Amplified Fragment-Length Polymorphism, a New Method for the Analysis of Brewer's Yeast DNA Polymorphism

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ABSTRACT

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This study sought to confirm the utility of amplified fragment-length polymorphism for identifying brewery yeast strains. Our results were promising, since single primer pairs can be used to distinguish most yeast strains. The use of several primer pairs, however, should still increase the method's reproducibility. Furthermore, this technique yields quantitative data on genetic polymorphism. Among the 26 strains studied, we calculated a 55% average of shared fragments. This similarity indicator was higher for bottom-fermenting strains (72%) than for top-fermentation yeasts (45%).

Keywords: Genetic fingerprint, Genetic similarity, *Saccharomyces cerevisiae*, Yeast strain

RESUMEN

AFLP, un nuevo método para el análisis del polimorfismo del ADN de la levadura cervecera.

Este estudio tenía como objetivo confirmar la utilidad en la identificación de cepas de levadura cervecera de la técnica del polimorfismo en las longitudes de fragmentos amplificados por medio de la reacción de la polimerasa en cadena. Nuestros resultados han sido prometedores puesto que con una única pareja de cebadores se pueden distinguir muchas cepas de levadura. No obstante, el empleo de diversas parejas de cebadores debería aumentar aún más la reproducibilidad del método. Además, esta técnica proporciona datos cuantitativos sobre el polimorfismo genético. Hemos calculado que, para las 26 cepas estudiadas, existe un promedio de fragmentos compartidos del 55%. Este índice de similitud era mayor en las cepas de fermentación baja (72%) que en las levaduras de fermentación alta (45%).

Palabras clave: Huella genética, Similitud genética, *Saccharomyces cerevisiae*, Cepa de levadura.

Because they are used in enzyme and antibiotic production, fine chemistry, and the food sciences, yeasts occupy an incomparable place in the bioindustries. They are responsible for alcoholic fermentation in the production of alcoholic beverages, both distilled (whiskey, rum, and sake) and undistilled (beer, wine, and cider). Their fermentation properties are also used to exploit wastes such as wood pulp and paper mass.

Although yeasts are attractive microorganisms, they remain agents that alter foodstuffs if their development is not adequately controlled. Protection from any contamination is obviously necessary. It is also important to know the microorganism used, and this inevitably requires a stable classification system in which the identity of each strain is defined and specific.

Unfortunately, the classification of yeasts has been reorganized many times, and numerous confusions arise from the lack of reliability, specificity, and sensitivity of the characters used (3,13). Phenotypic expression of fermentative characters fluctuates considerably, as it results from loci that may have several active genes or silent ones. Any classification based on such biochemical characters is exposed to revision, as in the case of the melibiose-fermenting strains of *Saccharomyces cerevisiae* and *S. bayanus* (9).

Brewers generally use the following names: *S. cerevisiae* (top fermentation), *S. carlsbergensis* (bottom fermentation), and *S. diastaticus* (contaminant yeast), while wine manufacturers handle *S. ellipsoideus*, *S. oviformis* or *bayanus*, and *S. pastorianus* (for low-temperature fermentation). On the basis of data obtained with new molecular techniques of investigation, these yeasts have been classified into four species: *S. bayanus*, *S. cerevisiae*, *S. paradoxus*, and *S. pastorianus*. The contaminant yeast, *S. diastaticus,* is grouped in the same taxonomic field as *S. cerevisiae* (13).

While defining and describing a species is difficult, it is even more difficult to characterize and identify a strain. Here, molecular marker techniques based on polymerase chain reaction (PCR) are leading to the replacement of classical markers such as morphological and biochemical characters. Among the molecular marker techniques, amplified fragment-length polymorphism (AFLP) seems particularly promising (13,16). It shows a high rate of polymorphism and good reproducibility at moderate cost (lower than with microsatellites). It reveals mutations found mainly in the noncoding region of the genome, such as replacements, insertions, and deletions, thus optimizing detection of polymorphisms. Thanks to the multiplex ratio (marker abundance) and high resolution of this method, it is possible to distinguish species, varieties, and individuals (4).

Although AFLP is mainly used in plant breeding, de Barros Lopes and coworkers (6) used it successfully to differentiate yeast species. Our study sought to validate the use of AFLP at the yeast strain level. After assessing the reproducibility of AFLP profiles for selected yeast strains, we used the technique to assess the DNA polymorphism of brewer's yeast strains. AFLP will not provide a phylogenetic tree for yeasts, but it should allow discrimination between closely related strains used in industrial processes.

