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Quantitative real time PCR assays for the enumeration of *Saccharomyces cerevisiae* and the *Saccharomyces sensu stricto* complex in human feces

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Abstract

There have been an increasing number of reports of yeast systemic infection involving *Saccharomyces cerevisiae* strains. The development of a rapid and reliable diagnostic tool is therefore warranted in order to explore the distribution of *S. cerevisiae* as an opportunistic pathogen in humans. In this study, we designed and validated five primer sets targeting the 26S rRNA gene of *S. cerevisiae* and the *S. sensu stricto* complex using 26 yeast strains. Among them, two sets of primers specifically amplified the 26S rRNA gene and the ITS region of *S. cerevisiae* strains, and three sets were specific for amplifying the same genes in the *S. sensu stricto* complex. After determining the optimal conditions of two primer pairs for quantitative real time PCR, human fecal samples were analyzed to examine the distribution of *S. cerevisiae* and the *S. sensu stricto* complex. It was possible to detect a single cell of *S. cerevisiae* in environmental sample. Qualitative PCR revealed that out of eleven fecal samples tested, one sample contained *S. cerevisiae* and four samples contained the *S. sensu stricto* complex. Quantitative real time PCR revealed that the target gene copy numbers of *S. cerevisiae* and the *S. sensu stricto* complex were 0.84 and 2.44 respectively, in 1 ng of DNA from the bulk fecal community.

Keywords: *Saccharomyces cerevisiae*; *Saccharomyces sensu stricto*; Real time PCR; Human feces

1. Introduction

The yeast *Saccharomyces cerevisiae* has been extensively studied and used in the baking and brewing industry. It has superior characteristics including easy cultivation, short generation time, and a high capacity for the production of beneficial compounds such as ethanol and citric acid (Landry et al., 2006). Recent advances in genetics and biochemistry have lead to the study of *S. cerevisiae* as a model eukaryotic organism for molecular and cellular research, in the same way that *Escherichia coli* is used as a model prokaryote. *S. cerevisiae* is therefore used as a model organism to describe basic biological processes in many other higher eukaryotes, including humans (Wang and Chen, 2006). In recent years, it has become apparent that *S. cerevisiae* exists in various habitats and thus harbors important genetic variation (Ciani et al., 2004; Zeyl, 2004).

To date, many strains of the genus *Saccharomyces* have been isolated from a variety of sources including vineyards, food, soil, and clinical samples (Sniegowski et al., 2002; Fay and Benavides, 2005; de Llanos et al., 2006a). With the development of molecular and biochemical classification techniques, *Saccharomyces* strains have undergone extensive taxonomic reclassification and their classification has thus been altered (Tornai-Lehoczki et al., 1996). Several members of this genus that are phylogenetically very close to *S. cerevisiae* (Van Der Walt, 1970), were grouped together as the *Saccharomyces sensu stricto* complex; a species associated with alcoholic fermentation (Rainieri et al., 2003). This complex is composed of seven

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species: *S. cerevisiae*, *S. paradoxus*, *S. mikatae*, *S. bayanus*, *S. cariocanus*, *S. pastorianus*, and *S. kudriavzevii* (Naumov, 1996; Naumov et al., 2000; Kurtzman and Robnett, 2003). Recent speciation studies make this as an interesting model group to investigate the importance of molecular mechanisms of eukaryotic speciation (Cliften et al., 2001, 2003; Greig et al., 2002; Deineri et al., 2003).

The *S. sensu stricto* complex is not readily distinguished from other members of the *Saccharomyces* group using classical microbiological methods because of its frequent exchange of genetic material, high genetic variability and the limited ribosomal RNA divergence (Mortimer et al., 1994; Wolfe and Shields, 1997; Groth et al., 2000; Monch and Stahl, 2000; Puig et al., 2000). Thus, various techniques have been developed for identifying yeast species including mitochondrial DNA restriction profiling (Aigle et al., 1984), DNA–DNA reassociation (Martini and Martini, 1987), pulsed-field electrophoresis karyotyping (Blondin and Vezinhet, 1988), mitochondrial DNA restriction endonuclease profiling (Guillamon et al., 1994), randomly amplified polymorphic DNAs (RAPDs) (Molnar et al., 1995), PCR amplification using primers based on intron splicing sites (De Barros Lopes et al., 1996), the analysis of the 18S rRNA gene (James et al., 1997), the analysis of the internal transcribed spacers (ITS) region (Montrocher et al., 1998), the analysis of the D1/D2 region of the 26S rRNA gene (Kurtzman and Robnett, 1998), amplified fragment length polymorphism (AFLP) based analysis (Azumi and Goto-Yamamoto, 2001), and microsatellite-based techniques (Hennequin et al., 2001). More recently, the development of DNA microarrays has allowed the study of genetic diversity and variation at a genomic level in

