Differentiation of Brewing and Related Yeasts Based on PCR Amplification and Restriction Fragment Length Polymorphism of Ribosomal DNA

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ABSTRACT

J. Inst. Brew. 108(2), 164–168, 2002

A study to differentiate commercially applied brewing yeasts selected from the culture collection of the University of the Free State from related yeasts of the genus *Saccharomyces* using PCR amplification and RFLP of the internal transcribed spacers region was conducted. Differentiation was dependent on the restriction enzymes used to digest the amplified rDNA. Digestion with *Hae* III , *Cfo* I, *Sau* 3AI and *Msp* I divided representatives of the genus *Saccharomyces* into several unique groups. With *Msp* I the DNA patterns for the two brewing strains were similar, but could be differentiated from *Sacch. cerevisiae* and other species tested. It was also possible to distinguish some members of the *Saccharomyces sensu stricto* group i.e. *Sacch. bayanus* and *Sacch. pastorianus* from *Sacch. cerevisiae* and *Sacch. paradoxus* using *Hae* III as well as *Sacch. paradoxus* from the other *sensu stricto* members using *Msp* I digestion.

Key words: Differentiation, rDNA, RFLP, yeast.

INTRODUCTION

Beer production is one of the oldest biotechnologies in the world. In this industry one thing that should not change and which is one of the pillars of this industry is the yeast that is used. A variety of methods have been used until recently to monitor contamination, mutation, and viability in brewing yeast cultures. These methods have been used, albeit not without criticism to estimate genotypic and phenotypic creep in the populations. Generally, differentiation of yeasts relies on a number of criteria that include among others the mode of sexual reproduction, carbohydrate utilisation, cell wall composition and serology. Many of these methods, even though they have been used for a long time, suffer from the lack of sensitivity and specificity to distinguish between the brewing strains and other wild yeasts or to differentiate one production strain from the other[1,6,10].

In recent times, there have been a number of reports describing the application of molecular genetic tools for the differentiation and characterisation of yeasts used in industrial fermentation[2,3,4,7,8,9,11]. Data from these studies show that RFLP patterns allow recognition of individual species as well as individual strains within a species.

It was reported that the restriction patterns produced from the region spanning the internal transcribed spacers (ITS 1 and ITS 2) and the 5.8S rRNA gene were useful, with a few exceptions, in identifying a total of 132 yeast species including some of the fermentative yeasts of the genus *Saccharomyces sensu stricto* i.e. *Sacch. bayanus, Sacch. cerevisiae, Sacch. paradoxus* and *Sacch. pastorianus*[3]. Here the size of the PCR products and the restriction patterns obtained by using endonucleases *Cfo* I, *Hae* III and *Hin* fI produced a unique pattern for many species. However, in the *sensu stricto* group, it was not possible to distinguish between *Sacch. bayanus* and *Sacch. pastorianus* while clear differences exist between *Sacch. cerevisiae* and *Sacch. bayanus.* This method proved to be reproducible and of use in the rapid identification of wine yeasts[4].

In this paper we have expanded the existing DNA fingerprint database based on PCR amplification of rDNA

TABLE I. *Saccharomyces* species and brewing strains used in this study.

Organism	Fig. 1 reference	Strain no.
Saccharomyces barnettii	n	CBS 5648**T**
Saccharomyces bayanus	b	CBS 0380**T**
Saccharomyces castellii	f	CBS 4309**T**
Saccharomyces cerevisiae	c	CBS 1171**NT**
Saccharomyces dairenensis	g	CBS 0421**T**
Saccharomyces exiguus	h	CBS 0379**T**
Saccharomyces kluyveri	k	CBS 3082**T**
Saccharomyces paradoxus	d	CBS 0406**T**
Saccharomyces pastorianus	e	CBS 1397**T**
Saccharomyces rosinii	o	CBS 7127**T**
Saccharomyces servazzii	l	CBS 4311**T**
Saccharomyces spencerorum	m	CBS 3019**T**
Saccharomyces transvaalensis	i	CBS 2186**T**
Saccharomyces unisporus	j	CBS 0398**T**
Brewing strain 1	p	UOFS Y-0494
Brewing strain 2	q	UOFS Y-0532

