.63	0.00	0.42	0.89	0.87	0.95
ChX-188.7	0.65	0.24	0.00	0.64	0.24	0.68
ChX-403.7	0.54	0.00	0.00	0.55	0.66	0.68
ChX-423.8	0.73	0.00	0.00	0.85	0.66	0.89
ChX-469.8	0.69	0.00	0.00	0.84	0.87	0.89
ChX-518.90	0.83	0.00	0.00	0.93	0.87	0.96
ChX-GRR	0.49	0.00	0.00	0.57	0.66	0.73
ChX-639.6	0.72	0.00	0.00	0.89	0.66	0.73
ChXI-126.1	0.76	0.00	0.42	0.90	0.66	0.90
ChXI-184.5	0.74	0.00	0.05	0.74	0.00	0.68
ChXI-576.1	0.77	0.00	0.00	0.95	0.00	0.68
ChXII-511.5	0.65	0.00	0.05	0.72	0.00	0.68
ChXII-823.4	0.74	0.00	0.05	0.91	0.66	0.68
ChXIII-CMP II	0.70	0.00	0.05	0.85	0.00	0.73
ChXIII-ORF4	0.65	0.00	0.05	0.76	0.66	0.89
ChXIII-388.6	0.53	0.00	0.05	0.53	0.00	0.09

Allele-combination diversity D was calculated for each ploidy level as $1 - \sum p_i^2$, where p_i is the frequency of genotype i (as described in *Statistical analysis* in MATERIALS AND METHODS). Zero allele-combination diversity means that all strains of the ploidy level have the same genotype.

[a]Expected allele combination diversity was calculated on the basis of observed allele frequencies for the ploidy level. The difference between observed and expected allele-combination diversity was not significant for diploids, but it was highly significant ($P < 0.0001$) for tetraploids for almost all loci, except XIII-388.60.

If H_0 is true then we expect that the statistic $\chi^2 = -2\ln(L_0/L_1)$ is asymptotically distributed as χ^2 with d.f. = 1.

Rank test of diversity values comparison: For diversity scores at ploidy levels I and II, the expected number of signs that "level I is greater than level II" in 19 pairs (corresponding to 19 marker loci) of diversity comparison is 9.5. In the case of diversity equivalence, the value $\chi^2 = (\text{no. ``}>\text{''} - 9.5)^2/9.5 + (\text{no. ``}\leq\text{''} - 9.5)^2/9.5$ has χ^2-distribution with d.f. = 1. Allele diversity was significantly higher in diploids than in tetraploids (*P*-value 0.005) and in tetraploids than in triploids (*P*-value 0.015). Allele-combination diversity significantly differed from the expected values in tetraploids (Table 4).

Sexual *vs.* clonal reproduction: An exceptional feature of the inspected population is the seemingly clonal structure of its higher ploidy parts. Namely, at both tri- and tetraploid levels, we observed a very limited number of different multilocus genotypes (clones), representing the majority of strains (Figure 2, Table 4).

We think that the tight clustering shown in Figure 2 reflects clonality. Even with recent origin, one would expect that just one to two cycles of sexual reproduction of tetraploids would generate various segregants per each heterozygous locus (see also Table 4) and recombinant genotypes, which we do see in diploids, but not in tri- and tetraploids.

Low similarity in allele content was found between strains of different ploidy levels, except diploid strain nos. 03, 05, 07, and 23 that share their alleles with the cluster of tetraploids and might be either offspring or parental strains of the tetraploid strains. As indicated above, an interesting effect was found in tetrad analysis: all tested tetraploid strains produced diploid progeny, but all efforts to obtain haploids from this progeny failed. By contrast, haploid progeny was easily obtained from diploid strains.