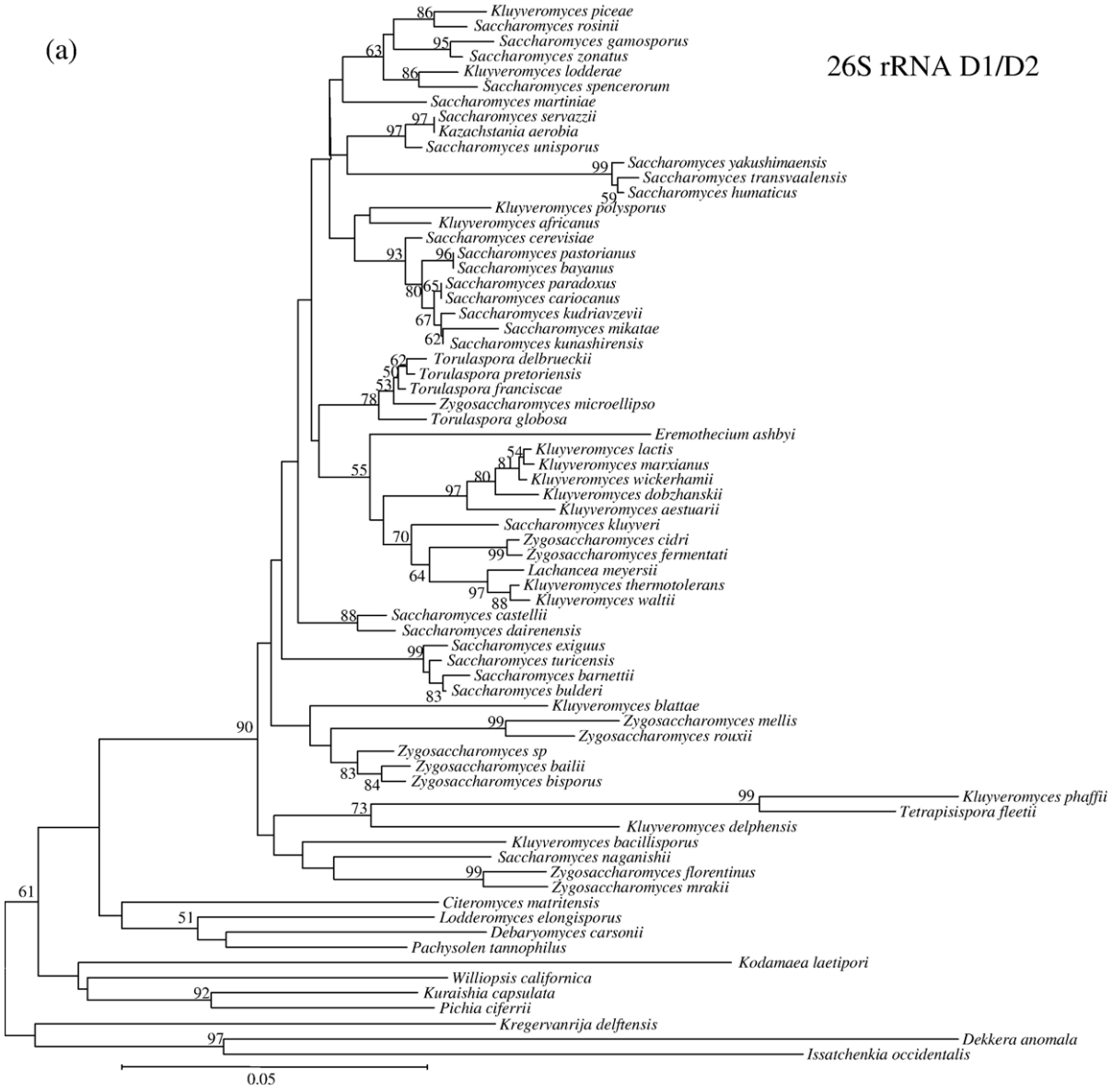

Fig. 1. Dendrogram showing the phylogenetic relationship of *Saccharomyces* and non-*Saccharomyces* species based on (a) 26S rRNA D1/D2 region and (b) ITS1-5.8S-ITS2 region sequences. The tree was generated by the neighbor-joining method. The numbers at the nodes indicate bootstrap values (1000 replications). Bar, 0.05 accumulated changes per nucleotide.

