A Mitochondrial Molecular Marker, *ori-rep-tra*, for Differentiation of Yeast Species

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Yeasts exhibit various mechanisms for the inheritance of their mitochondrial genomes. Differences among these mechanisms are based on variations within nuclear as well as mitochondrial genetic elements. Here we report diagnostic differences in the presence of biologically active mitochondrial intergenic sequences, *ori-rep-tra*, among related yeasts in the genera *Saccharomyces*, *Arxiozyma*, *Debaryomyces*, *Kluyveromyces*, *Pachytichospora*, *Torulaspora*, and *Zygosaccharomyces*. A molecular probe containing *ori-rep-tra* can be employed specifically for the differentiation and identification of isolates belonging to the species complex *Saccharomyces sensu stricto*.

To date, roughly 40 ascosporogenous yeast genera have been reported. Over the years, several methods have been used for differentiation of yeast strains. Originally, these comprised analysis of microbiological and physiological characters such as carbohydrate fermentation patterns, analysis of isoenzymes and serologically distinguishable components, analysis of the capacity of ascospore formation, and analysis of the ability to produce fertile offspring by hybridization among various isolates (for a review, see reference 2). However, the taxonomic relationship among numerous yeast isolates remained unclear, and little was known about the evolutionary origin of single yeasts. Introduction of molecular biological methods provided new views on yeast classification and changed the concept of a yeast species (1). Analyses of the genetic material, i.e., the nucleic acids, have been performed by determination of the average base composition of the genome, investigation of DNA-DNA reassociation kinetics, electrophoretic karyotype studies, and, recently, partial rRNA sequencing (5, 9, 15, 16, 22–24). In the near future, molecular taxonomy studies will presumably focus on specific molecular probes for differentiation and fast identification of single yeast strains as well as larger taxa.

When two yeast groups become sexually isolated, their sequences start to diverge. In general, the more changes that occur, the less related species become. However, changes are acquired at different rates by different genetic sequences. While some are “silent,” others suddenly lead to drastic changes in cellular metabolism. One cellular mechanism which apparently evolved and diverged at a high rate in yeasts is inheritance of the mitochondrial genome (mtDNA). Variations observed among various yeasts are due to different organization of nuclear genes as well as mtDNA (20, 21). The *Saccharomyces cerevisiae* mitochondrial genome consists of coding regions, introns, and intergenic regions. The intergenic regions represent two-thirds of the genome and are made up of

stretches formed by alternating and nonalternating adenine-thymine sequences and various guanine-cytosine clusters (for a review, see reference 20). A special class of intergenic sequences is formed by the *ori-rep-tra* sequences (4, 6, 18) present in several copies in the *S. cerevisiae* mitochondrial genome (mtDNA). These sequences are dispensable for expression of the respiratory phenotype (17, 19). However, they participate actively in cellular metabolism and play a role in determining a mode of mtDNA inheritance (for a review, see reference 20). In the present study, we have examined several yeasts that presumably are closely related to *S. cerevisiae* for the presence of the biologically active *ori-rep-tra* sequences.

The analyzed yeasts, representing typical strain isolates, are listed in Table 1. They belong to the genus *Saccharomyces* and to genera thought to be its close relatives (7–9). Some of the isolates, like *S. carlsbergensis*, *S. douglasii*, *S. monacensis*, and *S. uvarum*, are considered by some authors to be just synonyms of the “four-species” *S. cerevisiae sensu stricto* complex (1). However, because the taxonomic relationship within this group is still not completely clarified, the original nomenclature is followed in this publication. Yeast cultures were pregrown in a glucose medium (YPD [18]). To obtain sufficient amounts of mtDNA, mitochondrial derepression was induced by growth on nonfermentable media. Isolates were transferred to a glycerol-containing medium (GlyYP [18]) and cultured overnight at 25°C. A detailed procedure for mtDNA isolation can be found elsewhere (19). Cells were harvested by centrifugation, and protoplasts were formed with zymolyase (Seikagaku Kogyo) or lysing enzyme originating from *Trichoderma* spp. (Sigma). The protoplasts were disrupted with sodium dodecyl sulfate (SDS), and the supernatants were subjected to CsCl density gradient centrifugation. DNA bands were removed separately from the gradient. To verify the identity of the band containing the mtDNA fractions, the purified DNA bands were examined by molecular hybridization to several *S. cerevisiae* mitochondrial probes, such as 15S rRNA, 21S rRNA, *oxi1*, *oxi2*, *oxi3*, *oli1*, *oli2*, and *cob-box*. As expected, for all yeasts examined, the upper band was shown to represent mtDNA (data not shown). Purified mtDNA was digested with various restriction enzymes, and the digests were separated on agarose gels by following standard procedures (17). Several distinctive bands were observed in all cases examined (Fig. 1). mtDNA was blotted onto nylon membrane (Hybond N⁺; Amersham).

