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In Situ Accessibility of *Saccharomyces cerevisiae* 26S rRNA to Cy3-Labeled Oligonucleotide Probes Comprising the D1 and D2 Domains

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Fluorescence in situ hybridization (FISH) has proven to be most useful for the identification of microorganisms. However, species-specific oligonucleotide probes often fail to give satisfactory results. Among the causes leading to low hybridization signals is the reduced accessibility of the targeted rRNA site to the oligonucleotide, mainly for structural reasons. In this study we used flow cytometry to determine whole-cell fluorescence intensities with a set of 32 Cy3-labeled oligonucleotide probes covering the full length of the D1 and D2 domains in the 26S rRNA of *Saccharomyces cerevisiae* PYCC 4455^T. The brightest signal was obtained with a probe complementary to positions 223 to 240. Almost half of the probes conferred a fluorescence intensity above 60% of the maximum, whereas only one probe could hardly detect the cells. The accessibility map based on the results obtained can be extrapolated to other yeasts, as shown experimentally with 27 additional species (14 ascomycetes and 13 basidiomycetes). This work contributes to a more rational design of species-specific probes for yeast identification and monitoring.

In the last decade, fluorescence in situ hybridization (FISH) became the method of choice for the direct detection and identification of microorganisms in their natural environments (1, 3, 15). Even though FISH has been extensively used in ecological studies of bacteria (3) and other organisms (17), work with fungi has been restricted to the detection of *Aureobasidium pullulans* on the phylloplane (12, 19) and either clinically relevant or food spoilage yeasts (9, 10, 13, 14). Recently, a method using fluorescently labeled peptide nucleic acid probes was applied with success to the detection of *Dekkera bruxellensis* in wine (20), to the differentiation between *Candida albicans* and *Candida dubliniensis* (16), and to direct detection of *C. albicans* in blood culture bottles (18).

Preliminary studies with yeasts have shown that FISH assays are rapid and simple to carry out, do not require special cell permeation treatments and result in a high signal-to-noise ratio even when the cellular ribosome content is low, e.g., in late-stationary-phase cells (J. Inácio et al., unpublished data). However, a significant fraction of the probes designed yield low or no hybridization signals under optimal experimental conditions as assessed with a universal probe (10). One possible limitation of the method is associated with the target molecule, the rRNA. The targeted region of the ribosomes, which remain in the intact cell, might be structurally hindered or involved in molecular interactions, rendering it inaccessible to probe hybridization (3). Despite the development of procedures to improve the accessibility of those regions by using unlabeled helper oligonucleotides (6), a very useful clue when trying to design a good probe is to look for target sites located in rRNA regions already known to be accessible (7, 8).

The D1 and D2 domains at the 5′ end of 26S rRNA show a high degree of interspecies sequence variation for yeasts and are therefore frequently used for identification as well as in phylogenetic studies (5, 11). Due to the nucleotide sequence variability and to the large number of sequences available in public databases, this region provides an excellent basis to design species-specific FISH probes targeting the rRNAs of yeasts (16, 20).

The aim of the present study was to evaluate the accessibility of the D1 and D2 domains in the 26S rRNA to fluorescently labeled probes by using *Saccharomyces cerevisiae* as a model.

MATERIALS AND METHODS

Cultivation. *S. cerevisiae* PYCC 4455^T (Portuguese Yeast Culture Collection, Caparica, Portugal) was grown aerobically under continuous shaking in YM broth (malt extract, 0.3% [wt/vol]; yeast extract, 0.2%; peptone, 0.5%; glucose, 1%) at 25°C. Cells were harvested in the exponential growth phase (optical density of 2.5 at 600 nm) by centrifugation for 5 min at 4,500 × *g*. Cells were washed once with 1× phosphate-buffered saline (130 mM sodium chloride, 10 mM sodium phosphate buffer [pH 7.2]) and fixed for 4 h with 4% (vol/vol) paraformaldehyde at 4°C (2).

Probe design. Oligonucleotide probes were designed to cover the full length of the 26S rRNA D1 and D2 domains of *S. cerevisiae* (Fig. 1) (sequence retrieved from GenBank under accession number U44806). The sequences and positions of the 32 probes in the D1 and D2 domains are listed in Table 1. The standard probe length of 18 nucleotides was varied if the estimated dissociation temperature (T_d), according to the formula of Suggs et al. (21) [$T_d = 4 \times (G + C) + 2 \times (A + T)$], exceeded 60°C or was below 48°C.