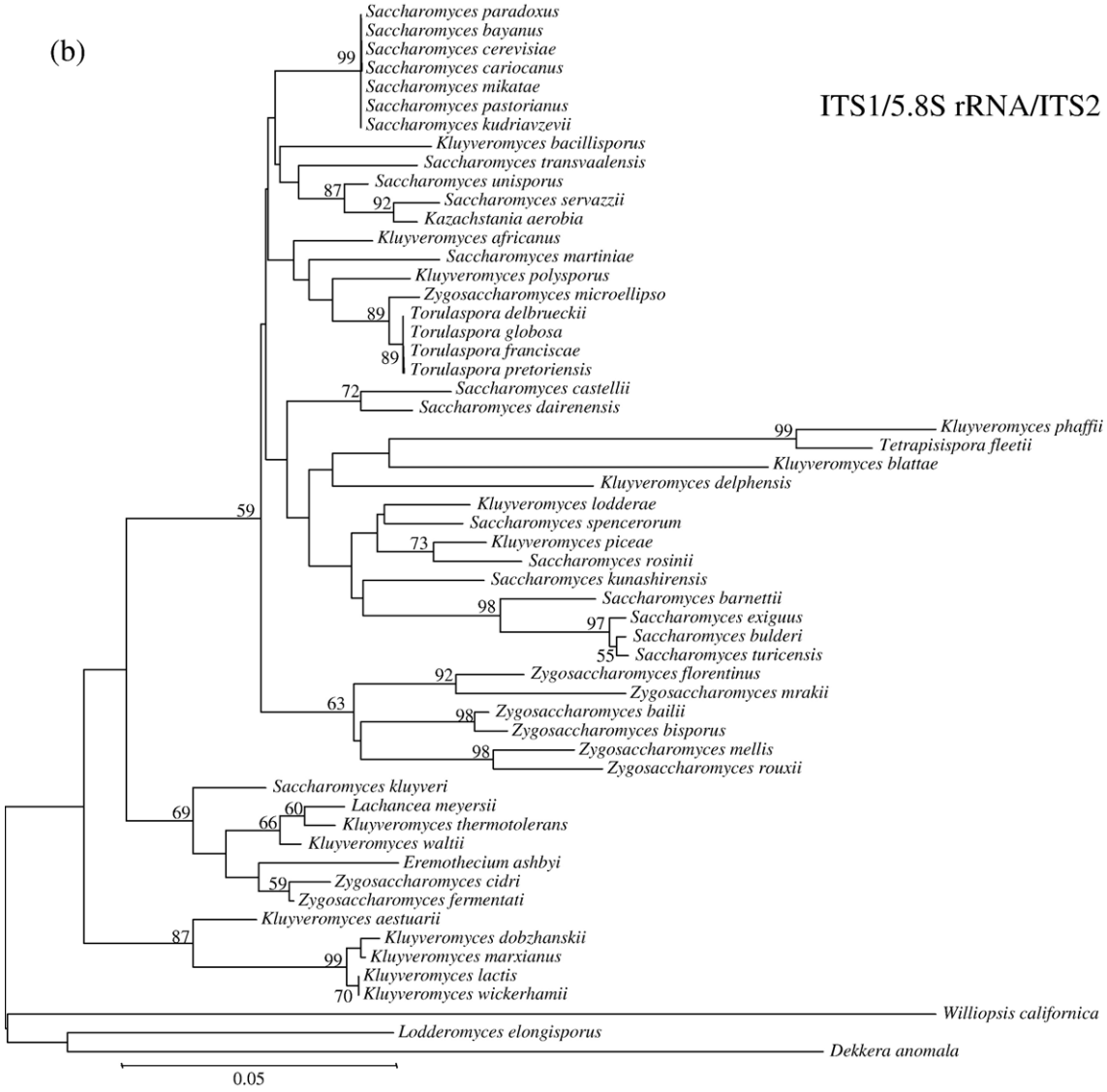

Fig. 1 (*continued*).

humans (Fortna et al., 2004). Until now, there has been no quantitative molecular tool for enumerating the species *S. cerevisiae* and the *S. sensu stricto* complex in their natural environment, despite their importance to the alcohol industry and to human health.

The quantitative real time PCR (qPCR) method has allowed researchers to detect and quantify certain microorganisms of interest, and has thus increased our understanding of the microbial dynamics in natural environments. This method has been applied to quantify yeast in fermented foods (Bleve et al., 2003; Castillo-Davis and Hartl, 2003; Delaherche et al., 2004; Martorell et al., 2005) and clinical samples (Trama et al., 2005), and has also been applied to enumerate *S. cerevisiae* in wines (Martorell et al., 2005; Hierro et al., 2006). However, they did not test specificity of designed primers for *S. sensu stricto* complex and compared only several species which were associated with wine fermentation. So, their method cannot be applied to other environment samples. Thus, there are no reports of a quantitative approach to enumerate the species *S. cerevisiae* and the *S. sensu stricto* complex either simultaneously or individually.

In the present study, we have developed two new species-specific PCR primer pairs to detect *S. cerevisiae*, and three complex-specific primer pairs to detect the *S. sensu stricto* complex based on the D1/D2 and ITS regions of the 26S rRNA gene. Specificity of the primers was examined using 26 yeast strains, and the quantification capability of the primers using a qPCR approach was demonstrated with several human fecal samples.

2. Materials and methods

2.1. Primer design

In order to design the primers, seventy sequences of the ITS1-5.8S-ITS2 and D1/D2 regions of the 26S rRNA gene of *Saccharomyces* and non-*Saccharomyces* species were downloaded from the NCBI nucleotide database and aligned using

the multiple sequence alignment program CLUSTAL_X (1.8) (Thompson et al., 1997). The phylogenetic relationship of these seventy strains is shown in Fig. 1. *S. cerevisiae*-specific and *S. sensu stricto* complex-specific regions were found from aligned sequences. Ten primers were designed based on the ITS1-5.8S-ITS2 and D1/D2 regions of the 26S rRNA gene sequences of *S. cerevisiae* and *S. sensu stricto* complex species including *S. cerevisiae*, *S. paradoxus*, *S. mikatae*, *S. bayanus*, *S. cariocanus*, *S. pastorianus*, and *S. kudriavzevii* (Table 1).

The primers SCDF, SCDR, and SCIF correspond to *S. cerevisiae* whereas the primers SDF, CDR, SI1F, SI4F, SI4R, SI5R, and SI7R correspond to the *S. sensu stricto* complex. The specificity of the primers was verified by homology searches for nucleotide sequences in the GenBank sequence database using the BLAST search program (http://www.ncbi.nlm.nih.gov/). Properties of the designed primers including the melting temperature, potential for hairpin formation, and mismatch were calculated using the oligonucleotide properties calculator program (http://www.basic.northwestern.edu/biotools/oligocalc.html).

2.2. Yeast strains and human fecal samples

Twenty six yeast strains were obtained from the Korean Collection for Type Cultures (KCTC) and the Centraalbureau voor Schimmelcultures (CBS) (Table 2). All strains were maintained and grown under conditions suggested by the collection from which they were sourced. Strains were grown in YM (Difco) and YPG (yeast extract 1%, bactopeptone 1% and glucose 2%) agar plates at 30 °C for 48 h, harvested at the exponential growth phase and frozen at −80 °C for the extraction of genomic DNA. Fecal samples were obtained from eleven healthy adults. All samples were collected in sterile plastic boxes and stored at −80 °C for further analysis.

2.3. DNA extraction

The genomic DNA from human fecal samples was extracted from approximately 1 g of sample that had been ground with a mortar and pestle in the presence of liquid nitrogen (Karakousis et al., 2006). The genomic DNA from yeast strains was extracted using the bead-beating method, as described previously (Yeates et al., 1998). All DNA samples were treated with RNase A (Sigma, St. Louis, MO) and then purified using the ethanol precipitation method (Karakousis et al., 2006). Extracted DNA was further purified using an UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA) with several modifications. The bead-beating step and the MD2 step were excluded, and the DNA solution was added to solution MD1 instead of the MicroBead solution. The DNA concentrations were determined in triplicate using a spectrophotometer (Nanodrop Technologies, Rockland, DE). We obtained approximately 10 mg of bulk DNAs from 1 g of fecal sample and after purification using an UltraClean Microbial DNA Isolation Kit, we acquired approximately 1 mg of DNA from each sample. The variation of DNA amount from each sample was very small indicating that there is little environmental DNA extraction bias.