[1] CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **T** = Type strain; **NT** = New Type strain; UOFS = University of the Orange Free State, Bloemfontein, South Africa

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Publication no. G-2002-0610-03R
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and subsequent digestion with various restriction enzymes not used before to differentiate between species within the *Saccharomyces sensu stricto* group and related yeasts. This technique was evaluated as a possible quality control method that may be used in the brewing industry to determine contamination with closely related yeasts.

MATERIALS AND METHODS

Strains used

Fourteen type strains representing the genus *Saccharomyces* obtained from the Centraalbureau voor Schimmelcultures, The Netherlands (CBS), were used in this study (Table I). Two commercial brewing *Saccharomyces* strains, i.e., UOFS Y-0494 and UOFS Y-0532, were also included. These strains are held in our culture collection at the University of the Free State.

DNA extraction and amplification

Yeast strains were grown in 5 mL YPD medium at 30°C for 20 h. The yeast cells were transferred to microfuge tubes and harvested by centrifuging for 15 min at 13000 rpm. DNA was isolated using CTAB according to Innes[5] and diluted to 1-50 ng/µL. rDNA region was amplified using a Hybaid thermal cycler. The primers ITS 4 and ITS 5 described by White et al.[12] were used to amplify the ITS region. The thermal cycling parameters were initial denaturing at 96°C for 2 min, followed by 35 cycles of denaturation at 96°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 2 min. A final extension was carried out at 72°C for 10 min. Products of each amplification reaction were resolved on 2% agarose gels, which contained 2.5 µL ethidium bromide (10 mg/mL) and visualised under ultra violet light. Negative controls (no DNA templates) were used in every reaction to test for the presence of contamination in reagents. The sizes of the DNA were compared to a commercial size standard (100 bp ladder) obtained from Promega.

TABLE II. A summary of yeast groups producing different DNA profiles upon *Hae* III digestion.

Group	Fig. 2 reference	Yeasts	Number of bands	Band sizes (bp)
1	b	*Sacch. bayanus*	3	468, 219, 139
	e	*Sacch. pastorianus*	3	460, 217, 145
2	c	*Sacch. cerevisiae*	4	275, 217, 162, 142
	d	*Sacch. paradoxus*	4	279, 215, 159, 140
3	f	*Sacch. castellii*	1	676
	g	*Sacch. dairenensis*	1	676
4	h	*Sacch. exiguus*	2	478, 239
5	i	*Sacch. transvaalensis*	2	480, 312
6	j	*Sacch. unisporus*	2	485, 119
7	k	*Sacch. kluyveri*	3	380, 204, 114
	m	*Sacch. spencerorum*	3	389, 190, 119
8	l	*Sacch. servazzii*	3	285, 220, 206
9	n	*Sacch. barnettii*	2	457, 208
	o	*Sacch. rosinii*	2	476, 219

