

Diversity of *Saccharomyces cerevisiae* strains isolated of the spontaneous fermentation of *cachaça* from northeastern Brazil**Diversidade de linhagens de *Saccharomyces cerevisiae* isoladas de fermentações espontâneas de *cachaça* do nordeste Brasileiro**

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ABSTRACT

Cachaça is a beverage obtained by distilling fermented sugar cane juice. The state of Bahia in northeastern Brazil is the second-largest producer of traditional cachaça, and this region has the potential to improve the quality and quantity of its beverage production. The aim of this study was to analyze the genetic diversity of *Saccharomyces cerevisiae* populations isolated from must in six distilleries in Bahia using mitochondrial DNA restriction fragment length polymorphism (mtDNA-RFLP). Among the three hundred and thirty *S. cerevisiae* strains isolated, mtDNA-RFLP analysis identified a total of 30 molecular patterns. Analysis of molecular variance (AMOVA) revealed that the greatest genetic variation was found among, rather than within, the populations. Population structure analyses showed the presence of three distinct gene pools, thereby corroborating the AMOVA results. This study represents an important contribution to better understanding the molecular characterization and genetic variability of *S. cerevisiae* strains during the fermentation of cachaça. The dominant molecular patterns identified here may be used to select *S. cerevisiae* strains that could improve the quality and volume of traditional cachaça production in Bahia.

Keywords: traditional cachaça, *Saccharomyces cerevisiae*, molecular characterization, mtDNA-RFLP

RESUMO

A cachaça é uma bebida obtida pela destilação do suco de cana de açúcar fermentado. O estado da Bahia, no nordeste do Brasil, é o segundo maior produtor de cachaça tradicional, e essa região tem potencial para melhorar a qualidade e a quantidade de sua produção de bebidas. O objetivo deste estudo foi analisar a diversidade genética de populações de *Saccharomyces cerevisiae* isoladas de mosto em seis destilarias da Bahia, utilizando polimorfismo de comprimento de fragmento de restrição de DNA mitocondrial (mtDNA-RFLP). Entre as trezentas e trinta cepas de *S. cerevisiae* isoladas, a análise do mtDNA-RFLP identificou um total de 30 padrões moleculares. A análise de variância molecular (AMOVA) revelou que a maior variação genética foi encontrada entre as populações, e não dentro delas. As análises da estrutura populacional mostraram a presença de três conjuntos genéticos distintos, corroborando os resultados da AMOVA. Este estudo representa uma contribuição importante para o melhor entendimento da caracterização molecular e variabilidade

genética de linhagens de *S. cerevisiae* durante a fermentação da cachaça. Os padrões moleculares dominantes identificados aqui podem ser usados para selecionar linhagens de *S. cerevisiae* que possam melhorar a qualidade e o volume da produção tradicional de cachaça na Bahia.

Palavras-chave: cachaça tradicional, *Saccharomyces cerevisiae*, caracterização molecular, mtDNA-RLPF

1 INTRODUÇÃO

According to Brazilian legislation, normative instruction number 13 of 29 June 2005, *cachaça* is a beverage with an alcoholic content of between 38 and 48 %, and it is made by distilling the must from fermented sugar cane juice in copper alembics. Brazil produces approximately 1.3 billion liters of industrial and traditional *cachaça* per year. The state of Bahia in northeastern Brazil is currently the second-largest producer of traditional *cachaça*. Bahia's market produces approximately 1.8 million liters of small-scale, craft-produced *cachaça*. It is estimated that around 300 small rural establishments are involved in the production of sugar cane derivatives (molasses, unprocessed brown sugar, and *cachaça*), employing approximately 60,000 people. Although current production is less than two million liters, the total production capacity of the state is 3.5 million liters per year (<http://www.sicm.ba.gov.br>).

Traditional distilleries in Brazil produce the beverage by spontaneous fermentation or, less frequently, using baker's yeast. The natural starter culture is prepared by various methods, including developing the fermentative microbiota in the sugar cane juice alone or mixing the sugar cane juice with crushed corn and rice or soy meal. This process occurs inside the fermentation vat and can last from 5 to 20 days (until the yeast population is sufficient to initiate the fermentative cycle). Fresh sugar cane juice diluted to 16° Brix is added to this natural starter culture, and after 18-30 hours, 4/5 of the fermented must is distilled in copper alembics, and fresh sugar cane juice is added to start a new fermentation cycle (eg. Pataro et al. 2000, 2002, Badotti et al. 2010, Barbosa et al. 2016).

The traditional alcoholic fermentation process for *cachaça* production involves many different yeast species with an overwhelming prevalence of *S. cerevisiae*. Different indigenous *S. cerevisiae* strains grow in the vat during the production period, and each of these strains may contribute to the quality of the *cachaça* (eg. Pataro et al., 2000; Gomes et al., 2007, Barbosa et al. 2016, Araujo et al. 2018).

The high genotypic diversity of microbes present during the *cachaça* fermentation season strongly indicates the need to differentiate *S. cerevisiae* strains. Molecular polymorphic analyses is essential to characterize individuals within a species. Mitochondrial DNA restriction analysis (mtDNA-RFLP) has been extensively used to characterize and differentiate indigenous *S. cerevisiae* strains (eg. Badotti et al., 2010, Barbosa et al. 2018). The main objectives of this study were to

determine the diversity of *S. cerevisiae* strains present in *cachaça* distilleries located in northeastern Brazil by mtDNA-RFLP.