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TABLE 1. Presence of the *ori-rep-tra* sequences in the mitochondrial genome of various yeasts

Species designation	Strain ^a	Hybridization to <i>ori-rep-tra</i>
<i>S. cerevisiae</i>	NRRL Y-12632	+
<i>S. uvarum</i>	CBS 395	+
<i>S. pastorianus</i>	CBS 1538	+
<i>S. bayanus</i>	CBS 380	+
<i>S. monacensis</i>	CBS 1503	+
<i>S. carlsbergensis</i>	CBS 1513	+
<i>S. paradoxus</i>	NRRL Y-17217	+
<i>S. douglasii</i>	CBS 2908	+
<i>S. servazzii</i>	NRRL Y-12661	—
<i>S. castellii</i>	NRRL Y-12630	—
<i>S. dairensis</i>	NRRL Y-12639	—
<i>S. exiguus</i>	NRRL Y-12640	—
<i>S. unisporus</i>	NRRL Y-1556	—
<i>S. kluyveri</i>	NRRL Y-12651	—
<i>Kluyveromyces wickerhamii</i>	NRRL Y-8286	—
<i>Torulaspora delbrueckii</i>	NRRL Y-866	—
<i>Torulaspora globosa</i>	IFO 1160	—
<i>Pachytichospora transvalensis</i>	CBS 2186	—
<i>Arxiozyma telluris</i>	NRRL YB-4302	—
<i>Zygosaccharomyces rouxii</i>	NRRL Y-229	—
<i>Zygosaccharomyces bisporus</i>	NRRL Y-12626	—
<i>Debaryomyces polymorphus</i>	NRRL Y-2022	—
<i>Debaryomyces vanriji</i>	NRRL Y-7430	—
<i>Debaryomyces occidentalis</i>	NRRL Y-10	—

^a All isolates are type strains from various culture collections. CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Ill. (the corresponding CBS numbers for these strains can be found in reference 9); IFO, Institute for Fermentation, Osaka, Japan.

The membrane was prehybridized and hybridized overnight at 55°C in 25 ml of the hybridization buffer containing 5× Denhardt's solution, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% SDS, and 0.5 mg of the denatured non-homologous DNA, as recommended by the manufacturer. DNA was labeled with ³²P by using the Boehringer random-primed DNA labelling kit and was then probed with pBB5 or with a fragment of mtDNA containing the *ori1* sequence (3). pBB5 is a pUC18 vector carrying a 0.7-kb insert containing *ori1* (3, 17). Thereafter, the membrane was washed under relatively mild conditions in 2× SSC–0.1% SDS for 15 min at room temperature and for 15 min at 55°C. In the case of eight yeast isolates, several fragments hybridized to the probe (e.g., *S. douglasii* [Fig. 1, lanes 1 and 2]), suggesting that several copies of the *ori* sequence are present in the mtDNA of this yeast. However, the mtDNA of some other yeasts did not exhibit any hybridization at all (e.g., *Torulaspora delbrueckii* [Fig. 1, lanes 4 and 5]). Positive hybridization signals were obtained from mtDNA belonging to *S. cerevisiae*, *S. bayanus*, *S. carlsbergensis*, *S. douglasii*, *S. monacensis*, *S. paradoxus*, *S. pastorianus*, and *S. uvarum*. These yeasts were previously shown to represent a complex of closely related species, *Saccharomyces sensu stricto* (for a review, see reference 1). Representative strains of yeasts belonging to the genera *Arxiozyma*, *Debaryomyces*, *Kluyveromyces*, *Pachytichospora*, *Torulaspora*, and *Zygosaccharomyces* did not exhibit any hybridization to the *ori-rep-tra* probe (Table 1). Surprisingly, even some yeasts designated as belonging to the *Saccharomyces* genus, like *S. castellii*, *S. dairensis*, *S. exiguus*, *S. kluyveri*, *S. servazzii*, and *S. unisporus*, did not hybridize to the *ori-rep-tra* probe under the test conditions (Table 1; Fig. 2, lane 2). Accordingly, *ori-rep-tra* represents a sequence which is specific only for a limited number of closely related species. These