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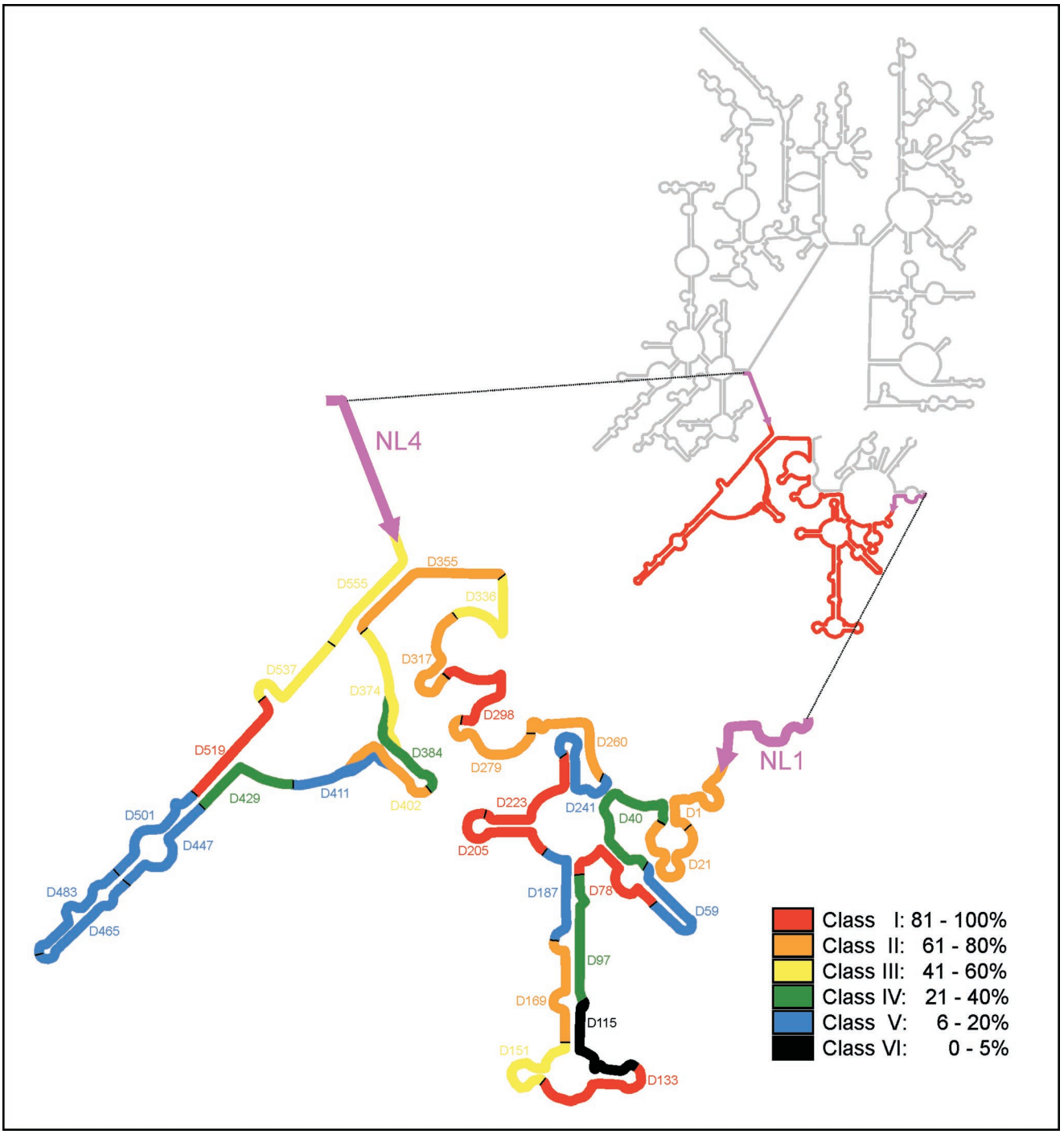

FIG. 1. Fluorescence intensities of all oligonucleotide probes, standardized to that of the brightest probe (D-223), indicated in a model of the *S. cerevisiae* 26S rRNA secondary structure in which the D1 and D2 domains (delimited by the NL1 and NL4 primer target sites) are enlarged. The color coding indicates differences in the level of Cy3 probe-conferred fluorescence. The secondary structure is adapted from the European rRNA database (http://rrna.uia.ac.be).