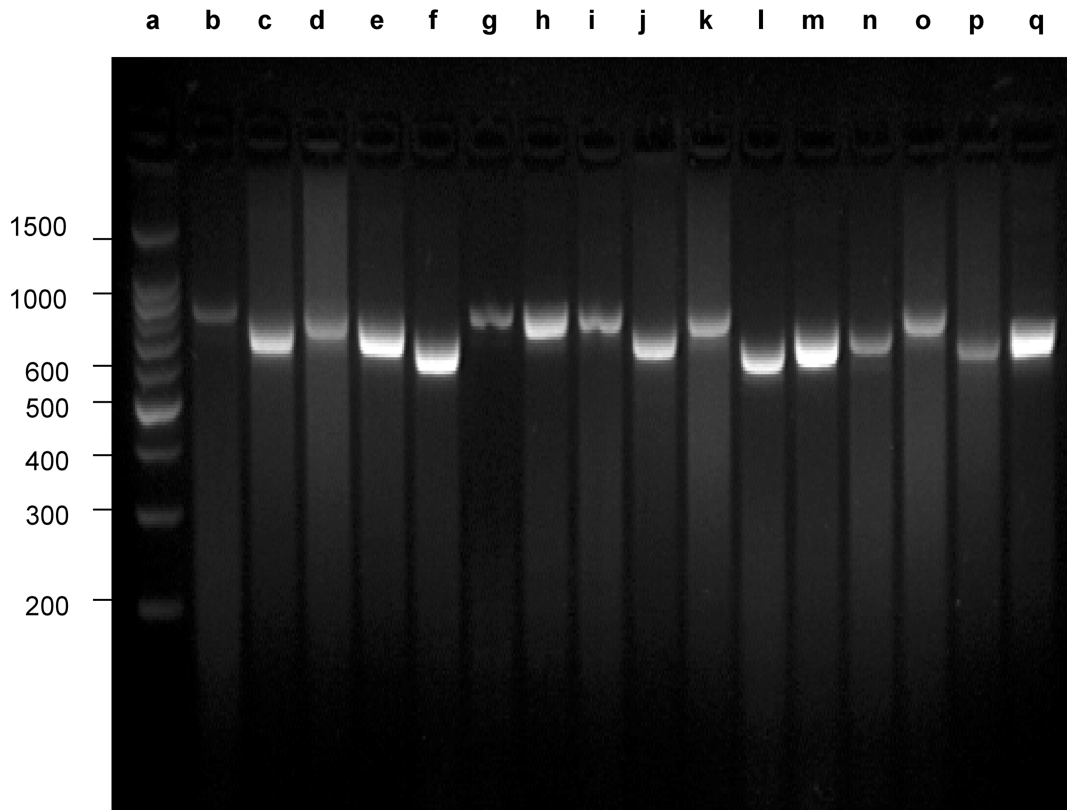

FIG. 1. Profile for the PCR based amplification of rDNA region of *Saccharomyces* species and the two brewing strains. a- is the standard ladder with bands corresponding to 200, 300, 400, 500, 600, 1000, 1500 base pairs.

Restriction digestion and RFLP analysis

PCR products were digested with restriction endonucleases *Hae* III, *Cfo* I, *Sau* 3AI and *Msp* I according to the supplier's instructions. The restriction fragments were electrophoresed on 2% agarose gel at 70 V for 90 min. The reaction fragment sizes were measured in base pairs calculated from comparison to a commercial standard (100 bp ladder).

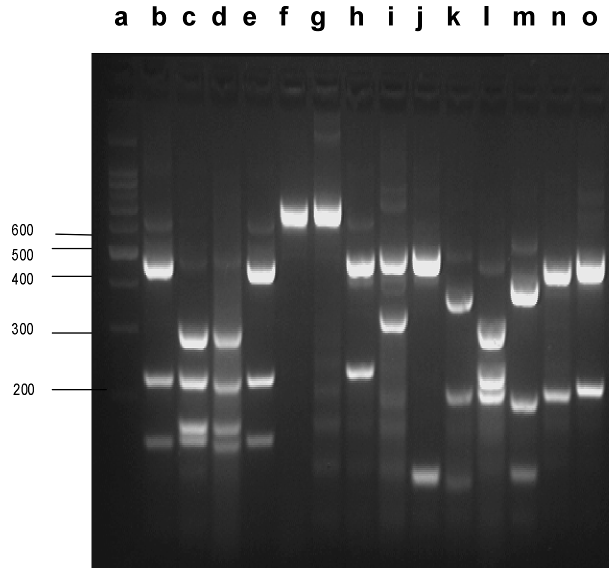

FIG. 2. Restriction pattern of the PCR-amplified rDNA region of the *Saccharomyces* type strains digested with *Hae* III restriction enzyme. a- is the standard ladder with bands corresponding to 200, 300, 400, 500, 600 base pairs.