In light of the foregoing findings, it is interesting to test whether the proportion of heterozygotes for SSR markers will show any pattern compatible with the assumed tendency of clonality. A simple test for deviation from Hardy–Weinberg (HW) proportions was, therefore conducted on diploids, the least clonal part of the population (Table 5). The observed number of homozygotes was found to exceed significantly the HW expectations, albeit the discrepancy varied among chromosomes and even within chromosomes. The majority of chromosomes displayed an excess of homozygotes despite the high polymorphism of the diploid part of the population. An exception was chromosome X with

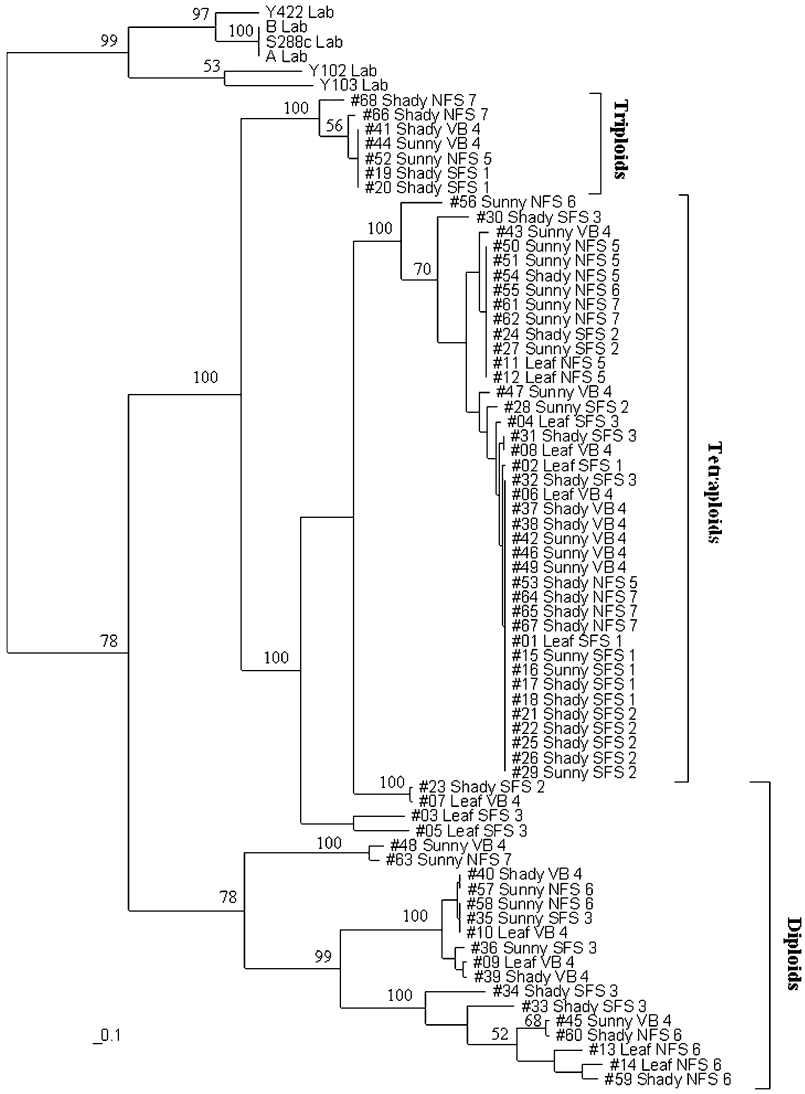

FIGURE 2.—Bootstrap consensus phylogenetic tree, demonstrating the relationship between *S. cerevisiae* isolates from Evolution Canyon and their relationship with laboratory strains. The tree was constructed on the basis of 19 SSR loci with distance matrices calculated as described in *Statistical analysis* in MATERIALS AND METHODS. The numbers above the branches are the percentages of bootstrap trees containing each bipartition (only values >50% are given).

only one marker of seven that showed deviation from HW proportions. Presumably, this may reflect selection against heterozygotes for loci of this chromosome or complex genetic architecture of modifiers of mating preferences (hence proportions of homozygotes). The fact that significant paucity of heterozygotes compared to HW expectation is found for loci distantly located from the centromere and near-centromeric loci indicates that mitotic recombination alone cannot be the only cause, and selfing should be a playing factor here. The results of the *HO* sequence test show that at least some of the strains are strictly heterothallic, whereas the HW test indicates that at least part should be or should have been homothallic.