2 MATERIALS AND METHODS

Isolation and physiological identification of *Saccharomyces cerevisiae* strains

The *S. cerevisiae* strains were isolated from must in six *cachaça* distilleries in the state of Bahia, Brazil (Figure 1). The distillery 1 is localized in Ibirataia (39° 40' 20" W and 13° 56' 48" S), the distillery 2 in Jaguaripe (38° 53' 45" W and 13° 06' 47" S), the distillery 3 in Ilhéus city (39° 11' 36" W and 14° 52' 59" S), the distillery 4 in Condeúba (41° 58' 19" W and 14° 54' 09" S), the distillery 5 in Caculé (42° 13' 23" W and 14° 30' 02" S) and the distillery 6 in Rio de Contas city (41° 48' 46" W and 13° 35' 11" S). The samples were collected at every 2° Brix decrease from the beginning of spontaneous fermentation until the end of the cycle. The initial sugar concentration in the fermentation process varied from 10° to 23° Brix in the distilleries studied. The concentration of soluble solids (°Brix) was measured using a saccharimeter. Serial 10-fold dilutions were aseptically spread in triplicate onto SCY agar (10 % sugar cane juice, 0.1 % yeast extract, 2 % agar and 0.01 % chloramphenicol). The plates were incubated at room temperature (25 ± 3° C) for 2 days. Plates containing 30 to 300 colonies were examined, and five isolates of the most prevalent morphotype were isolated and purified on the basis of cultural characteristics. When possible, at least three colonies of the other morphotypes were also selected for further analysis.

S. cerevisiae isolates were purified using modified Sabouraud agar (2 % glucose, 0.5 % yeast extract, 1 % peptone and 2 % agar). For cryopreservation, the pure cultures were inoculated in GYMP medium (2 % glucose, 0.5 % yeast extract, 1 % malt extract, and 0.2 % sodium phosphate) at 28 °C under agitation (200 rev.min⁻¹). After 24 hours (h) incubation, 15 % sterile glycerol was added, and the tubes were stored at -85 °C. The *S. cerevisiae* strains were identified using physiological characters and taxonomic keys by Kurtzman et al. (2011). The isolates were deposited in the Culture Collection of Microorganisms of Bahia (CCMB / UEFS, Bahia, Brazil).

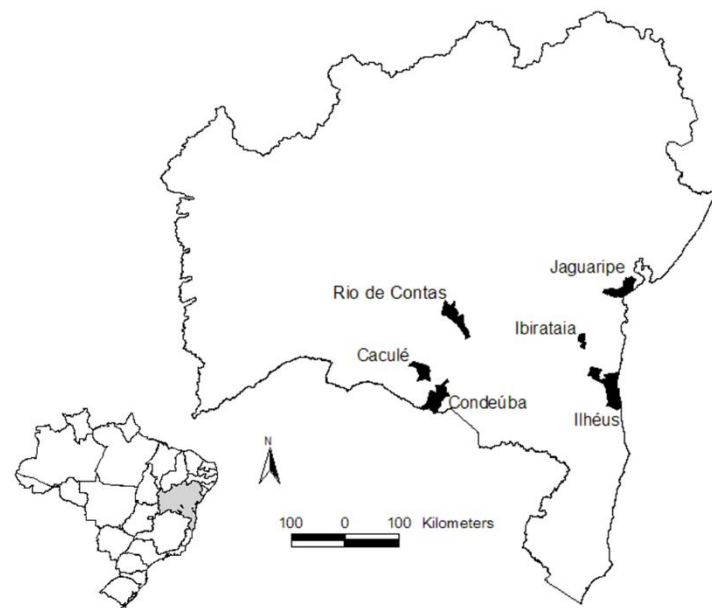


Figure 1. Map of the state of Bahia showing the regions (cities) where must samples were collected.

2.2 MITOCHONDRIAL DNA RESTRICTION ANALYSIS (MTDNA-RFLP)

Mitochondrial DNA (mtDNA) was isolated as described by Querol et al. (1992) with some modifications (Comi et al., 2000). The mtDNA was digested using the *Hinf*I restriction endonuclease (Invitrogen, Life technologies, USA), and the fragments were separated by agarose gel electrophoresis (1.5 % in TBE buffer), stained with ethidium bromide, visualized under UV light, and photographed.

2.3 ANALYSIS OF DIVERSITY OF THE *S. CEREVISIAE* POPULATIONS

The frequency of the molecular patterns was calculated as the percentage of patterns found relative to the number of colonies analyzed (Querol et al., 1994) for each collection period (°Brix). Abundance was assessed by the number of molecular patterns identified at each distillery. The molecular patterns were processed using the Gelcompar II software, version 5.0 (Applied Maths NV, Sint-Martens-Latem, Belgium) to normalize the bands to the molecular marker (1 Kb Plus DNA Ladder™; Invitrogen, Life technologies, USA). Data were scored for the presence (1) or absence (0) of bands using a binary matrix. The number of bands (N), the number of exclusive bands (NLE), and the proportion of polymorphic bands (P) were measured using GenAlex version 6.1 software (Peakall and Smouse, 2006), assuming Hardy-Weinberg equilibrium, as previously described Lynch and Milligan (1994). