Differentiation of European and Far East Asian Populations of *Saccharomyces paradoxus* by Allozyme Analysis

G. I. NAUMOV,¹ E. S. NAUMOVA,¹ AND P. D. SNIEGOWSKI^{2*}

*State Institute for Genetics and Selection of Industrial Microorganisms, 113545 Moscow, Russia,¹ and
Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824-1325²*

Allozyme electrophoresis was used to characterize 39 isolates belonging to the wild yeast species *Saccharomyces paradoxus* for variation at nine enzyme loci. The data revealed significant genetic differentiation between isolates from two geographically distinct regions, one including continental Europe and the other including the Russian Far East and Japan. The results are consistent with previous observations indicating that there is partial reproductive isolation between isolates collected from these regions, and they suggest the possibility that these two populations represent an early stage in speciation.

The evolutionary genetics of yeasts has received little attention compared with the classical and molecular genetics of these organisms, especially as developed in *Saccharomyces cerevisiae*. Indeed, *S. cerevisiae* is widely regarded as a domesticated organism and is seldom collected away from sources related to human activity, so that studies of wild *S. cerevisiae* populations are problematical. However, at least six sibling species have been identified in the *Saccharomyces sensu stricto* complex (*S. cerevisiae*, *Saccharomyces paradoxus*, *Saccharomyces bayanus* Hansen, one Brazilian *Saccharomyces* sp., and two Japanese *Saccharomyces* spp.) by genetic analysis (15, 18) and DNA-DNA reassociation studies (7, 33–35, 38), and some of these species appear to exist only in the wild state; that is, they are not found in any human baking or fermenting applications. Significantly, classification in the *Saccharomyces sensu stricto* complex is in accord with the biological species concept (12); interspecific matings yield viable hybrids, but these hybrids produce nonviable ascospores and are consequently sterile. In contrast, the products of intraspecific matings produce highly viable ascospores (13–15, 21, 23). The existence of interfertile populations of wild *Saccharomyces* yeasts opens the way to studies of the evolutionary systematics and population genetics of yeasts similar to studies conducted with higher eukaryotes and various other fungi (27, 28, 36, 37). In this paper, we describe a study of genetic variation in wild populations of *S. paradoxus*.

S. paradoxus Batchinskaya (synonyms, *S. cerevisiae* var. *tetrasporus* (Beijerinck ex Dekker) Phaff et al., *S. cerevisiae* var. *terrestris* Jensen, and *Saccharomyces douglasii* nom. nud. [14, 19]) is the closest relative of *S. cerevisiae* Hansen. The level of total DNA homology (as determined by reassociation studies) between *S. cerevisiae* and *S. paradoxus* is approximately 50%. (Some recent studies found considerably higher levels of homology between DNA sequences from individual genetic loci [1, 11]). The levels of total DNA homology between *S. cerevisiae* and the other sibling species are lower (7, 33–35, 38). While performing genetic identification studies of *S. paradoxus* isolates collected in various regions of the world, we noticed that isolates collected in Europe formed highly fertile hybrids with a reference tester strain of *S. paradoxus* from Denmark, whereas isolates collected from other regions, particularly from the Russian Far East and Japan (referred to as Far East

Asia below), showed substantially reduced ascospore viability when they were crossed with the same tester strain (16, 20, 23). Here we report the results of a biochemical characterization study of 39 *S. paradoxus* isolates from Europe and Far East Asia in which multilocus enzyme electrophoresis was used. Enzyme electrophoresis has been widely used in population genetic studies of bacteria and higher eucaryotes and in some fungi (8, 36, 37, 40, 41), but the method has not yet been used to study natural yeast populations to any appreciable degree. Limited studies of enzyme polymorphism in the *Saccharomyces sensu stricto* complex have been performed, for the most part, with the cultivated yeast *S. cerevisiae* (10, 32, 39).

MATERIALS AND METHODS

Yeast strains. The isolates used and their origins are listed in Table 1. A total of 28 isolates from the European continent (referred to as the EU isolates below) and 11 isolates from Far East Asia (referred to as the FE isolates below) were examined for protein electrophoretic variation. These isolates had been previously assigned to the biological species *S. paradoxus* on the basis of genetic hybridization analysis data. All of the isolates had been maintained as homozygotic homothallic cultures prior to this study.

Preparation of cell lysates for electrophoresis. Overnight cultures grown in 20 ml of YPD medium at 28°C were harvested by centrifugation at 5,000 × g for 10 min, washed once with 1 ml of Tris-glycine buffer (30 g of Trizma Base [Sigma] per liter, 144 g of glycine per liter; pH 8.0), resuspended in 0.5 ml of the same buffer, and frozen at –80°C. Frozen cell suspensions were thawed and immediately lysed with a sonicator (Heat Systems model W385; Ultrasonics, Inc., Farmingdale, N.Y.) at the maximum output for 20 min with continuous cooling at 2°C. After centrifugation at 4°C to remove debris, cell lysates were distributed into chilled Eppendorf tubes in 100-μl aliquots and were stored at –80°C until they were used for electrophoresis.

Electrophoresis and gel staining. Enzymes were separated by using the Zip Zone cellulose acetate system (Helena Laboratories, Beaumont, Tex.). For most enzymes, electrophoresis was carried out at room temperature at 185 V for 35 min. For arginine kinase (ARK), glucose-6-phosphate dehydrogenase (G6PDH), and malate dehydrogenase (MDH), electrophoresis was carried out at 100 V for 40 min. Gel staining was performed as described by Hebert and Beaton (5). Only enzymes that produced consistently scoreable, sharp bands were used in the analysis. All enzyme extracts were electrophoretically examined three times and produced consistent zymograms. The following nine enzymes were studied: alcohol dehydrogenase (ADH) (EC 1.1.1.1), ARK (EC 2.7.3.3), G6PDH (EC 1.1.1.49), glucose-6-phosphate isomerase (GPI) (EC 5.3.1.9), isocitrate dehydrogenase (IDH) (EC 1.1.1.42), lactate dehydrogenase (LDH) (EC 1.1.1.27), MDH (EC 1.1.1.37), mannose-6-phosphate isomerase (MPI) (EC 5.3.1.8), and phosphoglucomutase (PGM) (EC 5.4.2.2). We used side-by-side electrophoretic comparisons to confirm equivalence in enzyme mobilities between isolates. For each enzyme, distinct electromorphs were numbered in order of increasing anodal mobility. The electromorph with the greatest anodal mobility was designated electromorph 1, and the progressively more slowly migrating variants were assigned increasing numbers. Each isolate was characterized by its combination of electromorphs for the nine enzymes.

Data analysis. Population differentiation at individual loci was analyzed by the Fisher exact test. When the data could be represented by a 2-by-2 table of alleles within populations, the program FISH6 (3) was used to obtain a probability value for the table. When more than two alleles were observed at a locus, the program

* Corresponding author. Present address: Department of Biology, Leidy Laboratories, University of Pennsylvania, Philadelphia, PA 19104-6018.