Segregation analysis: We considered 27 segregation cases to test whether segregations of the tetraploid strain are random. Analysis was made only for 9 loci from the 19, because the others could produce only a $k = 1$ possible set of four diploid genotypes. It was found that only two loci CMP2 (XIII-159.9) and ORF4 (XIII-209.8) demonstrate significant deviation from random segregation (*P*-values 0.07 and 0.005 correspondingly, see Table 6). It can be noted that both of them are situated on chromosome XIII and the locus that demonstrated high significance is closer to the centromere.

Taxonomic consideration: Very high amplification efficiency of primer pairs designed for SSR loci on the basis of the published genome sequence of *S. cerevisiae* was achieved, while for *S. paradoxus*, a close relative of *S. cerevisiae*, it was reported recently that only 3 of 20 primer pairs, designed on the basis of *S. cerevisiae* genome sequence, resulted in amplimeres (JOHNSON *et al.* 2004). This is a strong indication that the collected isolates are indeed *S. cerevisiae*. However, no simple explanation seems to fit the observed complicated pattern of ploidy variation, SSR distribution among and within the stains, and the outcomes of tetrad analysis of diploid isolates compared to the diploid progeny of tetraploid strains. This motivated us to conduct in-depth specific taxonomic tests on the basis of sequence comparison at

TABLE 5

Discrepancy between the observed and expected (assuming panmixia) proportions of homozygotes among the diploid *S. cerevisiae* isolates from Evolution Canyon

Locus	*h* observed	*h* expected	*P*-value
ChII-ATP I	0.90	0.38	<0.001
Ch IV-1038.6	0.67	0.38	0.03
ChVI-FAB1	0.48	0.33	NS
ChIX-105.7	0.48	0.28	NS
ChX-188.7	0.57	0.51	NS
ChX-403.7	0.62	0.64	NS
ChX-423.8	0.19	0.30	NS
ChX-469.8	0.29	0.30	NS
ChX-518.90	0.43	0.20	0.02
ChX-GRR	0.43	0.59	NS
ChX-639.6	0.33	0.25	NS
ChXI-126.1	0.71	0.25	<0.001
ChXI-184.5	0.62	0.42	NS
ChXI-576.1	0.48	0.17	<0.001
ChXII-511.5	0.57	0.48	NS
ChXII-823.4	0.62	0.23	<0.001
ChXIII-CMP II	0.81	0.30	<0.001
ChXIII-ORF4	0.81	0.40	0.003
ChXIII-388.6	0.57	0.65	NS

NS, not significant. Expected homozygosity was estimated as the sum of squares of allele frequencies. The *P*-value was calculated using a χ^2-test with d.f. = 1.

two selected loci: ITS1-5.8S-ITS2 rDNA and translation EF-1 αA. Multiple sequence alignment for the representative isolates from Evolution Canyon with all available Saccharomyces *sensu stricto* sequences from GeneBank (http://www.ncbi.nlm.nih.gov) were conducted. For the translation EF-1 αA locus, the tested isolates showed high sequence similarity to *S. cerevisiae*. In fact, we found only one SNP out of 1127 bp.

For the ITS1-5.8S-ITS2 locus, sequencing of tetraploids, diploids, and their offspring yielded overlap of different sequences that could not be obviously merged to a single sequence. Only triploid strains tested formed a single sequence that could be analyzed. Most of the variation found between these triploid isolates and the members of the Saccharomyces *sensu stricto* group was due to mononucleotide repeats (MNRs). Our natural isolates tend to show a larger number of repeats compared to those in published sequences of three MNR tracts [all poly(T/A)] in the locus. Still, these results indicate that these isolates have closest homology to *S. cerevisiae*.

Microscale (microclimatic) differentiation: The differences in number of alleles, allele diversity, and allele-combination diversity were tested for association with microclimatic contrasts: (a) major localities SFS, NFS, and VB and (b) variation among niches/habitats *sun vs. shade vs. leaf surface*. Only tetraploid strains displayed significant interslope differentiation in their allele combinations that involved two loci, IX-107.70 and XI-126.10 ($\chi^2 = 11.1$, $P < 0.001$). No intraslope (*i.e.*, interstation) variation was detected. A slight tendency toward between-niche variation was found for diploids (for shade-derived strains *vs.* remainder strains $\chi^2 = 2.7$, $P < 0.1$; and for leaf *vs.* remainder $\chi^2 = 3.9$, $P < 0.05$). Additional tests, especially using candidate stress-related genes (for tolerance to high temperature, desiccation, UV, etc.), are needed to check whether the foregoing diversity reflects ecological adaptation to microclimatic variation.

