Use of an rRNA Internal Transcribed Spacer Region To Distinguish Phylogenetically Closely Related Species of the Genera *Zygosaccharomyces* and *Torulaspora*

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Analyses of the sequences of the small-subunit (18S) rRNA gene and two internal transcribed spacers (ITSs), ITS1 and ITS2, revealed that members of the yeast genera *Torulaspora* and *Zygosaccharomyces* are phylogenetically intermixed. Despite some minor differences in 18S rRNA-, ITS1-, and ITS2-derived trees, in general the patterns of the relationships inferred from the three chronometers were in good agreement. The ITS sequences of *Torulaspora* and *Zygosaccharomyces* species exhibited far greater interspecies differences than the 18S rRNA sequences and were better than 18S rRNA sequences for measuring close genealogical relationships. Despite the existence of interstrain ITS sequence variation in some species, it is possible to identify conserved regions in both ITSs that are useful in species differentiation.

Small-subunit rRNA sequencing is currently revolutionizing our knowledge about the natural relationships among bacteria (18). Small-subunit rRNA acts as a mosaic of different degrees of sequence conservation (from highly conserved to semiconserved to highly variable regions), which allows the molecule to measure distant evolutionary relationships, as well as closer evolutionary relationships. Compared with bacteria, there is relatively little information concerning yeast phylogeny based on this chronometer available (9). Although sequence analyses of small-subunit rRNA should make significant contributions to yeast systematics, the high overall level of conservation of the molecule may restrict its value in resolving the phylogeny of many yeast genera which exhibit smaller evolutionary depths than bacterial genera. The important food spoilage ascomycetous yeast genus *Zygosaccharomyces* is a case in point (7). Recent 18S rRNA gene sequence analyses revealed that the genus *Zygosaccharomyces* is relatively shallow in evolutionary terms (it exhibits an intrageneric level of divergence of approximately 1.8%) (7). Although the genus *Zygosaccharomyces* has been shown to consist of a number of subdivisions, the relatively high levels of sequence similarity observed between some species (e.g., *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus*) appear to limit the value of 18S rRNA for differentiating genotypically closely related species.

In this study we analyzed the sequence of the internal transcribed spacer (ITS) region as an alternative to 18S rRNA for delineating genealogically closely related yeasts. In yeasts, as well as in other eukarya, the ITS region is located between the 18S and 28S rRNA genes. The ITS region is subdivided into the ITS1 region, which separates the 18S and 5.8S rRNA genes, and the ITS2 region, which is found between the 5.8S and 28S rRNA genes. In principle, ITSs are attractive chronometers for determining the relationships of genotypically closely related species, as they have higher rates of divergence than 18S rRNA genes. At one time it was thought that these higher rates occurred because the spacers have no function, but recent findings of van der Sande et al. (16) and Musters et

al. (11) have shown that both ITS regions play a role in primary rRNA processing.

In this paper we describe the results of a comparison of the ITS1 and ITS2 sequences of members of the genus *Zygosaccharomyces* which showed that these sequences are useful for differentiating and delineating genealogically closely related species. In addition, we describe the phylogenetic intermixing of the genera *Zygosaccharomyces* and *Torulaspora* as determined by both 18S rRNA and ITS sequence analyses.

MATERIALS AND METHODS

Yeast strains. The *Zygosaccharomyces* and *Torulaspora* strains used in this study are shown in Table 1. All of the strains were cultivated on YM plates (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% glucose; pH 5.5) at 24°C.

PCR amplification and PCR product purification. PCR amplification of the 18S rRNA gene was performed as described by James et al. (7). The entire ITS region was amplified by using primers pITS1 (5'-TCCGTAGGTGAACCT GCGG) and pITS4 (5'-TCCTCCGCTTATTGATATG), which were derived from conserved regions of the 18S and 28S rRNA genes, respectively (17). The amplified products were purified by using a Wizard PCR Preps DNA purification system (Promega, Southampton, United Kingdom) according to the manufacturer's instructions.