TABLE 1. Wild isolates of *S. paradoxus* used in this study

Strain designation ^a		Source	Geographical origin	Reference	ET
Original	ATCC				
EU isolates					
CBS 406	ATCC 96988	Exudate of <i>Quercus</i> sp.	The Netherlands	14	1
CBS 432	ATCC 96965	Unknown	Unknown	14	1
CBS 5829	ATCC 96966	Mor soil	Denmark	14	2
INMIV 11/21	ATCC 96976	Poplar tree	Kiev, Ukraine	14	3
INMIV 544	ATCC 96979	Aspen leaves	Kiev, Ukraine	14	4
CECT 10176	ATCC 96989	<i>Noctua pronubula</i> (adult)	Spain	25	2
CECT 10178	ATCC 96972	<i>Noctua pronubula</i> (adult)	Spain	25	1
CECT 10308	ATCC 96967	Frass in <i>Quercus lusitanus</i>	Spain	25	1
CECT 10329	ATCC 96969	Frass in <i>Quercus ilex</i>	Spain	25	5
VKM Y-2472	ATCC 96975	Peat	Moscow, Russia	15	1
N7	ATCC 96885	Exudate of <i>Quercus robur</i>	St. Petersburg, Russia	15	1
N8	ATCC 96987	Exudate of <i>Quercus robur</i>	Moscow, Russia	15	1
N9	ATCC 96886	Exudate of <i>Quercus</i> sp.	Tashkent, Uzbekistan	15	6
N11	ATCC 96985	Exudate of <i>Quercus robur</i>	Novgorod, Russia	15	5
N12	ATCC 96978	Exudate of <i>Quercus</i> sp.	Lenkoran, Azerbaijan	15	1
N13	ATCC 96986	Exudate of <i>Quercus robur</i>	Moscow, Russia	15	1
N15	ATCC 96982	Exudate of <i>Quercus robur</i>	Moscow, Russia	15	1
N16	ATCC 96983	Exudate of <i>Quercus robur</i>	Moscow, Russia	15	1
N17	ATCC 96981	Exudate of <i>Quercus robur</i>	Tartastan, Russia	15	1
N18	ATCC 96984	Exudate of <i>Quercus robur</i>	Efremov, Russia	15	1
N25	ATCC 96973	Exudate of <i>Quercus robur</i>	Estonia	17	1
N34	ATCC 96980	Exudate of <i>Quercus robur</i>	Voronezh, Russia	15	1
N36	ATCC 96990	Exudate of <i>Quercus</i> sp.	Lithuania	24	1
N40	ATCC 96974	Exudate of <i>Quercus</i> sp.	Yalta, Ukraine	This study	1
N41	ATCC 96977	Exudate of <i>Quercus</i> sp.	Yalta, Ukraine	This study	1
ALKO 2638	ATCC 96968	Exudate of <i>Quercus</i> sp.	Finland	22	1
ALKO 2639	ATCC 96971	Exudate of <i>Quercus</i> sp.	Finland	22	1
DBVPG 1373	ATCC 96970	Soil	The Netherlands	22	1
FE isolates					
IFO 1804		Bark	Japan	23	9
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N42		Exudate of <i>Quercus mongolica</i>	Vladivostok, Russia	20	7
N43		Exudate of <i>Quercus mongolica</i>	Cape Peschanyi, Vladivostok, Russia	20	8
N44		Exudate of <i>Quercus mongolica</i>	Ternei, Russia	20	7
N45		Exudate of <i>Quercus mongolica</i>	Ternei, Russia	20	9
N46		Exudate of <i>Quercus mongolica</i>	Sikhote-Alinsky Reserve, Russia	20	7
N47		Exudate of <i>Quercus mongolica</i>	Alinsky Reserve, Russia	20	9
N48		Exudate of <i>Quercus mongolica</i>	v. Rjazanovka, Khazansky District, Russia	20	9
N49		Exudate of <i>Quercus dentata</i>	v. Rjazanovka, Khazansky District, Russia	20	9
N50		Exudate of <i>Quercus mongolica</i>	Chajka, Vladivostok, Russia	20	7

^a ATCC, American Type Culture Collection, Rockville, Md.; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; INMIV, Institute for Microbiology and Virology of the Ukraine Academy of Sciences, Kiev, Ukraine; CECT, Colección Española de Cultivos Tipo, Valencia, Spain; VKM, All-Russian Collection of Microorganisms, Moscow, Russia; ALKO, Research Laboratories of the Finnish State Alcohol Company, Helsinki, Finland; DBVPG, Industrial Yeasts Collection of the Department of Plant Biology, University of Perugia, Perugia, Italy; IFO, Institute for Fermentation, Osaka, Japan. Strains whose designations begin with N were obtained from the collection of G. I. Naumov. Additional FE isolates have been deposited in the American Type Culture Collection.

STRUC (30) was used to estimate the probability of the table. Pairwise genetic similarities among multilocus electrophoretic types (ETs) were estimated by calculating simple matching coefficients (31); genetic distances were calculated by subtracting these coefficients from 1. The program MEGA (9) was used to produce an unweighted pair group with mathematical average (UPGMA) dendrogram from the matrix of pairwise genetic distances.