EXPERIMENTAL

Reagents

Cineole and all biochemicals were purchased from Sigma-Aldrich (St Louis, MO), except RNAse A from ICN Biomedicals Inc. (Costa Mesa, CA). All chemicals were from Merck-Eurolab (Darmstadt, Germany), except phenol/chloroform/isoamyl alcohol solution from ICN Biomedicals Inc.

Yeast Strains

The *S. cerevisiae* strains used in this study are listed in Table I. All strains were from the BRAS and MUCL collections of the Université Catholique de Louvain, Louvain-la-Neuve, Belgium, except Σ1278b,

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which was from the Laboratoire de Physiologie Cellulaire et de Génétique des Levures of the Université Libre de Bruxelles, Belgium.

Media

For DNA extraction, cultures were grown in YPS medium (1% yeast extract, 0.5% peptone, 10% sucrose) at 28°C on a rotary shaker and collected in the exponential phase.

For fermentations, propagations were carried out in two steps (400 and 1,500 ml, respectively), with a pitching rate of 10^7 cells per milliliter each time. After collection (in the exponential phase) and washing, the yeast was pitched at the same level in a 12°P (1 g of extract per 100 g of wort) sterilized, industrial sweet wort at 18°C in 1-L EBC tubes for seven days.

Preparation of DNA Template for PCR

Yeast cells from a 5-ml culture grown in YPS medium were collected by centrifugation (4,000 rpm, rotor SS34, Sorvall, Kendro) in the exponential phase. After washing with deionized water at 4°C (Milli-Q water purification system, Millipore, Bedford, MA), the cell pellet was resuspended in 200 µl of breaking buffer (2% [v/v] Triton X-100, 1% sodium dodecyl sulfate, 100 m*M* NaCl, 10 m*M* Tris, 1 m*M* ethylene-diaminetetraacetic acid [EDTA, pH 8]) (2). The cells were homogenized by vortexing for 3 min with 300 mg of glass beads in 200 µl of phenol/chloroform/isoamyl alcohol (25:24:1) and 200 µl of TE buffer (10 m*M* Tris, 1 m*M* EDTA [pH 8]). The DNA was precipitated with ethanol at –30°C, centrifuged (10 min at 10,000 rpm, centrifuge 202MC, Sigma) and resuspended in 300 µl of TE buffer. RNA was digested by adding 3 µl of RNase A (10 mg/ml) and incubating for 5 min at 37°C. The DNA was extracted a second time with 200 µl of phenol/chloroform/isoamyl alcohol, then ethanol-precipitated and resuspended in 20 µl of TE buffer.

AFLP

The AFLP reactions (Fig. 1) were performed by the method of Vos et al (14) as modified by de Barros-Lopes et al (6).

One nanogram of the yeast DNA extract was digested with 5 units of *Eco*RI and 5 units of *Mse*I in 5 µl of RL buffer (10 m*M* Tris/acetate, 10 m*M* magnesium acetate, 50 m*M* potassium acetate, 5 m*M* dithiothreitol [pH 7.5]) for 150 min at 37°C. Adaptors (5′-GACGATGAGTCCTGAG-3′ and 5′-TACTCAGGACTCAT-3′ for *Mse*1, 5′-CTCGTAGACTGCGTACC-3′ and 5′-AATTGGTA-CGCAGTC-3′ for *Eco*RI) were ligated to the digested DNA for 3 hr at 20°C in RL buffer containing 1.2 m*M* adenosine 5′-triphosphate and 1 unit of T4 DNA ligase. The digested and ligated DNA was ethanol-precipitated and resuspended in 49.5 µl of Tris (10 m*M*)-EDTA (0.1 m*M*) buffer (pH 8.0).

The primers *Eco*RI–C (5′-AGACTGCGTACCAATTCC-3′) and *Mse*I–AC (5′-GATGAGTCCTGAGTCAAAC-3′) were used for the PCR reaction. For each AFLP reaction, 0.75 µl of the ligated DNA was amplified using 0.25 µl (5 µ*M*) of *Mse*I primer, 0.25 µl (1 µ*M*) of *Eco*RI primer, and 3.75 µl of AFLP core mix (AFLP ligation amplification kit, Perkin Elmer, Wellesley, MA). The reaction mix was then amplified: denaturation was at 94°C for 2 min and extension at 74°C for 2 min. The annealing temperature started at 74°C for 30 sec and was subsequently decreased by one degree every cycle until it reached 56°C. This was followed by 23 more cycles at 56°C and a final extention of 4 min at 72°C. To 2.05 µl of the completed reaction, 2.88 µl of gel loading buffer (52% formamide, 26% blue loading dye, 22% GS 500 Rx standard) was added. Samples were heated to 95°C for 5 min and cooled on ice. Products of each amplification reaction were resolved on a 6% polyacrylamide gel for 2 hr at 51°C, 3,000V, 60 mA, 200 W.