TABLE 6

Results of test on random segregation

Locus	χ^2	d.f.	*P*-value
IX-105.7	1.79	2	NS
X-423.8	3.37	2	NS
X-469.8	0.17	2	NS
X-518.9	1.25	4	NS
GRR (X-593.9)	2.89	2	NS
X-639.6	0.82	2	NS
XI-126.1	2.8	2	NS
CMP2 (XIII-159.9)	5.37	2	0.07
ORF4 (XIII-209.8)	10.67	2	0.005

The *P*-value was calculated using a χ^2-test (see *Segregation analysis* in MATERIALS AND METHODS). Only markers from chromosome XIII [both CMP2 (XIII-159.9) and ORF4 (XIII-209.8)] demonstrate significant deviation from random segregation. The locus that demonstrated higher significance is closer to the centromere (with coordinate 268.1 kb in the physical map).

DISCUSSION

The yeast *S. cerevisiae* is one of the most important organisms for biotechnology and the food industry. It is also the most studied eukaryote and an important model for cell biology. Thus, despite the huge amount of genetic and molecular studies on *S. cerevisiae*, only a narrow range of strains, which hardly represent the species, were characterized. Consequently, there is no understanding of the adaptive structure of yeast natural populations and evolutionary strategies (LITI and LOUIS 2005).

We assessed the genetic diversity in a natural population of *S. cerevisiae* from a well-studied natural model, called Evolution Canyon at Mount Carmel, where dozens of other species are analyzed at the phenotypic, genetic, and molecular-genetic levels. The collected *S. cerevisiae* strains were characterized for 19 SSR markers. Several findings are described in this article, including high SSR polymorphism, ploidy variation from diploid up to tetraploid, and a tendency toward a clonal rather than a sexual population structure. Our results seem to differ markedly from those reported in other studies of wild yeasts. In a population study of the yeast *S. paradoxus*, a close relative of *S. cerevisiae*, strains were

isolated from the bark of oak trees in southern England (Johnson *et al.* 2004). The isolation procedure in that study allowed getting *S. cerevisiae* yeast, but no strain of this species was found among the isolates. All *S. paradoxus* strains were found to be homothallic diploid, with a very low level of heterozygosity. These differences between the two studies can be explained by the environmental characteristics of extremely sharp spatial microclimatic contrasts at Evolution Canyon and relatively mild variation in Silwood Park and Windsor Great Park. In the studies of natural isolates of *S. cerevisiae* derived from noninoculated wine fermentations, most isolates were found to be homothallic diploids. The isolates displayed tremendous diversity, and 35% of isolates were completely homozygous to the assessed systems (Mortimer 2000).

Proving the taxonomic affiliation: The employed criteria for yeast isolation and characterization of the isolates were suitable for *S. cerevisiae* (Beech and Davenport 1971; Barnett *et al.* 2000). All SSRs were successfully amplified using specific primers designed on the basis of the published genome of *S. cerevisiae*, resulting in fragments of the expected size range for *S. cerevisiae*. For further validation, samples of the amplified products were sequenced and proved to coincide very well with the published sequence of the *S. cerevisiae* genome. One of the most popular genetic tools for differentiation among yeast species is sequence variation at the ribosomal DNA ITS1-5.8S-ITS2 locus (Kurtzman and Robnett 2003). Sequences of the ITS1-5.8S-ITS2 locus were successfully obtained only from triploid isolates. All other tested strains (tetraploids, diploids, and offspring of both) showed multiple sequences at this locus, presumably due to the large number of rDNA repeats in the yeast genome (100–200 repeats) (Johnston *et al.* 1997). Sequence variation in these ribosomal DNA regions arising from array duplication events is a known phenomenon (see detailed review by Alvarez and Wendel 2003). We hypothesize that triploids reproduce only clonally, and therefore all copies of the rDNA could have undergone a complete homogenization process (Gangloff *et al.* 1996). Multiple alignment of this sequence was conducted and a phylogenetic tree was generated for a triploid isolate and all Saccharomyces *sensu stricto* sequences from GenBank (not shown). The tested isolate showed the closest homology to *S. cerevisiae*. In addition, multiple alignment of the translation EF-1 αA locus was conducted, and all tested isolates showed good sequence similarity to *S. cerevisiae*. All these results give us high confidence that the studied material belongs to the *S. cerevisiae* species.