Direct PCR sequencing. Direct sequencing of the PCR-amplified 18S rRNA genes and ITSs was performed by using a Sequenase version 2.0 sequencing kit (United States Biochemical Corp., Cleveland, Ohio) as described by Hutson et al. (6). The primers used to sequence the 18S rRNA gene were the primers described by James et al. (7), and these primers generated approximately 97% of the 18S rRNA gene sequence for *Torulaspora globosa* and *Torulaspora pretoriensis*. To determine the sequences of the ITSs of the *Zygosaccharomyces* and *Torulaspora* strains shown in Table 1, we used two internal primers, primer pITS3 (5'-GCATCGATGAAGAACGCGAG), which targeted a conserved region at approximately positions 31 to 49 of the 5.8S rRNA gene, and primer p5.8Sr (5'-ATGACRCTCAAACAGGCAT), which targeted a conserved region at approximately positions 156 to 138 of the 5.8S rRNA gene, in addition to the two external amplification primers, primers pITS1 and pITS4.

Sequence analysis. The 18S rRNA gene sequences were aligned by using the multiple-sequence alignment program PILEUP (3) contained in the Genetics Computer Group software package (4), version 7.2. The alignments were adjusted manually. The ITS sequences were subjected to a pairwise analysis. Hypervariability, including extensive insertions and deletions, precluded the use of multiple sequence alignment programs for the ITS data sets. Levels of sequence similarity were calculated, and derived evolutionary distance (K_{nuc}) (5) values were used to produce unrooted phylogenetic trees by the neighbor-joining (14) and unweighted pair group with mathematical average (UPGMA) cluster analysis (15) methods. The confidence values of branches on the neighbor-joining tree were determined by performing a bootstrap analysis in which 500 replicates were used (2).

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TABLE 1. Yeast strains used and their ITS1 and ITS2 sequence accession numbers

Species ^a	Strain ^b	EMBL accession no.	
		ITS1 sequence	ITS2 sequence
<i>T. delbrueckii</i> (Lindner) Lindner	CBS 1146 ^{Tc}	Z48309	Z48311
<i>T. globosa</i> (Klöcker) van der Walt & Johannsen	NCYC 820 ^T	Z48335	Z48336
<i>T. pretoriensis</i> (van der Walt & Tschuschner) van der Walt & Johannsen	NCYC 524 ^T	Z48310	Z48312
<i>Z. bailii</i> (Lindner) Guillermond	NCYC 1416 ^T	X84640	X84641
	NCYC 573	X84640	X84641
	NCYC 1515	X84640	X84641
	NCYC 417	X87129	X87130
	NCYC 464	X87129	X87130
	NCYC 1427	X84732	X84733
	NCYC 1766	X84732	X84733
<i>Z. bisporus</i> Naganishi	NCYC 1495 ^T	X84642	X84643
	NCYC 171	X84642	X84643
	NCYC 1496	X84642	X84643
	NCYC 1555	X84642	X84643
<i>Z. cidri</i> (Legakis) Yarrow	NCYC 1567 ^T	X48347	Z48361
<i>Z. fermentati</i> Naganishi	CBS 707 ^T	Z48358	Z48362
<i>Z. florentinus</i> Castelli ex Kudriavzev	CBS 746 ^T	Z48348	Z48363
<i>Z. mellis</i> Fabian & Quinet	NCYC 2403 ^T	Z48349	Z48365
<i>Z. microellipsoides</i> (Osterwalder) Yarrow	CBS 427 ^T	Z48350	Z48364
<i>Z. mrakii</i> Capriotti (Osterwalder) Yarrow	CBS 4218 ^T	Z48351	Z48366
<i>Z. rouxii</i> (Boutroux) Yarrow	NCYC 568 ^T	X84644	X84645
	NCYC 381	X84644	X84645
	NCYC 565	X84644	X84645
	NCYC 579	X84644	X84645

^a Species descriptions are given in reference 19.^b Abbreviations: NCYC, National Collection of Yeast Cultures, Norwich, United Kingdom; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands.^c T = type strain.

Nucleotide sequence accession numbers. The nucleotide sequences of the 18S rRNA genes of *T. globosa* and *T. pretoriensis* have been assigned EMBL accession numbers X84639 and X84638, respectively. The EMBL accession numbers for the 28 ITS sequences which we determined are shown in Table 1.

FIG. 1. Unrooted dendrograms showing the genetic relationships among *Torulaspora* and *Zygosaccharomyces* species. The dendrograms were constructed by the neighbor-joining method with bootstrap analysis (A) and the UPGMA cluster analysis method (B). Bar = 0.001 K_{nuc} . *S. cerevisiae*, *Saccharomyces cerevisiae*.