From the AFLP profile shown in Figure 2, the presence/absence of AFLP markers was scored by Genescan (Applied Biosystem, Perkin Elmer, location) and analysis was carried out with Genotyper (Applied Biosystem, Perkin Elmer). Pairwise similarities were determined by the *F*-value (7), which represents the percentage of

$$F = \frac{2n_{ij}}{n_i + n_j}$$

TABLE I
***Saccharomyces* Yeast Strains Studied**[a]

Strains	Origin	ALLEV identification	Fermentation type	Conservation
BRAS12	Brewery	S. paradoxus (100%)	Bottom	4°C, agar-tube
BRAS25	Brewery	S. paradoxus (100%)	Bottom	4°C, agar-tube
BRAS26	Brewery	S. paradoxus (100%)	Bottom	4°C, agar-tube
BRAS291	Brewery	S. paradoxus (100%)	Bottom	4°C, agar-tube
BRAS299	Brewery	S. paradoxus (100%)	Bottom	4°C, agar-tube
BRAS33	Brewery	S. paradoxus (98.77%)	Bottom	4°C, agar-tube
BRAS40	Brewery	S. paradoxus (100%)	Bottom	4°C, agar-tube
BRAS41	Brewery	S. paradoxus (100%)	Bottom	4°C, agar-tube
BRAS52A	Brewery	S. paradoxus (100%)	Bottom	4°C, agar-tube
MUCL20479	Brewery	S. paradoxus (98.78%)	Bottom	Cryopreservation
BRAS212[b]	Brewery	*S. cerevisiae* (100%)	Top	4°C, agar-tube
BRAS213	Bakery	*S. cerevisiae* (100%)	Top	4°C, agar-tube
BRAS214	Brewery	*S. cerevisiae* (100%)	Top	4°C, agar-tube
BRAS223	Brewery	*S. cerevisiae* (100%)	Top	4°C, agar-tube
BRAS240A	Brewery	*S. cerevisiae* (100%)	Top	4°C, agar-tube
BRAS240B	Brewery	*S. cerevisiae* (100%)	Top	4°C, agar-tube
BRAS268	Brewery	*S. cerevisiae* (100%)	Top	4°C, agar-tube
BRAS270	Brewery	*S. cerevisiae* (100%)	Top	4°C, agar-tube
BRAS349	Brewery	*S. cerevisiae* (100%)	Top	4°C, agar-tube
MUCL28356	Brewery	*S. cerevisiae* (100%)	Top	Cryopreservation
MUCL28357	Brewery	*S. cerevisiae* (100%)	Top	Cryopreservation
MUCL28362[b]	Brewery	*S. cerevisiae* (100%)	Top	Cryopreservation
MUCL28734	Brewery	*S. cerevisiae* (100%)	Top	Cryopreservation
MUCL20475	Unknown	···	Not determined	Cryopreservation
MUCL39516	Laboratory	···	Not determined	Cryopreservation
Σ1278b	Laboratory	···	Not determined	Cryopreservation

[a] Bottom-fermenting strains are in bold.
[b] Same yeast strain stored using different techniques.

with n_{ij} = the number of common bands in the two fingerprints and $n_i + n_j$ = the sum of all bands.

Morphological and Physiological Profile Analysis

A morphological profile and a physiological analysis including 12 fermentation sugar tests, 44 assimilation tests of carbon compounds, nine assimilation tests of nitrogen compounds, and 10 growing tests without vitamins were performed. The profile obtained was then compared to identify the nearest reference species profile, following the ALLEV method (10).

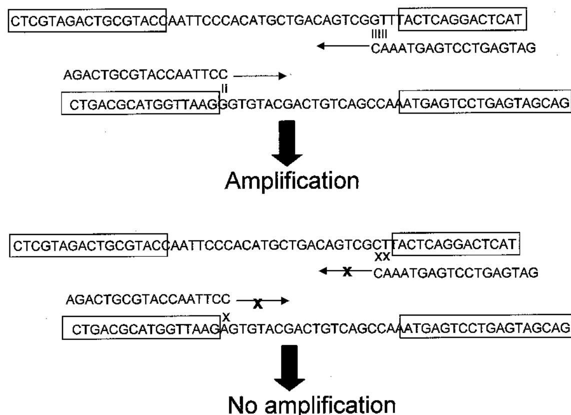

Fig. 1. Steps in the amplified fragment-length polymorphism fingerprint technique.