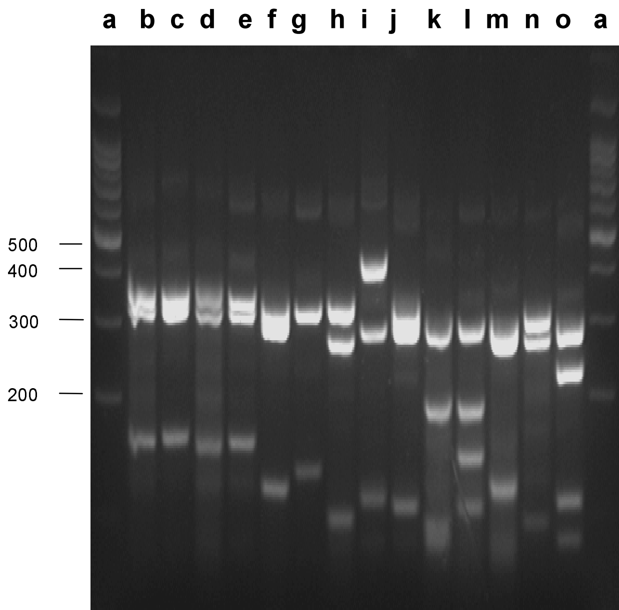

FIG. 3. Restriction patterns of the PCR-amplified rDNA region of the *Saccharomyces* type strains digested with *Cfo* I restriction enzyme. a- is the standard ladder with bands corresponding to 200, 300, 400, 500 base pairs.

RESULTS AND DISCUSSION

The organisms used for this study include 14 species of the genus *Saccharomyces* (Table I) and two commercial brewing strains. These yeasts were selected since they are phenotypically closely related and will therefore test the sensitivity of this molecular method as a quality control method to be used in industrial fermentation processes where these yeasts are used. By using the primers ITS 4 and ITS 5, the extracted DNA from all strains listed in Table I was amplified and the product examined after gel electrophoresis (Fig. 1). There was significant variation in the size of the amplified DNA as indicated by the vari-

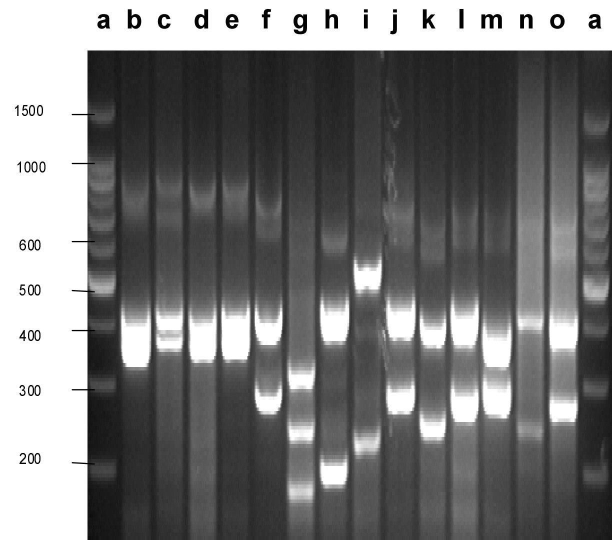

FIG. 4. Restriction patterns of the PCR-amplified rDNA region of the *Saccharomyces* type strains digested with *Sau* 3AI restriction enzyme. a- is the standard ladder with bands corresponding to 200, 300, 400, 500, 600, 1000, 1500 base pairs. Broad bands between 300 bp and 400 bp that appear as one, were found to be two upon closer inspection (see text).