2.4. PCR amplification for validation of primer specificity

To test the specificity of the designed primer pairs, PCR amplification was carried out in a final volume of 20 μl, comprising of 1 μl (10 ng/μl) of template, 10 mM Tris HCl (pH 9.0), 40 mM KCl, 250 μM deoxynucleoside triphosphates (dNTPs), 1 U of *Taq* polymerase, 1.5 mM $MgCl_2$, and 10 pmole each primer. Primers used in this study are summarized in Table 1. Amplification was carried out in a Peltier Thermal Cycler (DNA Engine DYAD™, MJ Research). The reactions were run for 30 cycles; denaturation at 95 °C for 1 m, annealing at 60 °C for 1 m, and extension at 72 °C for 1 m. The initial denaturation was performed at 95 °C for 5 m, and a final extension was performed at 72 °C for 7 m. The amplified PCR products were confirmed by analyzing 2 μl of product by agarose gel (2% w/v) electrophoresis and staining with ethidium bromide.

2.5. Optimization of quantitative real time PCR condition

To determine the detection limit of qPCR (DNA Engine OPTICON™2, MJ Research) for the designed specific primers,

Table 1
List of PCR primer pairs designed and used for this study

	Gene region	Primer name	Sequence	Amplicon length (bp)	Tm (°C)	Reference
Saccharomyces cerevisiae-specific primer	D1/D2	SCDF	5-AGG AGT GCG GTT CTT TG-3	310	47.5	This study
		SCDR	5-TAC TTA CCG AGG CAA GCT ACA-3		51.8	This study
	ITS	SCIF	5-GTG CTT TTG TTA TAG GAC AAT T-3	580	47.4	This study
		SI5R	5-AGA GAA ACC TCT CTT TGG A-3		44.7	This study
Saccharomyces sensu stricto complex-specific primer	D1/D2	SDF	5-TTG TAA TTT GGA GAG GGC A-3	510	50.2	This study
		CDR	5-CAG CAT CCT TGA CTT ACG-3		45.5	This study
	ITS	SI1F	5-TGA GAG CTT TTA CTG GGC AAG-3	580	53.7	This study
		SI4R	5-CAA TGA AAA GGC CAG CAA T-3		52.4	This study
	ITS	SI4F	5-ATT GCT GGC CTT TTC ATT G-3	200	52.4	This study
		SI7R	5-CGC CTA GAC GCT CTC TTC TTA T-3		53.7	This study
	D1/D2	NL-1	5-GCA TAT CAA TAA GCG GAG GAA AAG-3	600	57.2	Kurtzman
		NL-4	5-GGT CCG TGT TTC AAG ACG G-3		54.9	and Robnett (1998)
	ITS	ITS1	5-TCC GTA GGT GAA CCT GCG G-3	Irregular	58.0	White et al. (1990)
		ITS4	5-TCC TCC GCT TAT TGA TAT GC-3		51.7	White et al. (1990)

Table 2
List of strains used in this study and their detection using each primer pair

Number	Designation	Species	Primer pairs				
			SCDF/SCDR	SCIF/SI5R	SDF/CDR	SI1F/SI4R	SI4F/SI7R
1	KCTC 7915	*Saccharomyces cerevisiae*	+	+	+	+	+
2	KCTC 7966	*Zygosaccharomyces rouxii*	−	−	−	−	−
3	KCTC 7107	*Saccharomyces cerevisiae*	+	+	+	+	+
4	KCTC 7169	*Saccharomyces exiguus*	−	−	−	−	−
5	KCTC 7270	*Candida albicans*	−	−	−	−	−
6	KCTC 7296	*Saccharomyces cerevisiae*	+	+	+	+	+
7	KCTC 7445	*Saccharomyces cerevisiae*	+	+	+	+	+
8	KCTC 7805	*Saccharomyces kluyveri*	−	−	−	−	−
9	KCTC 7887	*Lodderomyces elongisporus*	−	−	−	−	−
10	KCTC 17009	*Saccharomyces castellii*	−	−	−	−	−
11	KCTC 17168	*Torulaspora delbrueckii*	−	−	−	−	−
12	KCTC 17171	*Schizosaccharomyces pombe* var. *pombe*	−	−	−	−	−
13	KCTC 17212	*Kluyveromyces marxianus*	−	−	−	−	−
14	KCTC 17223	*Saccharomyces bayanus*	−	−	+	+	+
15	KCTC 17224	*Saccharomyces paradoxus*	−	−	+	+	+
16	KCTC 17225	*Saccharomyces pastorianus*	−	−	+	+	+
17	KCTC 17226	*Saccharomyces servazzii*	−	−	−	−	−
18	KCTC 17256	*Williopsis californica*	−	−	−	−	−
19	CBS 8839	*Saccharomyces mikatae*	−	−	+	+	+
20	CBS 8840	*Saccharomyces kudriavzevii*	−	−	+	+	+
21	CBS 8841	*Saccharomyces cariocanus*	−	−	+	+	+
22	CBS 2517	*Kluyveromyces africanus*	−	−	−	−	−
23	CBS 7662	*Saccharomyces kunashirensis*	−	−	−	−	−
24	KCTC 7297	*Kluyveromyces polysporus*	−	−	−	−	−
25	KCTC 7807	*Saccharomyces transvaalensis*	−	−	−	−	−
26	KCTC 7691	*Saccharomyces unisporus*	−	−	−	−	−

+, PCR-amplified product with each primer pair detected; −, PCR-amplified product with each primer pair not detected.

purified genomic DNA from *S. cerevisiae* (KCTC 7445) was serially diluted 10-fold from 12.1 ng/μl to 12.1×10^{-6} ng/μl. The initial DNA concentration of 12.1 ng/μl was quantified using a spectrophotometer (Nanodrop Technologies, Rockland, DE). For the qPCR analysis, the DyNAmo™ HS SYBR® Green qPCR Kit (FINNZYMES, Seoul, Korea) was used. qPCR was performed in a total volume of 20 μl containing 1 μl of each template DNA, 10 pmole of each primer, 7 μl of nuclease-free water and 10 μl of 2× master mix (containing the hot start version of a modified *Tbr* DNA polymerase, SYBR Green I, optimized PCR buffer, 5 mM $MgCl_2$ and dNTP mix including dUTP). qPCR conditions were used with an initial denaturation step of 15 m at 95 °C followed by 45 cycles of 94 °C for 20 s, 58 °C for 30 s and 72 °C for 45 s with a final extension step of 5 m at 72 °C. A melting curve analysis followed PCR amplification at 95 °C to 65°C at a rate of 0.2°C/1 s with continuous acquisition of fluorescence decline. Standard curves were generated with the primer pairs SCDF/SCDR or SI4F/SI7R using the serially diluted *S. cerevisiae* (KCTC 7445) genomic DNAs by plotting threshold cycle (Ct) versus concentration of genomic DNA of *S. cerevisiae*.