Sulfite Quantification

Total sulfite quantification was performed in beers obtained from the 28 yeast strains by using the para-rosaniline method (11).

4-Vinylguaiacol Quantification

4-Vinylguaiacol was quantified from 10 ml of beer containing 500 ppb internal standard (cineole) and flushed thereafter through a Sep-Pak C18 cartridge (Waters Corporation, Milford, MA) pre-conditioned with 10 ml of water. Nonpolar compounds were eluted with 2 ml of dichloromethane. After the organic phase was dried on Na_2SO_4 and concentrated by a dry nitrogen flow, chromatographic analyses were conducted on a gas chromatograph (model 5890, Hewlett Packard, Brussels, Belgium), equipped with an automatic sampler (model 7673, Hewlett Packard), a cold on-column injector, a flame ionization detector, and an integrator (CR4A, Shimadzu, Kyoto, Japan). The separation of volatile compounds was performed on a 50 m × 0.32 mm, wall-coated, open tubular (CP-SIL5 CB, Chrompack, Antwerp, Belgium) capillary column (film thickness, 1.2 µm), which was preceded by a 1 m × 0.53 mm capillary column, coated with a thin film of methyl silicon phase (Hewlett Packard). The oven temperature was programmed to rise from 35 to 70°C at 25 degrees per minute, then to 140°C at 4 degrees per minute and to 240°C at 25 degrees per minute. The carrier gas was helium at a

TABLE II
***F*-Values (reproducibility, %) Obtained for Yeast Strains BRAS214, BRAS40, and BRAS52A[a]**

Strain	Intensity Threshold	Fragment Size (bp) 50–450	75–450	100–450	150–450	200–450
BRAS40	I>100	70	72	76	74	78
	I>200	80	80	84	81	89
	I>300	90	91	97	100	100
	I>400	90	92	96	100	100
BRAS52A	I>100	80	82	85	83	93
	I>200	82	83	86	84	90
	I>300	87	87	89	89	95
	I>400	91	92	92	92	100
BRAS212	I>100	68	70	72	69	72
	I>200	70	71	72	70	73
	I>300	74	77	77	78	79
	I>400	84	86	88	88	91

[a] I = intensity of fluorescence.

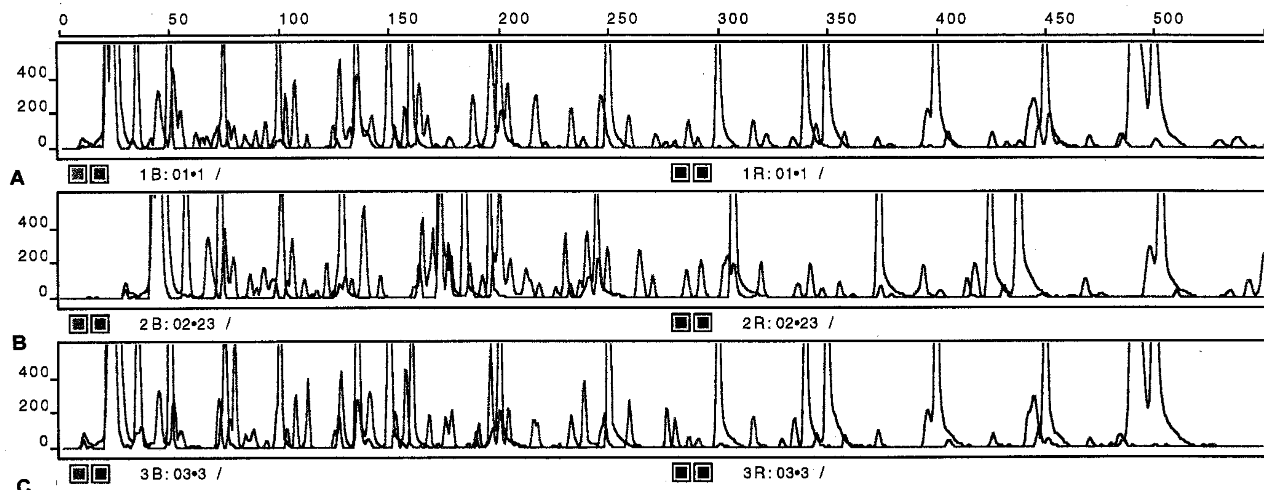

Fig. 2. Amplified fragment-length polymorphism profile of brewer's yeasts BRAS214 (a), BRAS40 (b), and BRAS52A (c). Each profile also includes its own calibration curve showing peaks at standard sizes of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bp.

flow rate of 1.5 ml/min. The injector temperature was maintained at 3 degrees above the oven temperature. The detector temperature was maintained at 260°C. The minimum peak area for data acquisition was set at 500 µV/sec.