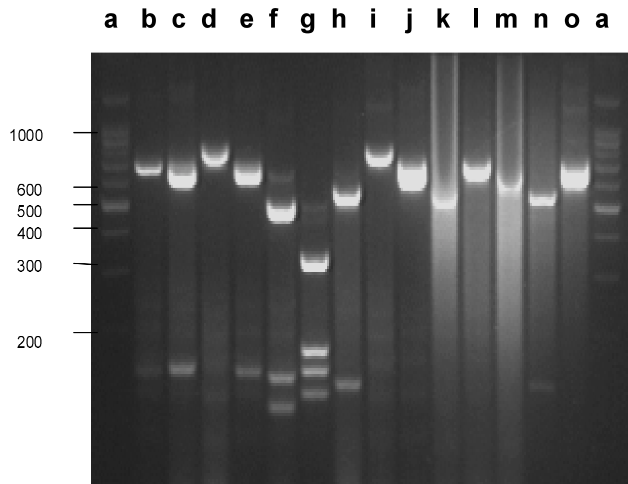

FIG. 5. Restriction patterns of the PCR-amplified rDNA region of the *Saccharomyces* type strains digested with *Msp* I restriction enzyme. a- is the standard ladder with bands corresponding to 200, 300, 400, 500, 600, 1000 base pairs.

ation in the position of the bands (Fig. 1). This amplified DNA was subsequently digested with restriction enzymes. Four restriction endonucleases, which recognise specific nucleotide sequences, were used to digest the PCR products. These digests were then examined by gel electrophoresis to determine whether unique patterns capable of differentiating between the yeasts in our cohort could be generated. As shown in Figures 2-6 and Tables II-V prominent restriction bands that reflect a high degree of length polymorphism were obtained.

Hae III digestion fragments

On the basis of DNA band profiles obtained through *Hae* III digestion (Fig. 2, Table II), the yeast species representing the genus *Saccharomyces* can be divided into nine separate groups, each with similar band patterns within each group. Consequently, it was possible to differentiate the *Saccharomyces sensu stricto* group from the *Saccharomyces sensu lato* group, i.e., *Sacch. barnettii, Sacch. castellii, Sacch. dairenensis, Sacch. exiguus, Sacch. kluyveri, Sacch. rosinii, Sacch. servazzii, Sacch. spencerorum, Sacch. transvaalensis* and *Sacch. unisporus*. It was also possible to separate *Sacch. bayanus* and *Sacch. pastorianus*, characterised by three bands each (Group 1), from *Sacch. cerevisiae* and *Sacch. paradoxus*, characterised by four bands each.

Cfo I digestion fragments

DNA band patterns obtained through Cfo I endonuclease (Fig. 3, Table III) divided the genus *Saccharomyces* into eight distinct groups. Here an overlap between the *Saccharomyces sensu stricto* and *sensu lato* groups was evident. As reported by Esteve-Zarzoso et al.[3], the *Saccharomyces sensu stricto* group could also not be separated using this digestion enzyme.

Sau 3AI digestion fragments

Digestion with Sau 3AI (Fig. 4, Table IV) yielded five groups. Here, the *Saccharomyces sensu stricto* group could be separated from the sensu lato group. However, the four species representing the *sensu stricto* group produced similar DNA patterns and could not be distinguished.

Msp I digestion fragments

With Msp I (Fig. 5, Table V) four groups were differentiated. According to our results, *Sacch. paradoxus* could be separated from the rest of the *Saccharomyces sensu stricto* group. Application of Msp I on the PCR products of the two brewing strains showed no difference between them (Fig. 6, c and d). Neither could the application of Cfo I, Hae III and Sau 3AI differentiate between the two brewing strains (results not shown). The profiles of the two brewing strains could be differentiated from the profile of *Sacch. cerevisiae* (Fig. 6, b) as well as other members of the *sensu stricto* and *sensu lato* groups (Fig. 6), i.e., the appearance of two extra bands with a size of approximately 116 and 12 base pairs.