Probe labeling and quality control. Probes were synthesized monolabeled at the 5′ end with Cy3 by Interactiva GmbH (Ulm, Germany). Aliquots of each probe were analyzed in a spectrophotometer (UV-1202; Shimadzu, Duisburg, Germany). The peak ratios of the absorption of DNA at 260 nm and the dye at 545 nm were determined in order to check the labeling quality of the oligonucleotides (7).

FISH. Approximately 10^6 cells were hybridized in 80 μl of hybridization buffer (0.9 M sodium chloride, 0.01% [wt/vol] sodium dodecyl sulfate, 20 mM Tris-HCl [pH 7.2]) with 1.5 ng of Cy3-labeled probe μl^{-1} at 46°C for 2 h. After incubation, cells were pelleted by centrifugation and the supernatant was discarded. Cells were resuspended in 100 μl of prewarmed hybridization buffer without probe. After washing for 30 min at 46°C, the suspension was mixed with 200 μl of 1× PBS, placed on ice, and analyzed within 3 h.

Flow cytometry. Fluorescence of hybridized cells was quantified with a FACStar Plus flow cytometer (BD Biosciences, Mountain View, Calif.). The argon ion

TABLE 1. Sequences, relative fluorescence intensities, and brightness classes of a set of Cy3-labeled oligonucleotide probes targeting the *S. cerevisiae* 26S rRNA D1 and D2 domains

Probe name	*S. cerevisiae* D1–D2 position (5′→3′)	Probe sequence (5′→3′)	Relative probe fluorescence (%)[a]	Brightness class
D-1	1–20	AAGGCAATCCCGGTTGGTTT	67	II
D-21	21–39	CGCTTCACTCGCCGTTACT	71	II
D-40	40–58	TTCAAATTTGAGCTTTTGC	32	IV
D-59	59–77	GGCACCGAAGGTACCAGAT	15	V
D-78	78–96	CTCTCCAAATTACAACTCG	96	I
D-97	97–114	AACGGCCCCAAAGTTGCC	39	IV
D-115	115–132	CAAGGAACATAGACAAGG	4	VI
D-133	133–150	CTCTATGACGTCCTGTTC	81	I
D-151	151–168	CCACACGGGATTCTCACC	44	III
D-169	169–186	AAAGAACCGCACTCCTCG	66	II
D-187	187–204	TCTTCGAAGGCACTTTAC	17	V
D-205	205–222	ATTCCCAAACAACTCGAC	84	I
D-223	223–240	CCACCCACTTAGAGCTGC	100	I
D-241	241–259	TAGCTTTAGATGGAATTTA	18	V
D-260	260–278	TCGGTCTCTCGCCAATATT	66	II
D-279	279–297	TCACTGTACTTGTTCGCTA	79	II
D-298	298–316	AGTTCTTTTCATCTTTCCA	84	I
D-317	317–335	TTTTTCACTCTCTTTTCAA	74	II
D-336	336–354	TTTCAACAATTTCACGTAC	55	III
D-355	355–373	CTGATCAAATGCCCTTCCC	69	II
D-374	374–392	AGGGCACAAAACACCATGT	48	III
D-384	384–401	AAGGAGCAGAGGGCACAA	30	IV
D-402	402–419	CGAGATTCCCCTACCCAC	61	II
D-411	411–428	AGTGAAATGCGAGATTCC	13	V
D-429	429–446	CAAAACTGATGCTGGCCC	24	IV
D-447	447–464	ATGGATTTATCCTGCCAC	7	V
D-465	465–482	GAGGCAAGCTACATTCCT	16	V
D-483	483–500	CAGGCTATAATACTTACC	6	V
D-501	501–518	CAGCTGGCAGTATTCCCA	7	V
D-519	519–536	CGTCGCAGTCCTCAGTCC	83	I
D-537	537–554	GCCAGCATCCTTGACTTA	48	III
D-555	555–572	GCGGCATATAACCATTAT	55	III