RESULTS AND DISCUSSION

Reproducibility of AFLP Profiles

Two independent DNA extractions and two independent AFLP analyses were made for separate cultures of each of three brewer's yeast strains (BRAS212, BRAS40, and BRAS52a). Statistical treatments applied to the AFLP profiles produced several "presence/absence" matrices according to the DNA fragment size (from 50 to 450 bp) and the minimum average intensity of each fragment (from 100 to more than 400 units). Percentages of shared fragments (*F*-values) are given in Table II.

The reproducibility of the BRAS40 and BRAS52A AFLP profiles was very promising when the average intensity threshold was 300 for fragments between 150 and 450 base pairs (bp); the *F*-value scores for these strains were, respectively, 100 and 89%. The score was only 78% for BRAS 212 when same intensity and fragment-length limits were applied. However, reproducibility was significantly improved (*F*-values increased to 91%) when more strict inclusion criteria were used (average intensity threshold: 400; fragment size between 200 and 400). Nevertheless, under these conditions the number of markers was obviously much lower. Therefore, we recommend increasing this number by using a second set of PCR primer pairs.

We also compared two strains of *S. cerevisiae* (MUCL28362 and BRAS212) derived from the same parental strain but conserved differently for several years, one in an agar tube at 4°C and one cryopreserved at –141°C in 15% glycerol. The proportion of shared fragments was as low as 46%, indicating potential genetic modification through conservation (S. Vanhulle and A. M. Corbisier, *personal communication*).

TABLE III
Amplified Fragment-Length Polymorphism Markers of Each *Saccharomyces cerevisiae* Strain Studied[a]

Fragment	Strain																									
	BRAS 12	BRAS 25	BRAS 26	BRAS 291	BRAS 299	BRAS 33	BRAS 40	BRAS 41	BRAS 52A	MUCL 20479	BRAS 212	BRAS 213	BRAS 214	BRAS 223	BRAS 240 A	BRAS 240 B	BRAS 268	BRAS 270	BRAS 349	MUCL 28356	MUCL 28357	MUCL 28362	MUCL 28734	MUCL 20475	MUCL 39516	Sigma 1278b	
151															-						-						2
152																				-			-				2
153	-	-	-	-	-		-	-	-	-		-		-		-		-	-			-		-	-		17
157			-														-			-	-		-				5
158	-	-	-	-	-	-	-	-	-	-																	10
160	-			-	-	-	-	-	-	-	-											-	-	-		-	13
162			-									-															2
164		-		-		-	-	-	-	-	-	-		-		-		-	-			-	-	-	-	-	18
165			-												-					-	-	-	-				6
168					-	-			-	-		-			-	-	-	-	-	-				-	-		13
172					-							-	-	-		-	-	-	-						-		8
175					-				-					-		-	-	-	-		-				-		9
178			-																							-	2
178																	-				-						2
181	-			-	-			-	-					-													6
185		-	-									-				-		-	-			-			-		8
186	-	-		-	-	-	-	-	-	-	-	-		-		-	-	-	-	-		-	-	-	-	-	22
189																	-			-			-				3
192		-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-		-	-	-	-	-	-	23
194	-	-	-	-		-		-	-	-	-	-			-	-	-	-	-		-		-	-	-		20
195					-		-							-							-						4
197	-	-	-		-	-	-	-	-	-		-					-	-	-		-	-	-	-	-	-	19
201			-						-													-					3
204			-									-						-	-		-		-		-		7
207		-	-	-	-			-	-	-	-	-		-	-	-	-		-		-		-	-	-	-	19
208	-		-		-	-	-						-				-	-				-					9
211																				-							1
217																				-						-	2
219															-												1
221	-	-	-	-	-	-	-	-	-	-		-	-	-		-	-	-	-		-	-	-	-	-	-	23
224																				-	-					-	3
227	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-		24
232		-			-	-	-		-	-		-				-		-	-	-	-	-	-	-			15
235	-		-	-	-	-		-				-		-		-	-	-	-		-	-	-	-			16
237	-					-			-	-										-							5
247															-					-							2
248			-																						-		2
250	-	-	-	-	-	-	-	-	-	-	-	-		-		-	-	-	-		-	-	-		-		21
253																				-						-	2
259												-														-	2
262												-											-				2
263	-			-	-		-							-						-				-			7
269	-	-	-	-	-	-		-		-		-	-			-	-	-	-		-	-	-	-	-		21
289																									-		1
291			-									-											-				3
298	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-		-	-	-	-	25
305																				-							1
314			-																								1
317			-																								1
319			-									-											-				3
329															-					-		-					3
334			-																	-			-				3
345												-								-	-		-				4
349																				-							1
360																				-							1
377																					-	-	-				3
388	-			-	-			-	-	-				-													7
396																				-							1
405																				-							1
	17	15	26	17	22	17	15	17	20	18	9	24	5	16	10	17	18	19	19	24	21	18	25	16	19	13	