2.6. qPCR of fecal samples and calibration

The qPCR conditions including annealing temperature and concentration of primers and Mg^{2+} were optimized by applying them in various combinations with purified human fecal DNA. After the optimization of suitable qPCR conditions for the quantitative analysis of *S. cerevisiae* and the *S. sensu stricto* complex in human feces, the purified bulk DNAs from eleven fecal samples were tested by qPCR using the specific primer pairs SCDF/SCDR and SI4F/SI7R. The samples identified were serially diluted 10-fold for qPCR analysis. All qPCR reactions were performed using the optimized conditions described previously. The quantity of *S. cerevisiae* and the *S. sensu stricto* complex was calculated from standard curve using SCDF/SCDR and SI4F/SI7R primer pairs from which the concentration of *S. cerevisiae* and the *S. sensu stricto* complex in the fecal samples was deduced. All experiments were performed in triplicate. In order to calibrate the gene copy number in DNA, the equation from a previously described method was used (Ritalahti et al., 2006). It was assumed that genome size of *S. cerevisiae* was 12.1 Mb and the 26S rRNA gene copy number was two, which were determined from the whole genome sequence of *S. cerevisiae* (GenBank genome project ID no. 9518).

3. Results

3.1. Specificity of designed primers

The specificity of the primers (listed in Table 1) was validated by PCR using the genomic DNA from 26 yeast strains (Table 2). One species-specific primer pairs, SCIF/SI5R, exactly

amplified the target regions of all *S. cerevisiae* strains, and two complex-specific primer pairs, SDF/CDR and SI1F/SI4R, also exactly amplified target regions of the *S. sensu stricto* complex members (Fig. 2). No PCR amplification products were detected from other related species or groups with the designed primer pairs. To distinguish between *S. cerevisiae* strains and the *S. sensu stricto* complex simultaneously, multiplex PCR analyses were carried out using the four primer combinations: SCDF, SCDR, SI4F, and SI7R. The multiplex PCR results showed that three distinct bands were obtained from *S. cerevisiae* strains and a single band was obtained from the *S. sensu stricto* complex (Fig. 2d). These PCR products were amplified by a combination of primer pairs SCDF/SCDR (D1/D2 region), SI4F/SI7R (ITS1-5.8S-ITS2 region), and SI4F/SCDR (ITS1-5.8S-ITS2-D1/D2 region).

Although combination of primer pairs SCDF/SCDR produced primer dimers, they were easily distinguished from the PCR amplicons of target strains. When the PCR annealing temperature was lower than 58 °C, the *S. cerevisiae* species-specific primer pairs SCIF and SI5R generated non-specific primer binding during amplification of the ITS region of *S. paradoxus* and *S. cariocanus*. This non-specific amplification seems to occur due to the dissimilarity of one base pair between the sequences of the SCIF primer and target ITS genes. This problem was solved by increasing the annealing temperature to 58–60 °C.

3.2. Sensitivity of designed primers in the qPCR assay

PCR sensitivity is another critical parameter which impacts the effectiveness of the qPCR-based approach for detecting genes in environmental samples. The minimum level of qPCR detection of the purified yeast genomic DNA with the primer pairs SCDF/SCDR and SI4F/SI7R was 12.1 fg with Ct values of 32.25 and 33.97, respectively. Primer dimers were detected if the DNA concentration was lower than 12.1 fg; however, this was not considered significant as the primer dimers were always observed at more than 35 cycles. The melting curves obtained from *S. cerevisiae* and *S. sensu stricto*-specific PCR-amplified products are shown in Fig. 3; the melting temperatures (Tm) were 80.2 °C and 77.4 °C, respectively. qPCR amplification represented by a melting curve with the expected Tm, was only observed for each specific target group of yeast, thus providing evidence of the specificity of the developed system. Non-specific amplification products and primer dimers were formed at lower Tms (data not shown). Ct values and standard errors of standard curves were also obtained from the serially diluted *S. cerevisiae* (KCTC 7445) DNA (Table 3). The small standard errors obtained using these replicates proved that the qPCR assay was reproducible and highly robust. Given that genomic DNA from a single cell of *S. cerevisiae* is about 18.0 to 19.9 fg (Sasaki, 1992), the detection