The ploidy challenge: The yeast *S. cerevisiae* can exist in haploid, diploid, or polyploid states. There are variations both within and between laboratory, commercial, and clinical strains and natural isolates (*e.g.*, our results). In recent decades, evolution of ploidy level became an important subject within the problems of genome molecular evolution and sex evolution. In particular, yeast may be a very relevant model organism for such studies (*e.g.*, Wolfe and Shields 1997; Mable and Otto 1998; Korona 1999; Mable and Otto 2001; Wolfe 2001; Zeyl 2004). It is noteworthy that the recent renaissance of the polyploidy studies is, to a large extent, due to the interest in the (presumably existing) mechanisms of (very) fast evolution of polyploid genomes toward diploidization and novel tools allowing us to address this "old" problem (Belyayev *et al.* 2000; Ozkan *et al.* 2001; Feldman and Levy 2005). The relevance of this problem to our project is caused both by the paleopolyploid nature of the yeast genome (Wong *et al.* 2002) and by the results presented here.

Our SSR analysis and subsequent test of the tetrads show that the isolated strains differ in ploidy level. Flow cytometry analysis confirmed the assessed ploidy levels based on the SSR analysis (diploids, triploids, and tetraploids) (see Figure 1). This result presumably reflects the transitory nature of the haploid stage in yeast in nature. We assume that these results indicate higher adaptive potential of heterozygous polyploids combined with higher flexibility of a population harboring genotypes with different ploidy levels. Indeed, it is known that a certain proportion of genes may be either repressed or induced in response to increased ploidy even if most genes will not be affected by variation in ploidy (Galitski *et al.* 1999).

An interesting pattern was revealed in our tetrad analysis. We managed to obtain diploid **a**/α progeny from tetraploid strains, but had no success in obtaining viable progeny of these diploids, despite the fact that a similar test on natural diploids did give viable haploid progeny. Possible explanations for this result can be the accumulation of a variety of epistatic relations between alleles in tetraploids strains or aneuploid "diploid" offspring that segregated to unbalanced haploids. Another possibility is an assumption of some hybridization event(s) that could have occurred in the past between cells of *S. cerevisiae* and a sibling yeast species, resulting in a fertile allotetraploid. Hybridizations between different species of Saccharomyces are known to occur and are a subject to a number of studies (*e.g.*, Naumov *et al.* 2000; de Barros *et al.* 2002; Greig *et al.* 2002a,b). Such an event may promote a fast intragenome evolution toward further perfection of diploid-like behavior (Shaked *et al.* 2001). Offspring of the evolved amphidiploids would not be able to undergo one more cycle of meiosis giving haploid products (Loidl 1995). Another consequence of such a hypothetical process would be a tendency for directed segregation.

To test the last hypothesis we conducted an analysis of the segregations of tetraploids to diploids. Only 9 of 19 SSR loci, located in chromosomes IX, X, XI, and XIII, could be tested. Our results (see Table 6) demonstrate that the segregation of chromosome XIII was nonrandom. For locus OFR4, which is close to the

centromere, significance was higher than for locus CMP2, situated more distantly from the centromere, fitting the expectation that direction of segregation is determined by the centromere and correlation with the centromere decreases by recombination toward the telomeres. It is possible that different chromosomes behave unlike each other due to a different level of sequence similarity. The nonsignificant results of other chromosomes can be also partially explained by different power of analysis, depending on tetraploid genotype and haplotypes. A larger sample size and more suitable genomic composition of targeted tetraploids could clarify whether or not segregations in other chromosomes are random.