FIG. 1. mtDNA samples were digested with restriction nucleases and then separated on a 1% agarose gel (A) and hybridized with *ori-rep-tra* (B). Lanes: 1, *S. douglasii* digested with *Msp*I; 2, *S. douglasii* digested with *Hae*III; 3, *S. douglasii* undigested; m, a *Bst*EII digest of lambda DNA; 4, *T. delbrueckii* digested with *Msp*I; 5, *T. delbrueckii* digested with *Hae*III.

yeasts have been previously shown in DNA-DNA reassociation kinetics studies, electrophoretic karyotype studies, and genetic studies of chromosomes (15, 22, 23) to belong to a group of the *S. cerevisiae*-like yeasts (1). Similarly, the recent determination of partial nuclear rRNA sequences showed that *S. bayanus*, *S. cerevisiae*, *S. paradoxus*, and *S. pastorianus* represent a complex of closely related species (9). In addition, the *ori-rep-tra*⁺ strains of yeast exhibit a distinctive mechanism of mtDNA inheritance and generation of petite mutations which differs from that of the *Saccharomyces* strains which are without *ori-rep-tra* (11, 21). When yeasts belonging to the *S. cerevisiae sensu stricto* complex are induced by chemical mutagens, spontaneous mitochondrial petite mutants arise at high frequencies approaching 100%. On the other hand, the *Saccharomyces* strains without *ori-rep-tra* generate only moderate frequencies

FIG. 2. Undigested total DNA from yeast isolates originating from human patients run on a 1% agarose gel (A) and blotted and hybridized with *ori-rep-tra* (B). Lanes: 1, standard strain of *S. cerevisiae* (positive control); 2, standard strain of *S. servazzii* (negative control); 3 to 8, clinical isolates (lanes 3, isolate 290; lanes 4, isolate 539; lanes 5, isolate 650; lanes 6, isolate 395; lanes 7, isolate 738; lanes 8, isolate 1578). The clinical isolates originated from a collection kept at the Statens Seruminstitut, Copenhagen.

of petite mutants after induction, and *S. kluyveri* seems to be a petite-negative yeast (21).

We propose that the *ori-rep-tra* sequences evolved in the mitochondrial genome relatively late in the evolution of the *Saccharomyces* group. Another interpretation is that the sequence diverged quite rapidly and cannot be detected in other species because of nucleotide substitutions. The appearance of *ori-rep-tra* clearly divides the present members of the genus into two subgroups. Therefore, these sequences may represent an ideal taxonomic marker for the identification of novel isolates of yeast strains and species that are close relatives of the examined *ori-rep-tra*⁺ yeasts.

Fungemia with members of the *Saccharomycetaceae* is rare. However, pathogenic yeasts have occasionally been isolated from patients with severe underlying disease (12, 14). Several clinical isolates originating from Danish patients were routinely identified as putative strains of *S. cerevisiae* by conventional nonmolecular methods (2). However, various commercial systems based on these methods sometimes give ambiguous results and do not always distinguish among different members of the *Saccharomyces* genus (10). Also, these methods are very time-consuming and provide results only after a couple of weeks.

In the following experiment, six yeast isolates obtained from clinical material were examined for the presence of *ori-rep-tra* sequences. The strains are listed in Fig. 2. Cultures of pure clinical yeast isolates were grown overnight in 2 ml of YPD medium at 25°C. Total cellular DNA was isolated by a modification of the quick method of Nasmyth and Reed (13, 18). Undigested DNA was separated on a minigel (Fig. 2A) and blotted onto a membrane (Hybond N⁺). Hybridization with the pBB5 probe was performed as described above. Strong hybridization to the probe was seen for five of the six isolates (Fig. 2B), while one isolate showed substantially weaker homology to the *ori-rep-tra* probe (Fig. 2B, lane 6). These results indicate that the collection examined did not contain a completely uniform group of yeasts. Furthermore, use of a molecular probe reduced the time needed for identification of *S. cerevisiae* and allied yeasts to only 3 days. This may be very important when an appropriate medical treatment must be instituted as soon as possible.

Yeasts are involved in many different processes in nature; however, their exact role is often only poorly understood, in part because of the problems of identifying species. Therefore, the development and employment of specific molecular probes, like *ori-rep-tra*, provide a convenient tool for monitoring quantitative and qualitative aspects of even complex microbiological processes.