[a] Bottom-fermenting strains are the first 10 strains (left to right).

Strain Discrimination on the Basis of the AFLP Profiles

Table III shows the markers detected by AFLP (average intensity >300; fragment length between 150 and 450 bp) for 26 *Saccharomyces cerevisiae* strains including top-fermentation, bottom-fermentation and haploid laboratory strains. This number is highly strain-dependent (from only five bands for BRAS214 to 26 for BRAS26). It can therefore be used as a first indicator of strain polymorphism.

Some fragments appeared to be shared by most of our strains (Table III). For instance, the 298-, 227-, 221-, 192-, and 186-bp fragments were detected in more than 80% of the AFLP profiles, while the 269-, 207-, 197-, 164- and 153-bp fragments were found in 75% of the fingerprints. On the other hand, we found fragments specific to strains BRAS26 (314-, 317-bp), MUCL28356 (211-, 305-, 349-, 360-, 396-, 405-bp), and MUCL39516 (289-bp).

Each yeast strain can be distinguished from the 26 other strains using the AFLP fragment profile. Table IV gives the *F*-values; among the 26 strains studied, the average percentage of shared fragments was 55%. The similarity indicator was higher when bottom-fermenting strains were compared (72%), and lower among top-fermentation yeasts (45%). Several authors (1,5,8,15) have also demonstrated higher similarity between lager yeasts, which have been used in breweries for only 150 years.

One marker (at 158-bp) proved typical of all bottom-fermenting yeasts (determined by the ALLEV method [ref. 10, Table 1]). This genotypic character was in all cases also associated with two important phenotypical properties for brewers (12): high sulfite excretion (>1 ppm, Fig. 3) and low production of 4-vinylguaiacol (<52 ppb in our unhopped wort, Fig. 4). On the other hand, the yeast strains lacking this 158-bp fragment in the AFLP profile were unable to excrete more than 1 ppm sulfite and produced larger amounts of 4-vinylguaiacol (generally more than 200 ppb).

CONCLUSION

Initially used for plant breeding, AFLP has been successfully applied to distinguishing brewer's yeast strains. However, to improve reproducibility by using more strict thresholds, it is advisable to use several sets of PCR primers. Differences were evidenced among yeasts preserved by different techniques. AFLP

TABLE IV

***F*-Values (proportion of shared fragments) Obtained by Pairwise Comparison for all *Saccharomyces cerevisiae* Strains[a]**