CONCLUSIONS

According to our results it was possible to differentiate the closely related *Sacch. bayanus* and *Sacch. pastorianus* (the latter two producing similar band patterns) from *Sacch. cerevisiae* and *Sacch. paradoxus* (the latter two pro-

TABLE III. A summary of yeast groups producing different DNA profiles upon *Cfo* I digestion.

Group	Fig. 3 reference	Yeasts	Number of bands	Band sizes (bp)
1	n	*Sacch. barnettii*	3	328, 270, 42
	b	*Sacch. bayanus*	3	367, 337, 151
	c	*Sacch. cerevisiae*	3	367, 327, 156
	d	*Sacch. paradoxus*	3	384, 323, 146
	e	*Sacch. pastorianus*	3	355, 327, 156
	f	*Sacch. castellii*	3	327, 309, 120
	j	*Sacch. unisporus*	3	327, 309, 111
2	g	*Sacch. dairenensis*	2	347, 125
3	h	*Sacch. exiguus*	3	355, 260, 50
4	i	*Sacch. transvaalensis*	3	457, 298, 112
5	k	*Sacch. kluyveri*	3	298, 176, 36
6	l	*Sacch. servazzii*	4	309, 179, 136, 100
7	m	*Sacch. spencerorum*	3	260, 119, 38
8	o	*Sacch. rosinii*	4	292, 221, 100, 30

TABLE IV. A summary of yeast groups producing different DNA profiles upon *Sau* 3AI digestion.

Group	Fig. 4 reference	Yeasts	Number of bands	Band sizes (bp)
1	b	*Sacch. bayanus*	2	396, 347
	c	*Sacch. cerevisiae*	2	410, 388
	d	*Sacch. paradoxus*	2	397, 370
	e	*Sacch. pastorianus*	2	415, 387
2	f	*Sacch. castellii*	2	397, 260
	j	*Sacch. unisporus*	2	401, 257
	k	*Sacch. kluyveri*	2	392, 245
	l	*Sacch. servazzii*	2	421, 257
	m	*Sacch. spencerorum*	2	392, 271
	n	*Sacch. barnettii*	2	427, 240
	o	*Sacch. rosinii*	2	398, 253
3	g	*Sacch. dairenensis*	3	320, 240, 154
4	h	*Sacch. exiguus*	2	421, 174
5	i	*Sacch. transvaalensis*	2	533, 210

TABLE V. A summary of yeast groups producing different DNA profiles upon *Msp* I digestion.

Group	Fig. 5 reference	Yeasts	Number of bands	Band sizes (bp)
1	b	*Sacch. bayanus*	2	676, 98
	c	*Sacch. cerevisiae*	2	610, 98
	e	*Sacch. pastorianus*	2	620, 98
	h	*Sacch. exiguus*	2	522, 86
2	d	*Sacch. paradoxus*	1	741
	i	*Sacch. transvaalensis*	1	767
	j	*Sacch. unisporus*	1	630
	k	*Sacch. kluyveri*	1	501
	l	*Sacch. servazzii*	1	653
	m	*Sacch. spencerorum*	1	550
	n	*Sacch. barnettii*	1	526
	o	*Sacch. rosinii*	1	630
3	f	*Sacch. castellii*	3	422, 86, 81
4	g	*Sacch. dairenensis*	4	320, 112, 98, 84

ducing similar band patterns) using Hae III (Fig. 2, Table II). Similar results within *Saccharomyces sensu stricto* using Hae III were obtained by Esteve-Zarzoso et al.[3]

It was also possible to distinguish *Sacch. paradoxus* from the *sensu stricto* members using Msp I digestion (Fig. 5, Table V). This is in accordance with the rather isolated position of *Sacch. paradoxus* when compared with the other members of the "cerevisiae" cluster based on PCR-RFLP and ITS sequence analyses as reported by Montrocher et al.[8]