[a] Fluorescence intensities expressed as a percentage of the value obtained for the brightest probe detected, D-223.

laser was tuned to an output power of 750 mW at 514 nm. Forward-angle light scatter (FSC) was detected with a 530 (± 30)-nm band pass filter (BD Biosciences). Fluorescence (FL1) was detected with a 620 (± 60)-nm band pass filter (Gesellschaft für dünne Schichten mbH, Hugo Anders, Nabburg, Germany). Cy3 probes were measured with deionized water as sheath fluid, and polychromatic, 0.5-μm-pore-size polystyrene beads (catalog no. 18660; Polysciences, Warrington, Pa.) were used to check the stability of the optical alignment of the flow cytometer and to standardize the fluorescence intensities of hybridized cells (7, 8).

Data acquisition and processing. The parameters FSC and FL1 were recorded, and for each measurement 10,000 events were stored in list mode files. The CellQuest software (BD Biosciences) was used for subsequent analysis. Probe-conferred fluorescence was determined as the mean of the fluorescence values of single cells recorded in a gate that was defined in an FSC-versus-FL1 dot plot. For every group of 10 measurements, the fluorescence of the reference beads was determined. The standardized cell probe-conferred fluorescence was obtained by dividing the probe values by the fluorescence values of the reference beads. All values were finally expressed relative to the value for the brightest probe detected (Table 1). FISH experiments were performed three times for each probe, on three different days, with independent triplicates in each experiment. Only triplicate values with a standard deviation of below 10% were accepted. The final value for each probe is the mean from at least two independent experiments, with a standard deviation of below 15%. This procedure was adopted to account for the daily variations due to the equipment (e.g., oven temperature and flow cytometer laser power) and user-dependent errors.

Estimation of nucleotide substitution rates for the D1 and D2 domains. The nucleotide substitution rate, defined as the number of nucleotide substitutions per site and per unit time in the DNA sequence, provides a relative measure of the conservation or variability of the positions analyzed. An alignment of 145 D1 and D2 sequences, reported for yeasts and fungi of different phylogenetic groups (Table 2), was obtained with Megalign (DNAStar, Madison, Wis.) and checked visually. The nucleotide substitution rate for each position in the alignment was estimated by using the software package TREECON (23) and the substitution rate calibration method reported by Van de Peer et al. (24).

Comparison of 26S rRNA accessibilities in different yeasts. To evaluate whether the accessibility data obtained for the region analyzed in the *S. cerevisiae* 26S rRNA could be extrapolated to other yeast species, a subset of the probes tested in this study were used in FISH experiments with several yeast species that presented a full complementary target site for those probes. The probes and yeast species selected are shown in Fig. 2. The EUK 516 (5′-ACCAGACTTGC CCTCC) (2) and NonEUB (5′-ACTCCTACGGGAGGCAGC) (25) probes were used as positive and negative controls, respectively. All of the yeast strains were grown, harvested, and paraformaldehyde fixed as described above. The FISH experiments were carried out as indicated above, and 10 μl of the final hybridization mixture was spotted onto microscopic slides, air dried in the dark, and mounted with Vectashield solution (Vector, Burlingame, Calif.). The slides were examined with an Olympus BX50 microscope fitted for epifluorescence microscopy with a U-ULH 100-W mercury high-pressure bulb and a U-MA1007 filter set for the fluorochrome Cy3 (Olympus). The fluorescence intensity of the hybridization signal was checked visually. Photomicrographs were obtained with a digital camera (Olympus C3030-ZOOM) and edited with standard software (Adobe Photoshop 6.0).