Microsatellite polymorphism: Nineteen SSR loci were amplified using specific primer pairs, to give amplimeres in the expected length range. The scored microsatellite loci showed two to nine alleles per locus (Table 1). However, no significant differences were found in the level of SSR variation in any ecological niche/habitat. Allele diversity varied with ploidy level (see Table 3). In particular, maximum diversity was observed among diploids and minimum among triploids. We found no correlation between the chromosomal position and the inter- and intrastrain diversity of the scored SSR, as one would expect for a sexual population with a recombination gradient within the chromosome caused by a centromeric effect on recombination (Lambie and Roeder 1988). This expectation derives from the mechanisms of selective sweep (caused by spreading of new favorable mutations) and background selection (which considers the consequences of elimination of deleterious mutations) (Maynard Smith and Haigh 1974; Charlesworth *et al.* 1993). Under these models, sequence polymorphism at the assayed loci is interpreted mainly as a result of their linkage to other negatively or positively selected loci; *i.e.*, the assayed sequences themselves are considered as neutral ones. Recombination reduces the dependence of the majority of neutral sequence variation on the minority of sporadically appearing new variants at the selected loci. Hence, lower diversity is expected in genomic regions with a low recombination rate. However, multilocus selection can cause opposite, either positive or negative, effects of recombination on sequence polymorphism (Kirzhner *et al.* 2003). Therefore, the absence of any positional (relative to centromere) pattern seemingly corroborates the hypothesis about a tendency toward asexual reproduction of yeast in Evolution Canyon.

Another interesting possibility of employing "chromosomal gradients" is to look for similar patterns in the excess of homozygosity (h) observed for some loci in diploid strains (see Table 5). Namely, it would be instructive to learn whether h increases with deviation from the centromere. Such a tendency would indicate simultaneously the shaping role of mitotic recombination and asexual reproduction. To address this question, we calculated rank correlation between homozygosity and the chromosomal position of the markers relative to the centromere. Because of the difference in allele diversity at different loci, we normalized observed homozygosity as $h_{\text{norm}} = (h - h_e)\sqrt{n/h_e(1 - h_e)}$, where $n = 21$ is the number of diploid strains. Rank of marker chromosomal position was calculated according to the percentage of physical distance to the centromere from the length of the corresponding chromosomal arm. Rank order correlation was $+0.42$ ($P = 0.07$). A similar trend was obtained for nonnormalized tests (not shown). This result indicates that the observed excess in homozygosity might be a consequence of mitotic recombination upon asexual reproduction. Indeed, even with a high rate of mitotic recombination and a corresponding increase of homozygosity (compared to Hardy–Weinberg expectations) with distance from the centromere, just one cycle of outbreeding would be sufficient to recover the expected proportions.

Population structure: The inspected population shows a clonal structure of its higher ploidy part. Both the tri- and the tetraploid subgroups are composed of a very limited number of different multilocus genotypes (clones), representing the majority of strains. In other words, the polyploid part of the population can be characterized as a set of a few clusters with high proximity of strains within clusters and relatively high genetic distance between the clusters (see Figures 2 and 3). No correlation was found between polymorphism level and chromosomal position of loci relative to the centromere. This may also point to predominance of asexual reproduction in the studied population. An additional argument for this assumption may be very low allele-combination diversity (relative to the expected level) displayed in triploids and tetraploids (Table 4). Analysis of SSR loci across the genome showed that even for the diploid part of the population, a significant excess of homozygotes is characteristic despite a high level of polymorphism. For asexual reproduction, there is no expectation of changing homozygote:heterozygote ratios. In other words, the homozygosity test (deviations from HW) asks actually how frequent the meiosis events are followed by selfing *vs.* outcrossing. The fact that a significant paucity of heterozygotes compared to the HW expectation is found for loci located both distantly from the centromere and near the centromere indicates that mitotic recombination alone cannot be the only explanation and selfing should be a playing factor here. Seemingly, this fact may reflect the homothallic nature of the collected natural strains, but this explanation does not fit our tests that showed high mating-type stability of the derived **a**- and α-strains in the lab and our *HO* sequence results. Apparently, these results indicate that at least some of the strains are strictly heterothallic, whereas at least part should be or should have been homothallic.