RESULTS AND DISCUSSION

18S rRNA gene sequence analysis. In a previous comparative 18S rRNA sequence study (7), *Torulaspora delbrueckii*, the type species of the genus *Torulaspora*, was found to be phylogenetically closely related to certain species belonging to the genus *Zygosaccharomyces*. Therefore, the 18S rRNA gene sequences of the other species of the genus *Torulaspora*, *T. globosa* and *T. pretoriensis*, were determined to elucidate the phylogenetic relationships of these organisms. Phylogenetic trees that were constructed by using both UPGMA cluster analysis (15) and neighbor-joining methods (14) and depict the relationship of species belonging to the genera *Zygosaccharomyces* and *Torulaspora* are shown in Fig. 1. High levels of sequence similarity were observed among the *Torulaspora* species (range, approximately 99.7 to 99.8%), and the *T. delbrueckii* and *T. pretoriensis* sequences differed by only three bases. Similar high levels of sequence similarity have been observed for some *Zygosaccharomyces* species (e.g., *Z. bailii* and *Z. bisporus*) (7). Phylogenetically, the three *Torulaspora* species were closely related to *Zygosaccharomyces* species (range of levels of sequence similarity, approximately 98.4 to 99.9%) and exhibited particularly close associations with *Zygosaccharomyces mrakii* and *Zygosaccharomyces microellipsoides* (levels of sequence similarity, 99.7 to 99.9%). The results of our treeing analysis (Fig. 1) demonstrated that the *Zygosaccharomyces* and *Torulaspora* species are intermixed phylogenetically and that *Z. microellipsoides*, *Z. mrakii*, and the three *Torulaspora* species form a distinct subgroup that is separate from other *Zygosaccharomyces* species (Fig. 1). This significant association was more apparent on the UPGMA tree than on the neighbor-joining tree; the latter exhibited a marked lack of ultrametricity.

ITS sequence analysis. The nucleotide sequences of ITS1 and ITS2 regions of all of the strains shown in Table 1 were

[illegible]

TABLE 3. Levels of ITS2 sequence similarity for *Torulaspora* and *Zygosaccharomyces* species

Taxon	% ITS2 sequence similarity												
	<i>Z. bailii</i> type B	<i>Z. bailii</i> type C	<i>Z. bisporus</i>	<i>Z. cidri</i>	<i>Z. fermentati</i>	<i>Z. florentinus</i>	<i>Z. mellis</i>	<i>Z. microellipsoides</i>	<i>Z. mrakii</i>	<i>Z. rouxii</i>	<i>T. delbrueckii</i>	<i>T. globosa</i>	<i>T. pretoriensis</i>
<i>Z. bailii</i> type A	98.8	96.1	87.0	75.8	75.0	81.1	68.1	72.4	72.2	56.0	72.1	72.4	71.8
<i>Z. bailii</i> type B		96.4	87.4	73.3	73.3	81.1	67.8	72.1	71.4	53.0	71.7	71.3	71.4
<i>Z. bailii</i> type C			88.2	77.1	77.8	82.5	59.7	69.9	70.2	60.8	72.4	72.0	70.6
<i>Z. bisporus</i>				71.8	72.2	81.0	65.8	71.4	73.0	67.3	72.9	73.0	72.5
<i>Z. cidri</i>					97.6	74.1	77.3	80.6	79.9	68.9	79.5	80.2	79.9
<i>Z. fermentati</i>						74.5	77.3	78.5	80.3	69.4	81.0	80.2	80.3
<i>Z. florentinus</i>							82.9	81.2	79.1	80.2	79.1	77.9	79.1
<i>Z. mellis</i>								66.6	66.7	80.4	64.5	67.7	66.7
<i>Z. microellipsoides</i>									89.6	54.4	88.8	88.8	89.6
<i>Z. mrakii</i>										56.6	97.3	93.4	100.0
<i>Z. rouxii</i>											55.1	59.3	53.4
<i>T. delbrueckii</i>												94.6	96.9
<i>T. globosa</i>													93.4

quence variation was observed among *Z. bailii* strains. Within *Z. bailii*, three distinct ITS sequence types were identified; type A included strains NCYC 1416 (the type strain), NCYC 573, and NCYC 1515; type B included strains NCYC 1427 and NCYC 1766; and type C included strains NCYC 417 and NCYC 464. A pairwise analysis of the three *Z. bailii* ITS types revealed similarity values ranging from 89.8 to 93.3% for ITS1 and from 96.1 to 98.8% for ITS2. Despite the considerable intraspecific sequence variation in the ITSs of *Z. bailii*, we