RESULTS AND DISCUSSION

The results obtained for the in situ accessibility of *S. cerevisiae* 26S rRNA to Cy3-labeled oligonucleotide probes covering the full length of the D1 and D2 domains are shown in Fig. 1 and Table 1. Fluorescence intensities for each probe were quantified by flow cytometry, expressed as a percentage of the fluorescence signal of the brightest probe detected (D-223), and grouped into different accessibility classes (7). The fluorescence intensity obtained for probe D-223 was of the same order of magnitude as the signal shown by the universal eukaryote probe EUK 516, which is targeted to the 18S rRNA. About 44% of the probes tested belong to the higher-accessibility classes (I and II), and 28% were poorly binding (brightness classes V and VI). To evaluate whether the probes belonging to the most inaccessible classes (IV, V, and VI) would show better fluorescent signals under different hybridization conditions, a subset of these probes was chosen and hybridization reactions were performed at different temperatures. The use of hybridization conditions with different stringencies did not significantly improve the fluorescence intensities (data not shown), in accordance with previous studies (7). The overall results indicate that, despite its short length of approximately 600 nucleotides, the D1 and D2 domains include potentially good targets for yeast probe design. However, care should be taken when selecting target sites complementary to the most variable areas of the D1 and D2 domains (Fig. 3), where it is easier to find species-specific sequences. The data obtained show that the most conserved stretches of the studied region are more accessible (Fig. 3) (e.g., positions 200 to 350), and the most variable areas often show medium to low accessibility (e.g., the region between nucleotides 415 and 510). A similar trend has been observed in a previous accessibility study conducted for *Escherichia coli* 16S rRNA (7).

As for other probes belonging to the weaker accessibility classes (IV, V, and VI), whose low probe-conferred fluorescence signals may be due to the rRNA secondary structure

TABLE 2. GenBank accession numbers of the D1 and D2 sequences of a variety of yeast species and related fungi, used to estimate nucleotide substitution rates

Phylum, class, and species	Accession no.
Ascomycota (n = 65)	
Archiascomycetes	
Schizosaccharomyces pombe	U40085
Taphrina deformans	U94948
Euascomycetes, *Aureobasidium pullulans*	AF050239
Hemiascomycetes	
Arxula terrestris	U40103
Blastobotrys nivea	U40110
Candida bombi	U45706
Candida cariosilignicola	U70188
Candida caseinolytica	U70250
Candida castellii	U69876
Candida fennica	U45715
Candida galacta	U45820
Candida humilis	U69878
Candida insectorum	U45791
Candida nemodendra	U70246
Candida norvegica	U62299
Candida quercitrusa	U45831
Candida quercuum	U70184
Candida rugosa	U45727
Candida sake	U45728
Candida santjacobensis	U45811
Candida shehatae	U45761
Candida torresii	U45731
Candida tropicalis	U45749
Candida vini	U70247
Clavispora lusitaniae	U44817
Clavispora opuntiae	U44818
Debaryomyces castellii	U45841
Debaryomyces udenii	U45844
Dekkera anomala	U84244
Dipodascus albidus	U40081
Dipodascus ingens	U40127
Eremothecium coryli	U43390
Galactomyces geotrichum	U40118
Issatchenkia orientalis	U76347
Issatchenkia terricola	U76345
Kluyveromyces lodderae	U68551