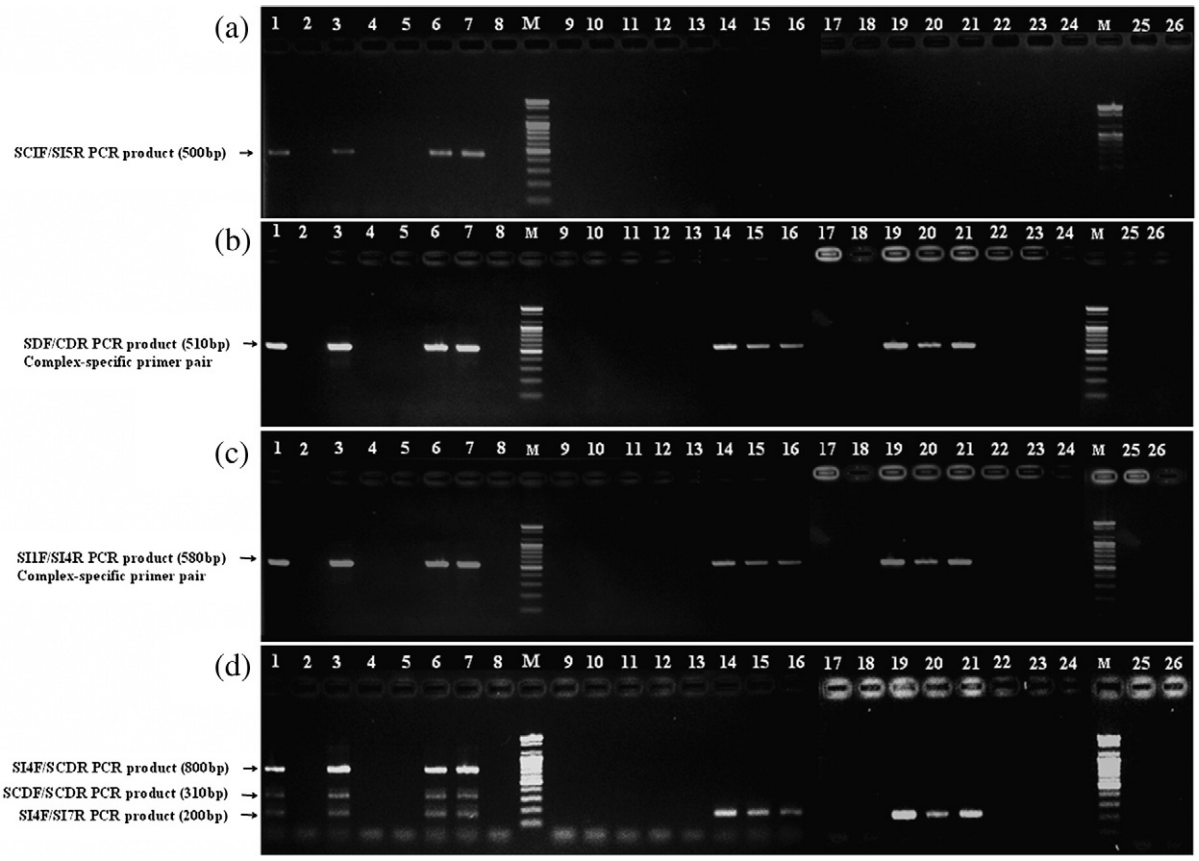

Fig. 2. Gel electrophoresis of PCR products amplified using *S. cerevisiae*-specific primers and *S. sensu stricto* complex-specific primers designed in the present study. Lane: M, 100 bp marker; 1–26, number in shown inTable 2. (a) SCIF/SI5R primer pair specific for *S. cerevisiae*. (b) SDF/CDR primer pair specific for the *S. sensu stricto* complex. (c) SI1F/SI4R primer pair specific for the *S. sensu stricto* complex. (d) SCDF, SCDR, SI4F and SI7R primers for multiplex PCR.