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REFERENCES

1. Barnett, J. A. 1992. The taxonomy of the genus *Saccharomyces* Meyen ex Reess: a short review for non-taxonomists. *Yeast* **8**:1–23.
2. Barnett, J. A., R. W. Payne, and D. Yarrow. 1990. *Yeasts: characteristics and identification*. Cambridge University Press, Cambridge.
3. Bingham, C. G., and P. Nagley. 1983. A petite mitochondrial DNA segment arising in exceptionally high frequency in a *mit*[−] mutant of *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **740**:88–98.
4. Blanc, H., and B. Dujon. 1980. Replicator regions of the yeast mitochondrial DNA responsible for suppressiveness. *Proc. Natl. Acad. Sci. USA* **77**:3942–3946.
5. Carle, G. F., and M. V. Olson. 1985. An electrophoretic karyotype for yeast. *Proc. Natl. Acad. Sci. USA* **82**:3756–3760.
6. DeZamaroczy, M., G. Baldacci, and G. Bernardi. 1979. Putative origins of replication in the mitochondrial genome of yeast. *FEBS Lett.* **108**:429–432.
7. James, S. A., M. D. Collins, and I. N. Roberts. 1994. Genetic interrelationship among species of the genus *Zygosaccharomyces* as revealed by small-subunit rRNA gene sequences. *Yeast* **10**:871–881.
8. Kurtzman, C. P. (U.S. Department of Agriculture). 1994. Personal communication.
9. Kurtzman, C. P., and C. J. Robnett. 1991. Phylogenetic relationship among species of *Saccharomyces*, *Schizosaccharomyces*, *Debaryomyces* and *Schwannomyces* determined from partial ribosomal RNA sequences. *Yeast* **7**:61–72.
10. Lin, C. C. S., and D. Y. C. Fung. 1987. Conventional and rapid methods for yeast identification. *Crit. Rev. Microbiol.* **14**:273–289.
11. McArthur, C. R. 1980. M.Sc. thesis. Australian National University, Canberra.
12. McCusker, J. H., K. V. Clemons, D. A. Stevens, and R. W. Davis. 1994. Genetic characterization of pathogenic *Saccharomyces cerevisiae* isolates. *Genetics* **136**:1261–1269.
13. Nasmyth, K. A., and S. I. Reed. 1980. Isolation of genes by complementation of yeast: molecular cloning of a cell-cycle gene. *Proc. Natl. Acad. Sci. USA* **77**:2119–2123.
14. Nielsen, H., J. Stenderup, and B. Bruun. 1990. Fungemia with *Saccharomycetaceae*. *Scand. J. Infect. Dis.* **22**:581–584.
15. Pedersen, M. B. 1986. DNA sequence polymorphism in the genus *Saccharomyces*. IV. Homoeologous chromosomes III of *Saccharomyces bayanus*, *S. carlsbergensis* and *S. uvarum*. *Carlsberg Res. Commun.* **51**:185–202.
16. Pedersen, M. B. 1988. The use of nucleotide sequence polymorphism and DNA karyotyping in the identification of brewer's yeast strains in microbiological control, p. 180–194. In H. F. Linskens and J. F. Jackson (ed.), *Modern methods of plant analysis, new series*. Springer Verlag KG, Berlin.
17. Piškur, J. 1988. Transmission of yeast mitochondrial loci to progeny is reduced when nearby intergenic regions containing *ori/rep* sequences are deleted. *Mol. Gen. Genet.* **214**:425–432.
18. Piškur, J. 1989. Transmission of the yeast mitochondrial genome to progeny: the impact of intergenic sequences. *Mol. Gen. Genet.* **218**:161–168.
19. Piškur, J. 1989. Respiratory-competent yeast mitochondrial DNAs generated by deleting intergenic region. *Gene* **81**:165–168.
20. Piškur, J. 1994. Inheritance of the yeast mitochondrial genome. *Plasmid* **31**:229–241.
21. Piškur, J. Unpublished data.
22. Vaughan-Martini, A., and C. P. Kurtzman. 1985. Deoxyribonucleic acid relatedness among species of the genus *Saccharomyces* sensu stricto. *Int. J. Syst. Bacteriol.* **35**:508–511.
23. Vaughan-Martini, A., A. Martini, and G. Cardinali. 1993. Electrophoretic karyotyping as a taxonomic tool in the genus *Saccharomyces*. *Antonie Leeuwenhoek* **63**:145–156.
24. Yarrow, D., and T. Nakase. 1975. DNA base composition of the genus *Saccharomyces*. *Antonie Leeuwenhoek* **41**:81–88.