In addition, the *Saccharomyces sensu stricto* group could easily be differentiated from the *Saccharomyces sensu lato* group when comparing band patterns after *Sau* 3A1 digestion (Fig. 4, Table IV). It was also possible to distinguish the brewing strains from all the other *Saccharomyces* species tested on the basis of the two extra bands that differentiated the brewing yeasts from *Sacch. cerevisiae* (Fig. 6). These results show promise in the construction of a quality control system where it is necessary to distinguish brewing strains from closely related yeasts. However, more commercial brewing strains and strains from other yeast genera known to be contaminants should be analysed to make a thorough evaluation if the brewing strains remain unique on the basis of this characteristic. It is also important to apply this method to mixed cultures, i.e., brewing strain mixed with other closely related species, in order to determine at what level contaminants can be detected.

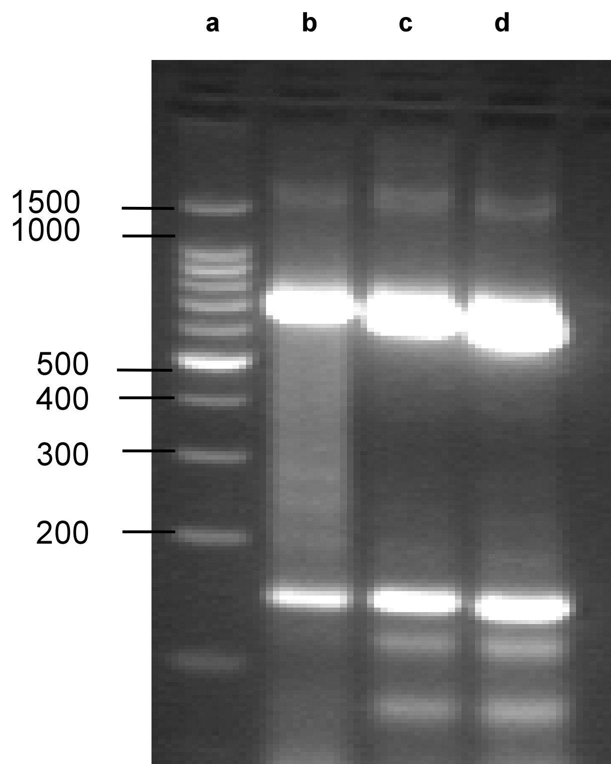

FIG. 6. Restriction pattern of the PCR-amplified rDNA region of *Saccharomyces cerevisiae* and the two brewing strains digested with *Msp* I restriction enzyme. a- is the standard ladder with bands corresponding to 200, 300, 400, 500, 1000, 1500 base pairs. b- *Sacch. cerevisiae,* c- brewing strain UOFS Y-0494, d- brewing strain UOFS Y-0532.

ACKNOWLEDGEMENTS

The authors wish to thank the National Research Foundation in South Africa for funding.

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(Manuscript accepted for publication March 2002)

APPENDIX

Appendix. List of abbreviations and terminology used

CBS	Centraalbureau voor Schimmelcultures
Cfo 1	Restriction enzyme obtained from *Clostridium formicoaceticum.*
CTAB	Hexadecyl trimethyl ammonium bromide
DNA	Deoxyribose nucleic acid
rDNA	Ribosomal deoxyribose nucleic acid
EDTA	2-ethylene-diamine-tetra-acetate
Hae 111	Restriction enzyme obtained from *Haemophilus aegyptius*
ITS	Internal transcribed spacer
Msp 1	Restriction enzyme obtained from *Moraxella sp.*
DNTP	Deoxyribose nucleoside triphosphate
PCR	Polymerase chain reaction
ppm	parts per million
RFLP	Restriction fragment length polymorphisms
Sau 3A1	Restriction enzyme obtained from *Staphylococcus aureus*
T E buffer	Tris, 2-ethylene-diamine-tetra-acetate buffer
YPD medium	Yeast extract 1%, Peptone 2%, Glucose 2%