identified regions of homology in types A, B, and C that could be used to distinguish this species from *Z. bisporus* and *Z. rouxii* (Fig. 2 and 3). Alignments of the ITS1 and ITS2 regions of the three *Torulaspora* species, *Z. microellipsoides*, and *Z. mrakii* are shown in Fig. 4 and 5. A pairwise analysis revealed ranges of levels of sequence similarity of 77.1 to 99.3% for ITS1 and 88.8 to 100% for ITS2. For both spacers, the highest levels of sequence similarity were observed with *Z. mrakii* and *T. pretoriensis* (99.3% for ITS1 and 100% for ITS2). Other very similar ITS sequences worthy of note are the sequences of *Zygosaccharomyces cidri* and *Zygosaccharomyces fermentati* (levels of sequence similarity for ITS1 and ITS2, 95.2 and

18S rDNA	
Tdel	AGGATCATTAGAGAAATCTATATGAATGAAGTTAGAGGACGCTCTAAAGAT
Tglob	G.G.....
Tpret	C.....T.....A.G.....
Zmicro	T.....A.....C.....G.A.T.....TA.G.....
Zmrak	C.....TC.....A.G.....
Tdel	-ACTGTA-AGAGAGGATCAGGTTTC-AAGACAGCGCTTAATTGCGCGTT
Tglob	T....C.G...GA...A.CTGTA...GT...T...T.....G.....
Tpret	TTT.....
Zmicro	.G...A.G...A...TC...TTTGG.....G.....
Zmrak	TTT.....
Tdel	GCGGCTTGGTTCGCTTTTTCGGAACAT---GCTCTTCTCGTTGTTAA
Tglob	...TGCAA...C.AA.C...T...A...AAAT...CG.....C...
Tpret	...A.....T.....G.....C.....
Zmicro	TT...TGA.AG.A.TA...AT...TT.TAC-N...C...T.....
Zmrak	...A.....T.....N.....C.....
Tdel	CTCTACTTCAACTTCTACACACTGTGGAGTTTCTACACAACCTT--TC
Tglob	A...AACTT..
Tpret	...G.....A.....
Zmicro	...A.....A.....CT..
Zmrak	...G.....A.....C.....
Tdel	TTCTTTGGGAA---GATA---CGTCTT-GTGGCT-GCTCCAGAGGTGAC
Tglob	G.TAA.T...GT...T...C.AAT.ACTT.C.....
Tpret	G.....A.....C.....
Zmicro	G.....T.....C.....T.....A.....
Zmrak	...NN...G.....A.....C.....
Tdel	AAACACAACAACCTTTTATTATT-ATAAACAGCTCAAAA-CCAATTTG
Tglob	TA.C.....T.....A.....
Tpret	A.....T.....
Zmicro	T.....TT.....
Zmrak	A.....T.....
5.8S rDNA	
Tdel	TTAT-GAAATTAATAA-TATTTAAACTTCA
Tglob	...T.A.....A.....C.....
Tpret	
Zmicro	
Zmrak	

FIG. 4. Alignment of the ITS1 sequences of *T. delbrueckii* (Tdel), *T. globosa* (Tglob), *T. pretoriensis* (Tpret), *Z. microellipsoides* (Zmicro), and *Z. mrakii* (Zmrak).

5.8S rDNA	
Tdel	AGCGTCAATTCCTTCTCAAAACATCATGTTGGTAGTGAGTACTCTG
Tglob	
Tpret	
Zmicro	TC.....A
Zmrak	
Tdel	TCAAGGGTTAACTTGAAATTCGTAGCCTGTTATTGGTTGTGATTTTGCT
Tglob	...TC.....
Tpret	
Zmicro	A.T.....
Zmrak	
Tdel	GGCTTGGATGACTTTGTCCAGTCTAGCTAATACCGAATGTCGTATTAGG
Tglob	C.....
Tpret	G.....
Zmicro	T.....G.....
Zmrak	G.....
Tdel	TTTTACCAACTTCGGCAGACTGTCTGTGGCTCGGCGCTTTAAAGACTT
Tglob	C.....A.....T.....A.....G
Tpret	T.....T.....
Zmicro	A.....TA.....A.T.TA...T.A.....AA
Zmrak	T.....
Tdel	TGTCGTAACGATTATCGTTTGTGAGCTTTT-GCATACGCAATCCGG
Tglob	A.....G.....C.....
Tpret	G.....A.....A.....G.....
Zmicro	GA.....A.T.A...C...G.....C.AA
Zmrak	G.....A.....A.....G.....
28S rDNA	
Tdel	CGAACAATACTCTCAAGTTTGACCTCA
Tglob	GT.....
Tpret	
Zmicro	
Zmrak	