Kluyveromyces thermotolerans	U69581
Lipomyces starkeyi	U45824
Metschnikowia reukaufii	U44825
Myxozyma mucilagina	U94945
Myxozyma udenii	U76353
Nadsonia commutata	U73598
Pichia angophorae	U75521
Pichia anomala	U74592
Pichia cactophila	U75731
Pichia euphorbiae	U73580
Pichia farinosa	U45739
Pichia inositovora	U45848
Pichia japonica	U73579
Pichia membranifaciens	U75725
Pichia onychis	U75421
Pichia opuntiae	U76203
Pichia quercuum	U75416
Pichia toletana	U75720
Saccharomyces cerevisiae	U44806
Saccharomycopsis capsularis	U40082
Saturnispora dispora	U94937
Stephanoascus smithiae	U76531
Torulaspora delbrueckii	U72156
Williopsis mucosa	U75961
Williopsis salicorniae	U75968
Yarrowia lipolytica	U40080
Zygoascus hellenicus	U40125
Zygosaccharomyces mellis	U72164
Zygozyma smithiae	U84242
Basidiomycota (n = 80)	
Hymenomycetes	
Agaricus arvensis	U11910
Apiotrichum porosum	AF189833
Auricularia auricula-judae	L20278
Boletus rubinellus	L20279
Bullera crocea	AF075508
Bullera oryzae	AF075511
Bulleromyces albus	AF075500
Calocera cornea	AF291302
Cryptococcus albidus	AF075474
Cryptococcus curvatus	AF189834
Cryptococcus diffluens	AF075502
Cryptococcus gastricus	AF137600
Cryptococcus heveanensis	AF075467
Cryptococcus humicola	AF189836
Cryptococcus laurentii	AF075469
Cryptococcus magnus	AF181851
Cryptococcus skinneri	AF189835
Cryptococcus terreus	AF075479
Cystofilobasidium capitatum	AF075465
Fellomyces borneensis	AF189877
Fellomyces fuzhouensis	AF075506
Filobasidiella neoformans	AF075526
Filobasidium capsuligenum	AF075501
Ganoderma australe	X78780
Mrakia frigida	AF075463
Tremella aurantia	AF189842
Tremella tropica	AF042251
Trichosporon aquatile	AF075520
Trichosporon montevideense	AF105397
Trichosporon mucoides	AF075515
Udeniomyces pyricola	AF075507
Urediniomycetes	
Aurantiosporium subnitens	AF009846
Bensingtonia phyllada	AF189894
Eocronartium muscicola	L20280
Erythrobasidium hasegawianum	AF189899
Helicogloea variabilis	L20282
Kondoa aerea	AF189901
Kurtzmanomyces tardus	AF177410
Leucosporidium felli	AF189907
Leucosporidium scottii	AF070419
Melampsora lini	L20283
Occultifur externus	AF189909
Pachnocybe ferruginea	L20284
Rhodosporidium kratochvilovae	AF071436
Rhodotorula aurantiaca	AF189921
Rhodotorula bogoriensis	AF189923
Rhodotorula ferulica	AF189927
Rhodotorula fujisanensis	AF189928
Rhodotorula glutinis	AF070430
Rhodotorula hordea	AF189933
Rhodotorula minuta	AF189945
Rhodotorula vanillica	AF189970
Sporidiobolus ruineniae	AF070438
Sporidiobolus salmonicolor	AF070439
Sporobolomyces coprosmae	AF189980
Sporobolomyces coprosmicola	AF189981
Sporobolomyces dracophylli	AF189982
Sporobolomyces falcatus	AF075490
Sporobolomyces gracilis	AF189985
Sporobolomyces roseus	AF070441
Sporobolomyces ruber	AF189992
Sporobolomyces sasicola	AF177412
Sporobolomyces singularis	AF189996
Sporobolomyces tsugae	AF189998
Sterigmatomyces elviae	AF177415
Ustilaginomycetes	
Doassinga callitrichis	AF007525
Entorrhiza aschersonia	AF009851
Entyloma calendulae	AJ235296
Exobasidium rhododendri	AF009856
Malassezia furfur	AJ249955
Melanotaenium endogenum	AJ235294
Pseudozyma fusiformata	AJ235304
Rhodotorula bacarum	AF190002
Rhodotorula phylloplana	AF190004
Thecaphora amaranthi	AF009873
Tilletia caries	AJ235308
Tilletiaria anomala	AJ235284
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Ustacystis waldsteiniae	AF009880
Ustilago maydis	AJ235275