	BRAS 12	BRAS 25	BRAS 26	BRAS 291	BRAS 299	BRAS 33	BRAS 40	BRAS 41	BRAS 52A	MUCL 20479	BRAS 212	BRAS 213	BRAS 214	BRAS 223	BRAS 240 A	BRAS 240 B	BRAS 268	BRAS 270	BRAS 349	MUCL 28356	MUCL 28357	MUCL 28362	MUCL 28734	MUCL 20475	MUCL 39516	Sigma 1278b
BRAS 12	1,00																									
BRAS 25	0,64	1,00																								
BRAS 26	0,53	0,67	1,00																							
BRAS 291	0,77	0,80	0,59	1,00																						
BRAS 299	0,81	0,77	0,63	0,89	1,00																					
BRAS 33	0,85	0,72	0,59	0,77	0,81	1,00																				
BRAS 40	0,72	0,67	0,61	0,72	0,85	0,80	1,00																			
BRAS 41	0,77	0,88	0,65	0,92	0,89	0,85	0,80	1,00																		
BRAS 52A	0,74	0,85	0,63	0,81	0,79	0,81	0,69	0,89	1,00																	
MUCL 20479	0,81	0,85	0,57	0,89	0,86	0,81	0,69	0,89	0,93	1,00																
BRAS 212	0,48	0,42	0,48	0,48	0,54	0,56	0,58	0,48	0,38	0,38	*1,00*															
BRAS 213	0,60	0,83	0,74	0,73	0,71	0,67	0,62	0,80	0,71	0,71	*0,41*	*1,00*														
BRAS 214	0,65	0,73	0,62	0,77	0,75	0,71	0,67	0,84	0,75	0,75	*0,40*	*0,74*	*1,00*													
BRAS 223	0,61	0,82	0,58	0,87	0,83	0,70	0,73	0,87	0,75	0,75	*0,55*	*0,74*	*0,71*	*1,00*												
BRAS 240 A	0,29	0,50	0,41	0,48	0,36	0,38	0,20	0,48	0,45	0,45	*0,30*	*0,40*	*0,38*	*0,44*	*1,00*											
BRAS 240 B	0,64	0,92	0,67	0,88	0,77	0,72	0,67	0,88	0,77	0,77	*0,50*	*0,83*	*0,73*	*0,91*	*0,50*	*1,00*										
BRAS 268	0,64	0,74	0,67	0,71	0,76	0,79	0,74	0,79	0,69	0,69	*0,74*	*0,69*	*0,67*	*0,72*	*0,43*	*0,74*	*1,00*									
BRAS 270	0,77	0,88	0,71	0,77	0,81	0,85	0,80	0,85	0,74	0,74	*0,56*	*0,80*	*0,71*	*0,78*	*0,38*	*0,88*	*0,79*	*1,00*								
BRAS 349	0,69	0,96	0,71	0,85	0,81	0,77	0,72	0,92	0,81	0,81	*0,48*	*0,87*	*0,77*	*0,87*	*0,48*	*0,96*	*0,79*	*0,92*	*1,00*							
MUCL 28356	0,31	0,26	0,25	0,31	0,30	0,31	0,26	0,25	0,30	0,36	*0,32*	*0,28*	*0,38*	*0,28*	*0,37*	*0,26*	*0,35*	*0,25*	*0,25*	*1,00*						
MUCL 28357	0,50	0,59	0,61	0,57	0,55	0,57	0,52	0,64	0,55	0,55	*0,59*	*0,63*	*0,61*	*0,56*	*0,52*	*0,59*	*0,73*	*0,57*	*0,64*	*0,35*	*1,00*					
MUCL 28362	0,67	0,69	0,69	0,67	0,79	0,74	0,85	0,74	0,71	0,64	*0,46*	*0,65*	*0,63*	*0,67*	*0,27*	*0,69*	*0,62*	*0,81*	*0,74*	*0,24*	*0,48*	*1,00*				
MUCL 28734	0,58	0,67	0,67	0,71	0,69	0,71	0,67	0,77	0,69	0,69	*0,53*	*0,74*	*0,72*	*0,64*	*0,46*	*0,67*	*0,79*	*0,65*	*0,71*	*0,49*	*0,73*	*0,63*	*1,00*			
MUCL 20475	0,72	0,83	0,61	0,88	0,85	0,80	0,75	0,96	0,85	0,85	0,50	0,76	0,80	0,82	0,50	0,83	0,74	0,80	0,88	0,26	0,59	0,69	0,73	1,00		
MUCL 39516	0,64	0,92	0,67	0,72	0,69	0,64	0,58	0,80	0,77	0,77	0,42	0,76	0,67	0,73	0,40	0,83	0,67	0,80	0,88	0,19	0,59	0,62	0,60	0,75	1,00	
Sigma 1278b	0,38	0,56	0,41	0,54	0,59	0,54	0,48	0,62	0,59	0,59	0,40	0,53	0,65	0,87	0,29	0,48	0,50	0,46	0,54	0,38	0,43	0,44	0,52	0,64	0,48	1,00

[a] The first 10 strains (in bold) are bottom-fermenting yeasts.

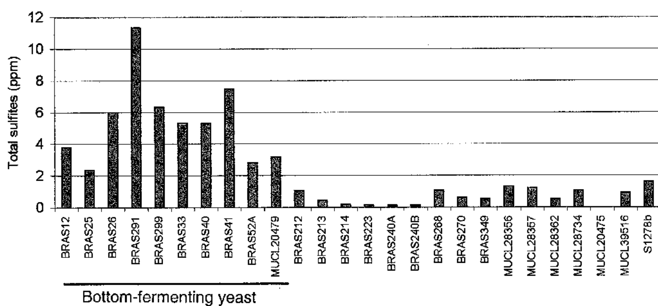

Fig. 3. Total sulfite excretion (ppm) by the 26 yeast strains assayed (bottom-fermenting yeasts on the left).