RESULTS

Genetic variability among all of the isolates. Nine different multilocus ETs were observed among the 39 *S. paradoxus* isolates examined. Table 1 shows the ET to which each isolate belonged. No ETs were shared by the EU isolates and the FE isolates. Six ETs (designated ETs 1 through 6) were identified in the sample containing the 28 EU isolates, and three ETs

(designated ETs 7 through 9) were identified in the sample of FE isolates. The number of alleles and gene diversity (26) at each locus for the full data set, including all 39 EU and FE isolates, are shown in Table 2. Although the proportion of polymorphic loci observed across all isolates was relatively high (0.66), most of the variation observed was due to differences between the EU and FE isolate collections in allelic composition at the six variable loci. Similarly, although several nonrandom allelic associations between pairs of loci were suggested by the results of an inspection of the full data set for haplotypes (data not shown), these associations also were attributable to four apparently fixed or nearly fixed allelic differences between the isolate collections. We describe the allelic compositions of the EU and FE isolate collections separately below.

TABLE 2. Genetic variability at nine enzyme loci for 39 isolates of *S. paradoxus*

Enzyme	EU and FE isolates (n = 39)		EU isolates alone (n = 28)		FE isolates alone (n = 11)	
	No. of alleles	Gene diversity	No. of alleles	Gene diversity	No. of alleles	Gene diversity
ADH	1	0	1	0	1	0
ARK	1	0	1	0	1	0
G6PDH	2	0.22	1	0	2	0.54
GPI	2	0.18	2	0.20	2	0.19
IDH	2	0.39	1	0	1	0
LDH	2	0.39	1	0	1	0
MDH	1	0	1	0	1	0
MPI	3	0.43	2	0.07	1	0
PGM	3	0.48	3	0.20	1	0
Mean	1.89	0.23	1.44	0.05	1.22	0.08

EU isolates. The number of alleles and gene diversity at each locus for the EU isolate collection are shown in Table 2. Six of the nine enzymes examined (LDH, IDH, G6PDH, ADH, MDH, and ARK) were not variable in the EU isolate collection. Three electrophoretic phenotypes were identified for GPI activity. A common fast allele, *Gpi-1*, was observed in 25 isolates; a slow allele, *Gpi-2*, was observed in 2 isolates; and 1 isolate, INMIV 544, carried both allele *Gpi-1* and allele *Gpi-2*, suggesting that duplication of the *Gpi* gene had occurred. A fast MPI allele, *Mpi-1*, was present in one isolate; all other isolates harbored the slow *Mpi-2* allele. PGM migrated cathodally in all of the isolates studied (both the EU isolates and the FE isolates); staining for PGM revealed two bands in all of the isolates except CECT 10176 and CBS 5829, in which the upper band was absent. A fast cathodally migrating *Pgm* allele was observed in isolate INMIV 11/21.

FE isolates. The number of alleles and gene diversity at each locus for the FE isolate collection are shown in Table 2. Three enzymes, ADH, ARK, and MDH, were detected in the same allelic form in the FE isolate collection and in the EU isolate collection and were not variable. The slow GPI allele *Gpi-2*, which had been observed in two EU isolates, was present in one FE isolate; all of the other isolates harbored the most common allele, *Gpi-1*. For G6PDH, six isolates exhibited the common electromorph, and five isolates (N42, N43, N45, N47, and N50) had a variant phenotype with two bands that was unique to the FE isolate collection. A PGM variant observed in all 11 isolates of the FE isolate collection was present in only one EU isolate, INMIV 11/21. The remaining three enzymes (IDH, LDH, MPI) were monomorphic within the FE isolate collection, but were detected as alleles that were different from the alleles observed in the EU isolate collection.