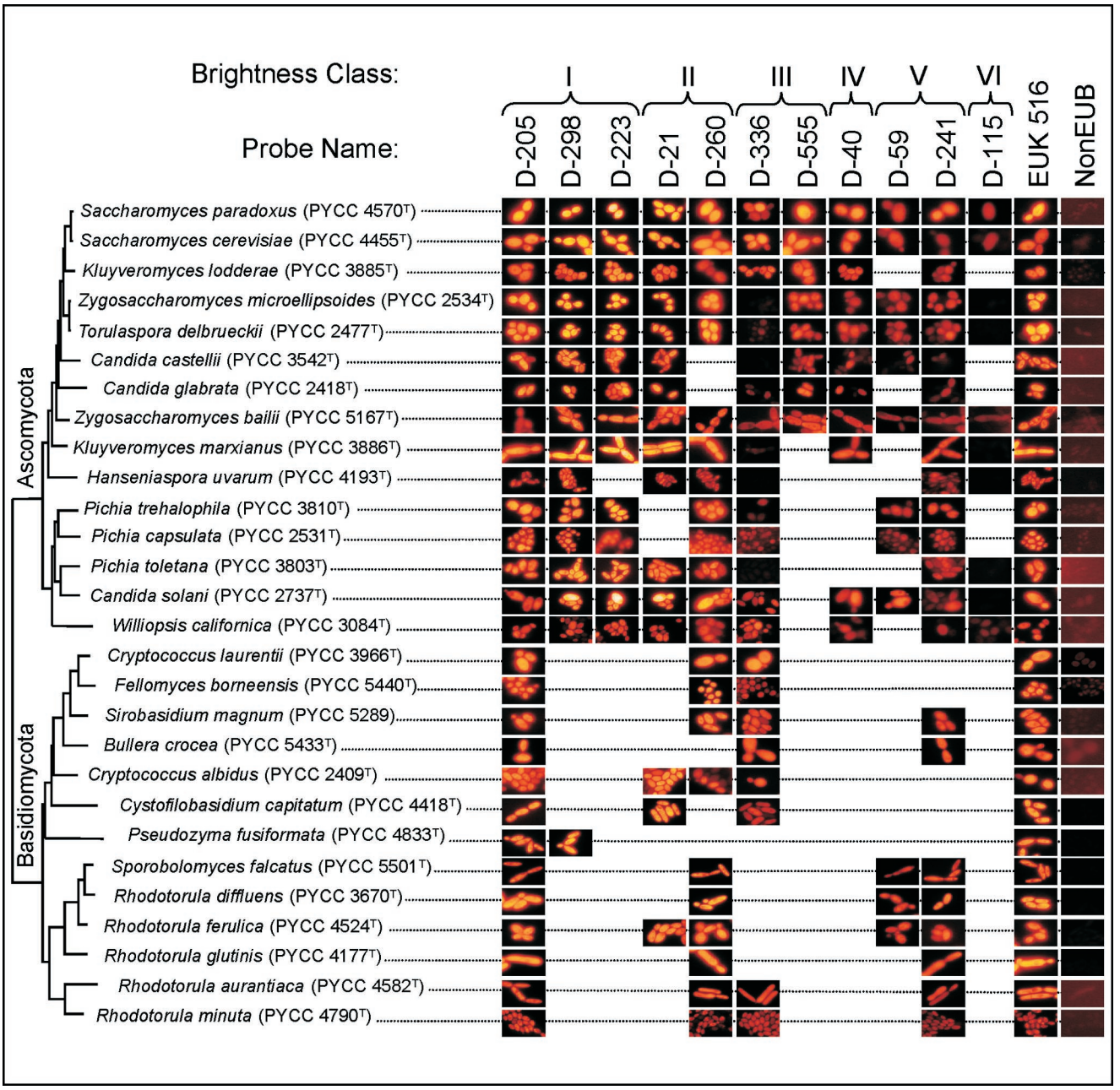

FIG. 2. Comparison of in situ hybridization signals for *S. cerevisiae* and other yeast species. The probes selected fall into different accessibility classes in the *S. cerevisiae* 26S rRNA D1 and D2 domains and have an identical target site in that region for all of the yeasts indicated.

and/or protein-rRNA interactions, the weaker signal of probe D-59 can additionally be attributed to a significant degree of self-complementarity in its sequence, an aspect to be taken into consideration when designing species-specific probes. Another possible explanation for the less intense fluorescence signals observed with some FISH probes is the quenching due to the presence of guanine residues adjacent to the 3′ ends of the rRNA targets (4, 22). We observed no significant correlation between the probe-conferred fluorescence intensities and this nucleobase position in the vicinity of the 3′ ends of the respective rRNA target sites (data not shown). This observation agrees with those of Torimura and colleagues (22), who observed the quenching phenomenon for fluorescein isothiocyanate-labeled oligonucleotides but not for Cy3-labeled ones.

Interestingly, a comparative analysis of the in situ accessibility of the first 350 nucleotides in *E. coli* 23S rRNA to Cy3-labeled oligonucleotide probes (8) and the data obtained in this work for *S. cerevisiae* show some striking similarities (Fig. 4). Although the probes used have different target sequences in the two microorganisms, the accessibilities follow the same general trend. On the other hand, the probes belonging to the higher accessibility classes (I and II) in *S. cerevisiae* also yielded