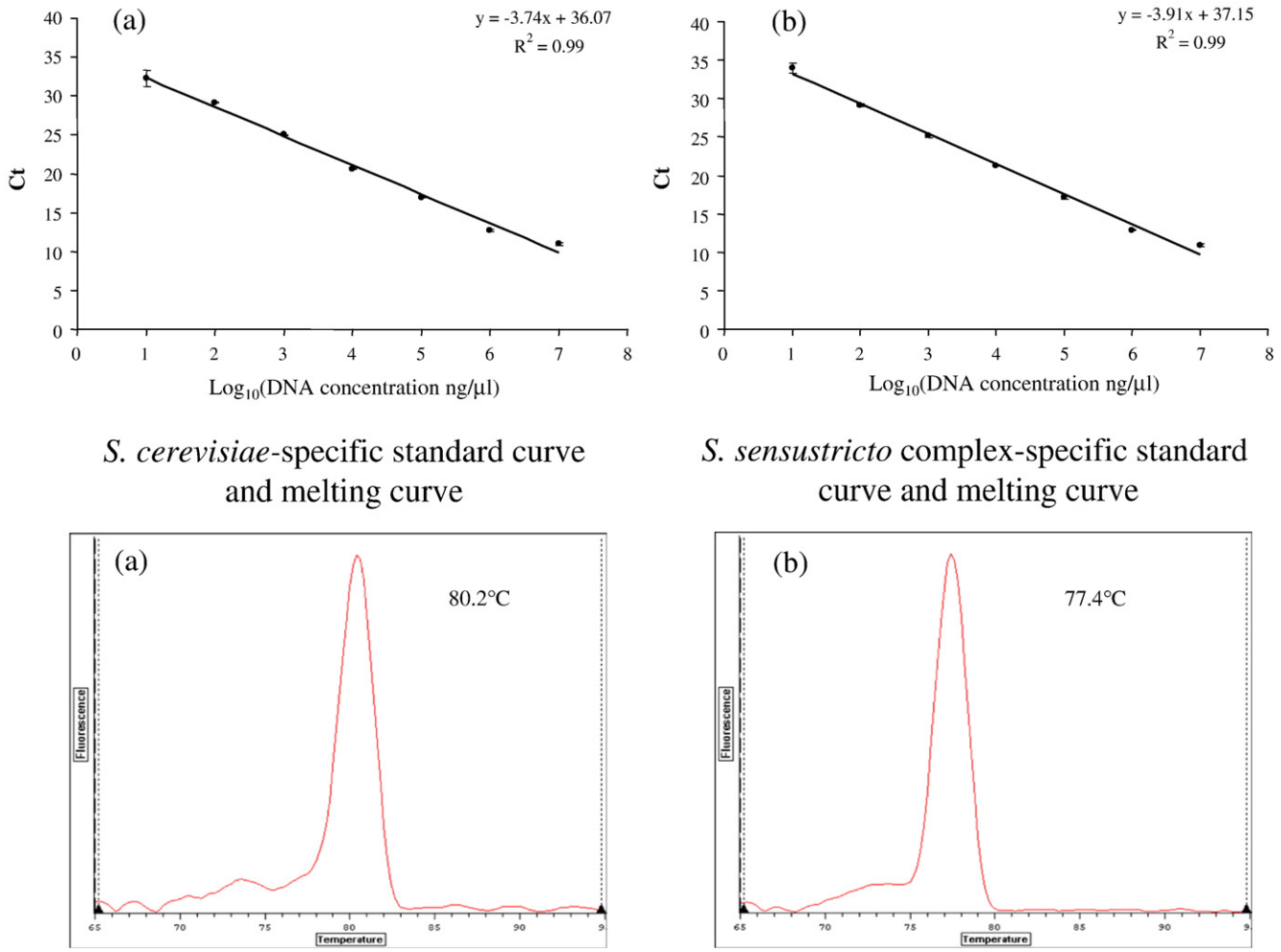

Fig. 3. Standard curve obtained from a 10-fold serial dilution series of purified DNA of from the *S. cerevisiae* strain (KCTC 7445) and melting curve analysis of the amplified PCR product using (a) the *S. cerevisiae*-specific primers SCDF and SCDR; (b) the *S. sensu stricto* complex-specific primers SI4F and SI7R. Ct values and standard error are calculated from triplicate experiments. Each error bar indicates the standard error.

sensitivity (12.1 fg) obtained with this qPCR assay reached that of a single cell of *S. cerevisiae*.

3.3. qPCR enumeration of S. cerevisiae and the S. sensu stricto complex in human feces

To quantify *S. cerevisiae* in human feces, the designed primers SCDF/SCDR and SI4F/SI7R were used for qPCR since these primer sets produced appropriate product size (100 to 300) for qPCR using SYBR green as suggested by qPCR kit manufacturer's instruction. Eleven fecal samples were analyzed using the SCDF/SCDR primer pair and of these, PCR product was only obtained from sample F11 and chromatogram of amplified PCR product showed clear single sequence. Using the SI4F/SI7R primer pair however, PCR product was detected from samples F2, F5, F6 and F11. Given the fact that the *S. sensu stricto* complex-specific primer (SI4F/SI7R) was designed to detected more yeast species than the *S. cerevisiae*-specific primer (SCDF/SCDR), an increased detection frequency of PCR product with the SI4F/SI7R primer is reasonable. The gene copy number of *S. cerevisiae* and the *S. sensu stricto* complex in the human fecal samples was estimated from the standard curve (Fig. 4). The four positive samples identified were analyzed with each specific primer pair and quantitative results were obtained (Table 4). The gene copy number ranged from 0.47 to 2.44 in 1 ng of human fecal DNA. qPCR using each specific primer pair

Table 3
Determination of Ct values of *S. cerevisiae* and the *S. sensu stricto* complex in each standard curve by real time PCR

DNA concentration (ng/µl)	Gene copy number	Ct (mean±SE)	
		S. cerevisiae	*S. sensu stricto* complex
12.1	1.82×10^{6}	10.96±0.20	10.93±0.17
12.1×10^{-1}	1.82×10^{5}	12.76±0.11	12.89±0.03
12.1×10^{-2}	1.82×10^{4}	16.96±0.07	17.18±0.20
12.1×10^{-3}	1.82×10^{3}	20.64±0.10	21.24±0.06
12.1×10^{-4}	1.82×10^{2}	25.00±0.10	25.15±0.21
12.1×10^{-5}	1.82×10^{1}	29.17±0.08	29.14±0.18
12.1×10^{-6}	1.82	32.25±1.06	33.97±0.59

The standard error (SE) was obtained from triplicate experiments. The standard curve of *S. cerevisiae* and the *S. sensu stricto* complex was obtained by SCDF/SCDR and S14F/S17R primer pairs, respectively.

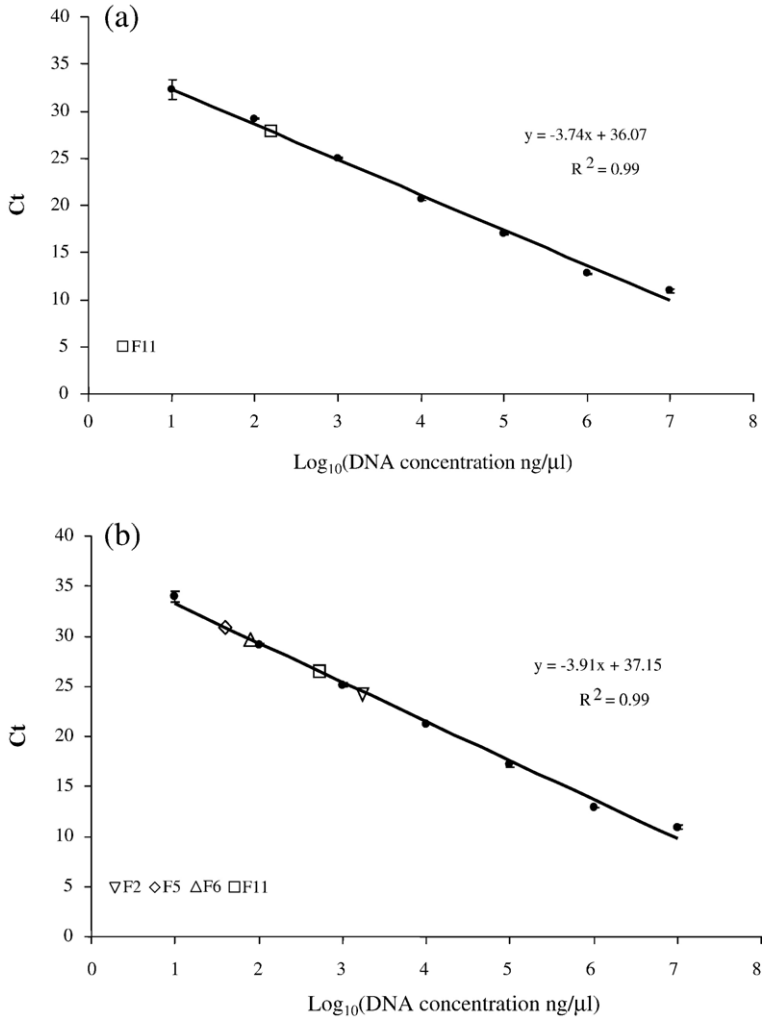

Fig. 4. Quantification of *S. cerevisiae* and the *S. sensu stricto* complex in fecal samples by real time PCR using correlation between the standard curve from each specific primer pair, and DNA from fecal samples. (a) Correlation between the standard curve obtained using the SCDF/SCDR primer pair and DNA (36 ng/μl) from sample F11; (b) correlation between the standard curve obtained from the SI4F/SI7R primer pair and DNA (42 ng/μl) from sample F2, DNA (13.1 ng/μl) from sample F5, DNA (15.1 ng/μl) from sample F6, and DNA (36 ng/μl) from sample F11.