FIG. 5. Alignment of the ITS2 sequences of *T. delbrueckii* (Tdel), *T. globosa* (Tglob), *T. pretoriensis* (Tpret), *Z. microellipsoides* (Zmicro), and *Z. mrakii* (Zmrak).

FIG. 6. Unrooted dendrograms constructed by the neighbor-joining method from ITS1 sequences (A) and ITS2 sequences (B).

97.6%, respectively) (Tables 2 and 3). Species belonging to the genus *Zygosaccharomyces* have been shown to be distinct as determined by nuclear DNA-DNA pairing (8). The presence of unique ITS sequences in all nine species of this genus is consistent with the genotypic distinctiveness of these organisms. The DNA-DNA pairing data for *Torulaspora* species are limited (13). However, the comparative species data that do exist are consistent with the ITS distinctiveness of these taxa.

Figure 6 shows the genealogical relationships of *Zygosaccharomyces* and *Torulaspora* species determined by using ITS1 and ITS2 sequences. Although ITS1 generally exhibited more sequence divergence than ITS2 (Tables 2 and 3), the tree topologies derived from the two data sets were for the most part in good agreement. For example, consistent with previously recognized phenotypic traits (1, 8, 19), *Z. bailii* (types A to C) and *Z. bisporus* formed a distinct subgroup, as did the species *Z. cidri* and *Z. fermentati*. Some differences in the pattern of the relationship between *Z. mellis* and *Z. rouxii* were evident on the trees derived from 18S rRNA and ITS sequences. The 18S rRNA data indicated that *Z. mellis* and *Z. rouxii* are closely related to each other and significantly related to the *Z. bailii*-*Z. bisporus* subgroup (7). On the ITS1-derived tree, *Z. mellis* did not exhibit a close genealogical relationship to *Z. rouxii* (Fig. 6A), although these species were closely associated on the ITS2-derived tree, in accordance with the 18S rRNA data. The close genealogical relationship among *T. delbrueckii*, *T. pretoriensis*, and *Z. mrakii* inferred from the comparative 18S rRNA analysis (Fig. 1) was confirmed by both the ITS1 tree and the ITS2 tree (Fig. 6). It is clear that *T. delbrueckii*, *T. globosa*, *T. pretoriensis*, *Z. mrakii*, and *Z. microellipsoides* form a tight phylogenetic subgroup and should be placed in the same genus. These results are consistent with the phenotypic similarities of these species (1, 19). Although it is tempting to suggest that these findings support reclassification of *Z. microellipsoides* and *Z. mrakii* in the genus *Torulaspora*, we believe that such a

move would be premature. Several other yeast genera may also be phylogenetically intermixed with the genus *Zygosaccharomyces* (e.g., the genera *Saccharomyces* [7] and *Kluyveromyces* [unpublished data]). Consequently, additional genealogical comparisons will be necessary before any phylogenetic rationale for subdividing this group of highly related ascomycetous yeasts can be put forward with confidence.

Despite some minor incongruencies in the 18S rRNA, ITS1, and ITS2 data, in general the patterns of relationships derived from the three sequence data sets were in good agreement. Although 18S rRNA is an immensely powerful chronometer for elucidating phylogenetic relationships, its high overall level of sequence conservation seems to limit its value for measuring and resolving close relationships. It is evident from our data that ITS sequences exhibit far greater interspecies sequence divergence and because of this are superior to 18S rRNA for measuring very close genealogical relationships (i.e., relationships of closely related species or species groups). However, the hypervariability of ITSs and the associated alignment problems (increasing noise and error) preclude the use of ITS for assessing more distant relationships. Hence, whenever possible, it is advisable to combine information from both types of data sets when close phylogenetic relationships are studied. It is pertinent to note that despite considerable interstrain ITS sequence variation in some species (e.g., *Z. bailii* types A, B, and C) (12), it is clearly possible to identify conserved regions ("core sequences") in both spacers which can be used for species delineation. Such regions could also be invaluable for rapid species identification with probes.

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