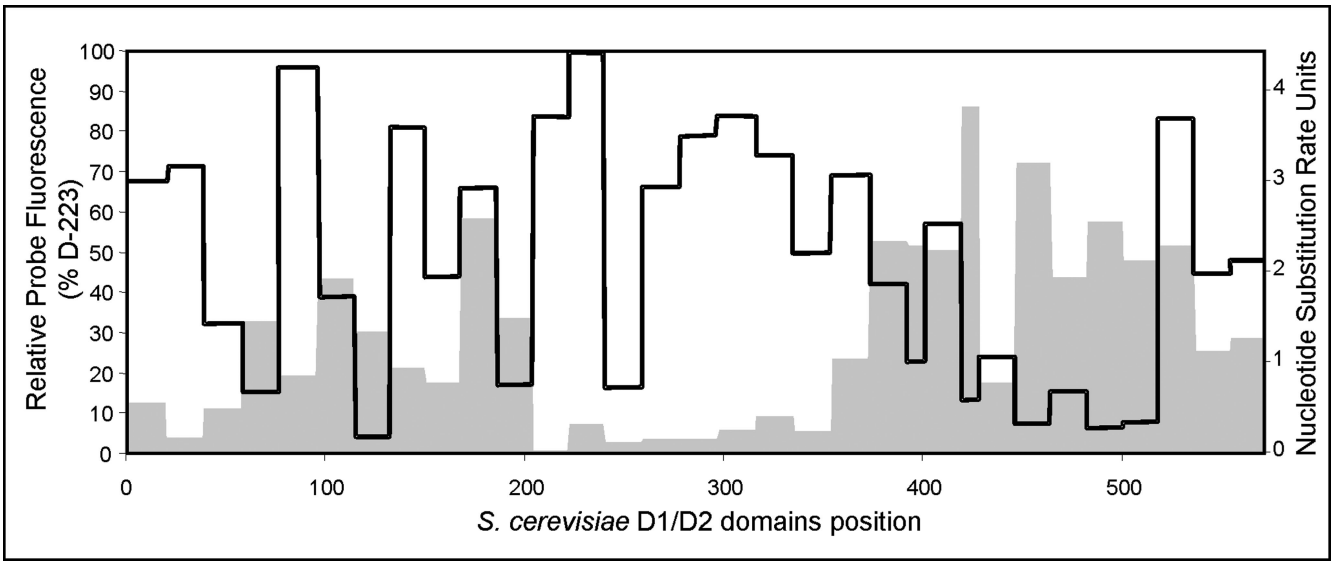

FIG. 3. Comparison of the relative in situ accessibilities (black line) of the *S. cerevisiae* 26S rRNA D1 and D2 domains and the average nucleotide substitution rates (gray) in yeasts.

strong hybridization signals with species belonging to different phylogenetic groups, including the distantly related basidiomycetous yeasts (Fig. 2). This suggests that the D1-D2 accessibility map presented here for *S. cerevisiae* provides useful guidance for the design of species-specific probes for other yeasts, maybe even for other fungi or eukaryotic microorganisms. However, the design of probes for more distantly related organisms would probably require a different model.

With this study we hope to contribute to a more rational design of fluorescently labeled probes for yeast identification that will stimulate the use of FISH-based methods in a wide range of applications, including studies on the ecology of yeasts.

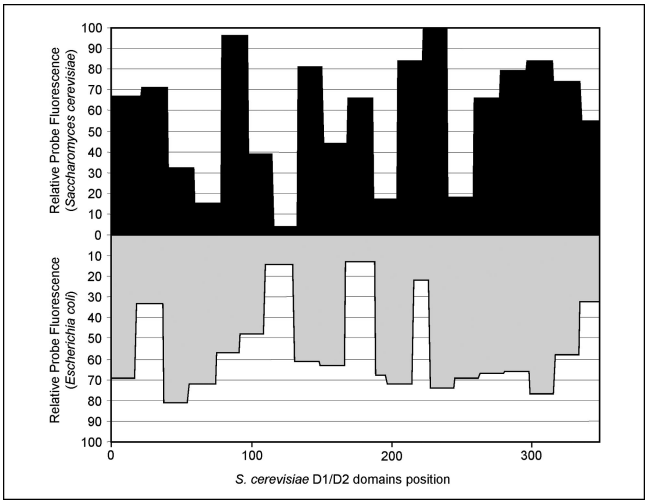

FIG. 4. Comparison of the accessibilities of homologous regions in *S. cerevisiae* 26S rRNA and *E. coli* 23S rRNA to Cy3-labeled probes.

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