showed that the total rRNA gene copy number of the *S. sensu stricto* complex in sample F11 was about 3 times more than that of *S. cerevisiae.* In sample F2, the gene copy number of the *S. sensu stricto* complex was about 3.5 times greater than that detected in sample F11; however, we could not detect the presence of *S. cerevisiae*. These results show that qPCR with the designed primers is a sensitive detection method for the enumeration of *S. cerevisiae* and the *S. sensu stricto* complex in environmental samples, and that the environmental niche for *S. cerevisiae* is not tightly related to that of the other members of the *S. sensu stricto* complex.

Table 4
Quantification of *S. cerevisiae* and *S. sensu stricto* complex in human fecal samples by real time PCR

Primer pairs	Sample (1 ng/μl)	Log_{10}(DNA concentration ng/μl)	Ct value	Gene copy number
SCDF/SCDR	F11	2.12	27.85	0.84
SI4F/SI7R	F2	3.26	24.39	7.30
	F5	1.59	30.92	0.47
	F6	1.91	29.69	0.86
	F11	2.73	26.48	2.44

4. Discussion

The *S. sensu stricto* complex includes most of yeast strains which are used to produce ethanol. Among these strains, *S. cerevisiae* is predominantly found in the natural environment and in human activities associated with fermentation processes (Vaughan-Martini and Martini, 1995). It is well studied among yeasts because it is an important microorganism in the food and beverage fermentation industry. Although *S. cerevisiae* is widely considered to be a non-pathogenic yeast, it has recently been described as an opportunistic pathogen, part of the microbial flora in the human intestine which causes an emerging infectious disease in humans (Malgoire et al., 2005). Moreover, there is an increasing evidence that some strains of *S. cerevisiae* have been isolated from the blood or sterile regions of debilitated patients

with malignancies, transplant recipients and AIDS patients (Eschete and West, 1980; Dougherty and Simmons, 1982; Cimolai et al., 1987; Sethi and Mandell, 1988; Manzella et al., 1989; Tawfik et al., 1989; Aucott et al., 1990; Nielsen et al., 1990; Oriol et al., 1993; McCusker et al., 1994; Enache-Angoulvant and Hennequin, 2005; Munoz et al., 2005). These results implicated *S. cerevisiae* but not the *S. sensu stricto* complex, as an emerging opportunistic human pathogen (Hennequin et al., 2001; Malgoire et al., 2005). *S. cerevisiae* has also been isolated from human feces; this is indicative of opportunistic invasive infection in the intestine (Nielsen et al., 1990; Munoz et al., 2005; de Llanos et al., 2006b).

The human intestine is estimated to contain approximately 10^{14} microbial cells from a variety of different organisms (Ley et al., 2006). Most of these are anaerobic bacteria belonging to two phylum, the Firmicutes and the Bacteroidetes, and a few are archaea belonging to the class *Methanobacteria* (Eckburg et al., 2005). Yeast such as *Candida* and *Saccharomyces* species are also members of the microflora in the human intestine (Pecquet et al., 1991; Dieterich et al., 2002); however, the effect of the presence of these yeasts on human health is not well understood. Most fungal pathogens associated with human infection were *Candida* species found as endogenous flora in the gastrointestinal tract of humans, as well as *Aspergillus* species which caused invasive disease. Fungal pathogens were also detected together with *S. cerevisiae*; these were considered to be opportunistic pathogens and were detected from immunocompromised patients. *S. cerevisiae* in the human intestine frequently react with intestinal epithelial cells and stimulate the mucosal immune system (Saegusa et al., 2004). In this study, *S. cerevisiae* was detected from only one fecal sample in the qPCR assay using designed species-specific primer pairs. This showed that *S. cerevisiae* is not widely distributed in normal human intestine, although we did not analyze a large number of human fecal samples. It is likely that the probiotic yeast *S. cerevisiae* subsp. *boulardii* was present in the sample that we determined to be positive using *S. cerevisiae* species-specific primers. *S. cerevisiae* subsp. *boulardii* is a well known probiotic yeast and is widely used in the prevention and treatment of intestinal illnesses such as diarrhea.

Since members of the *S. sensu stricto* complex are most commonly used in the food industry to produce ethanol, PCR systems for the specific identification of these strains would be very helpful to monitor them in the brewing and fermenting industry. The merit of this method using qPCR is that it avoids labor-intensive work and offers a rapid and accurate assay. In addition, it could be used for screening new strains belonging to the *S. sensu stricto* complex from diverse natural environments. These primers can be used to evaluate the environmental source for a novel member of the *S. sensu stricto* complex prior to enrichment, and to identify the colonies on agar plates used for seeking the novel yeast without sequencing the PCR products by the rapid colony-PCR assay.

We conclude that this qPCR assay using the primers we have designed is a suitable method for the enumeration of *S. cerevisiae* and the *S. sensu stricto* complex in human feces. This method can provide high reproducibility, specificity, sensitivity and quantification without cultivation. Moreover, it will be used for detecting *S. cerevisiae* and the *S. sensu stricto* complex in various natural environments, as well as for exploring them as a novel bioresource.

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