Genetic differentiation between the EU and FE isolates. The FE and EU isolate collections differed significantly in allelic composition at the following five loci: *G6pdh*, *Pgm*, *Idh*, *Ldh*, and *Mpi* ($P \leq 0.001$ in every case, as determined by two-tailed Fisher exact tests). Combining probability estimates for the individual tests at all six informative loci (*G6pdh*, *Pgm*, *Idh*, *Ldh*, *Mpi*, and *Gpi*) by the method of Fisher (4) gave a highly significant χ^2 ($P < 0.001$; $df = 12$), which supported the conclusion that the two isolate collections represent genetically distinct subpopulations. (The method of combining probabilities relies on the independence of the individual significance tests. The independence of the loci examined in this study could in principle be supported by nonsignificant linkage disequilibria within each population; however, insufficient varia-

FIG. 1. UPGMA dendrogram based on pairwise genetic distances (see Materials and Methods) among the nine ETs observed in this study. ETs 1 through 6 are unique to the EU isolates; ETs 7 through 9 are unique to the FE isolates.

tion was observed for meaningful tests of within-population linkage disequilibrium.)

Figure 1 is a UPGMA dendrogram illustrating the genetic relationships among the nine ETs observed in this study. As expected given the genetic differences described above, ETs 7 through 9, which represented the entire FE isolate collection and were unique to it, clearly form a group distinct from the group made up of ETs 1 through 6, which were observed solely in EU isolates.

DISCUSSION

The allozyme data presented here indicate that the EU and FE isolates belong to genetically distinct geographical populations of the biological species *S. paradoxus*, a European population (including one isolate from Uzbekistan, isolate N9) and a Far East Asian population. The EU isolates differed almost completely from the FE isolates in allelic composition at four enzyme loci (*Idh*, *Ldh*, *Mpi*, and *Pgm*) and differed significantly in allele frequencies at a fifth locus, *G6pdh*.

In previous genetic hybridization studies we have noted that the viability of ascospores derived from crosses within the biological species *S. paradoxus* depended on the geographic origin of the parent isolates crossed. Fifty *S. paradoxus* isolates from a number of sites in Europe exhibited generally high ascospore viability ($67.1\% \pm 1.1\%$) in intraspecific crosses with European tester strain CBS 5829 isolated in Denmark (14–18, 22, 24, 25), whereas 12 isolates from the Russian Far East and 2 isolates from Japan exhibited markedly lower ascospore viability ($42.3\% \pm 2.3\%$) in crosses with the same tester strain (16, 20, 23). Taken together with the data on genetic variability presented here, these data suggest that geographically separated populations of *S. paradoxus* could represent early stages in allopatric speciation. Further research examining the fertility of crosses among isolates from the same and different geographical regions will be necessary to test this possibility.

Although in our study we examined a small number of enzymes, the results suggest that genetic variation within the European and Far East Asian populations of *S. paradoxus* is relatively low. In contrast, other studies have shown that the cultivated yeasts *S. cerevisiae*, *S. bayanus*, and *S. pastorianus* are very polymorphic for many enzymes (10, 32, 39). For example, among 18 *S. cerevisiae* isolates taken from stock cultures of wine yeasts, no two yeasts had identical electrophoretic patterns for five enzymes studied (32). It is likely that the low genetic variation within the two wild *S. paradoxus* populations studied here is related to the degree of inbreeding. No estimates of selfing rates in wild yeasts are available. However, wild isolates of *S. paradoxus* are homothallic and readily produce monosporic diploid clones in the laboratory through mating type switching, and thus a high level of selfing in natural populations seems likely. A number of evolutionary processes

can account for reduction in neutral genetic variability within highly inbred populations, including genetic hitchhiking (6), background selection against deleterious mutations (2), and genetic drift associated with decreases in effective population size (29). Future research in which higher-resolution molecular techniques are used to characterize genetic variation in *S. paradoxus* may provide important insight into the extent of selfing in wild yeast populations and its role in shaping genetic variation.

This is the first study to document geographical genetic differentiation within a *Saccharomyces* species classified according to the biological species concept. The results suggest that evolutionary genetic studies of wild *Saccharomyces* populations are both feasible and likely to be of general interest. Indeed, such studies may provide the evolutionary context essential for a fuller understanding of important phenomena (e.g., transposable elements, mating type switching) first discovered and studied in the classical and molecular genetics of *S. cerevisiae*.

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