Low similarity in allele content was found between strains of different ploidy levels, except diploid strain

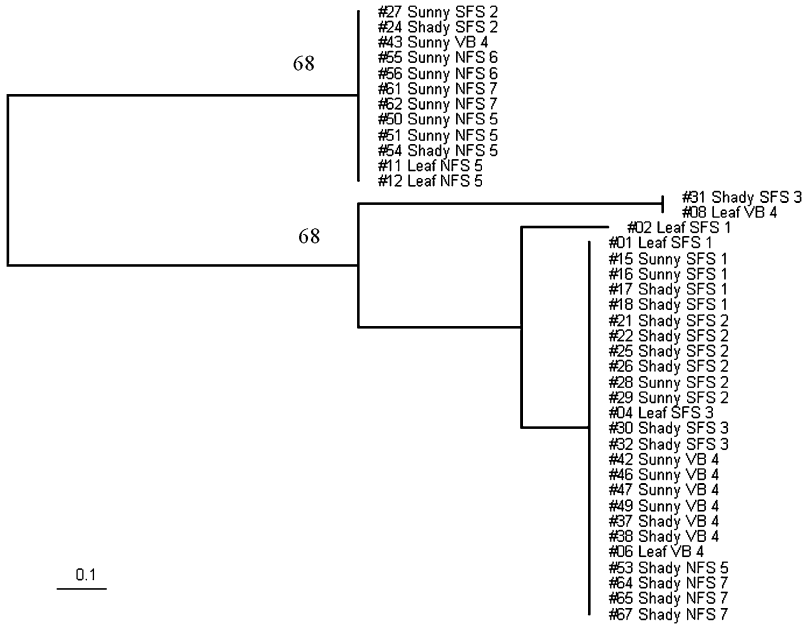

FIGURE 3.—Phylogenetic analysis of tetraploid *S. cerevisiae* isolates from Evolution Canyon. The tree is based on three SSR loci differentiating between tetraploids [FAB1 (VI-186.2), IX-105.70, and XI-126.10]. The numbers above the branches are the percentages of bootstrap trees containing each bipartition (only values >50% are given). Excluding locus FAB1 from the analysis leads to the increasing clade credibility of main bipartition up to 100%.

nos. 03, 05, 07, and 23 that share their alleles with the tetraploid cluster. In tetrad analysis all tested tetraploid strains produced diploid progeny, but all efforts to obtain haploids from this progeny failed. By contrast, haploid progeny was easily obtained from diploid strains. These results allow us to speculate that clonal reproduction tends to predominate in the polyploid part of the tested population of *S. cerevisiae*.

Unlike the results obtained on other species studied in Evolution Canyon (KRUGMAN *et al.* 2001; MICHALAK *et al.* 2001; SINGH *et al.* 2005; ZAMORZAEVA *et al.* 2005; RASHKOVETSKY *et al.* 2006), no interslope divergence was found in this study, except in two loci in tetraploids. It is possible that analysis of adaptive traits may be a much better source of evidence for interslope differential selection than genetic distances estimated using molecular markers. Differentiation for adaptively valuable gene complexes can better withstand destruction by migration and recombination due to the protective effect of differential selection. However, such adaptive differentiation would not necessarily be sufficient to preserve allelic combinations of selectively neutral markers, unless the latter are in linkage disequilibrium with selected loci. This last condition can also persist despite migration, but only under very tight linkage and strong selection. On the other hand, it is possible that from the yeast's perspective our classification of the different habitats by location and niches was too broad.

Conclusions: *S. cerevisiae* yeasts were isolated from soil and leaf samples in Evolution Canyon at Mount Carmel, Israel. Analysis of 19 SSR loci showed one to four alleles per strain, indicating different ploidy levels, diploids, triploids, and tetraploids, confirmed by flow cytometry tests. Maximum genetic diversity was observed among diploids and minimum among triploids. Clonal structure of triploid and tetraploid subpopulations was revealed using SSR data. In tetrad analysis, all tested tetraploids gave viable diploid progeny. No viable haploids were obtained from this progeny in a second round of tetrad dissection, while natural diploids gave high viability of haploid offspring. These results suggest that clonal reproduction tends to predominate in the polyploid subpopulation of the studied natural population of *S. cerevisiae*.

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