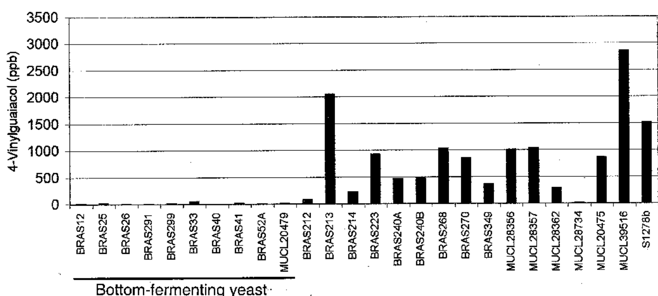

Fig. 4. 4-Vinylguaiacol production (ppb) by the 26 yeast strains assayed (bottom-fermenting yeasts on the left).

should therefore help brewers to monitor yeast evolution in the course of conservation.

Among the 26 strains studied, the average percentage of shared fragments was 55%. The similarity indicator was higher for bottom-fermenting strains (72%) than for top-fermenting yeasts (45%). A 158-bp fragment was found only in bottom-fermentation yeasts, which were also characterized by low levels of 4-vinylguaiacol and high sulfite levels.

LITERATURE CITED

1. Aigle, M., Erbs, D., and Mool, M. Some molecular structures in the genome of lager brewing yeast. *J. Am. Soc. Brew. Chem.* 43:1, 1984.
2. Ausubel, F. M., Brent, R., Klingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. *Current Protocols in Molecular Biology,* Vol.2. Wiley and Sons, New York, 1994.
3. Barnett, J. A., Payne, R. W., and Yarrow, D. *Yeast, Characteristics and Identification*, 2nd ed. Cambridge University Press, Cambridge, U.K., 1992.
4. Breyne, P., Boerjan, W., Gerats, T., Van Montagu, M., and Van Gijsel, A. Applications of AFLP in plant breeding, molecular biology and genetics. *Belg. J. Bot.* 129:107-117, 1997.
5. Casey, G. P, Pringle, A. T., and Erdmann, P. A. Evaluation of recent techniques used to identify individual strains of Saccharomyces yeast. *J. Am. Soc. Brew. Chem.* 48:100-106, 1990.
6. de Barros Lopes, M., Rainieri, S., Henschje, P. A., and Langridge, P. AFLP fingerprinting for analysis of yeast genetic variation. *Int. J. Syst. Bacteriol.* 49:915-924, 1999.
7. De Riek, J., Dendauw, J., Mertens, M., De Loose, M., Heursel, J., and Van Blockstaele, E. Validation of criteria for the selection of AFLP

markers to assess the genetic variation of a breeder's collection of evergreen azaleas. *Theoret. Appl. Genet.* 99:1155-1165, 1999.

8. Lee, S. Y., and Knusden, F. B. Differentiation of brewery yeast strains by restriction endonulcease analysis of their mitochondrial DNA. *J. Inst. Brew.* 91:169-173, 1985.
9. Naumov, G. I. Genetic idendification of biological species in *Saccharomyces* sensu stricto complex. *Can. J. Microbiol.* 42:335-339, 1996.
10. Robert, V., de Bien, J. E., Buyck, B., and Hennebert, G. L. ALLEV, a new program for computer-assisted identification of yeast. *Taxon* 43:433-439, 1994.
11. Stone, I., and Laschiver C. American Society of Brewing Chemist. Report of Subcommittee on SO_2 in Beer. *Am. Soc. Brew. Chem. Proc., 1960*, p. 210.
12. Thalacker, R., Ihring, H. H., Muskat, E., and Wagner, B. *Monatsch. Brau*. 27:166, 1974.
13. Vaughan-Martini, A., and Martini A. Facts, myths and legends on the prime industrial mucroorganism. *J. Ind. Microbiol.* 14:514-522, 1995.
14. Vos, P., Hogers, R., Bleekers, M., Reijans, M., van de Lee, T., Hornes, M., Freijters, A., Pot Peleman, J., Kuiper, M., and Zabeau, M. AFLP™: A new technique for DNA fingerprinting. *Nucleic Acid Res.* 21:4407-4414, 1995.
15. Walmsley, R. M. DNA fingerprinting of yeast. *Ferment* 7:231-234, 1994.
16. Zabeau, M., and Vos, P. Selective restriction fragment amplification: A general method for DNA fingerprinting. European patent application EP 0534858, 1993.

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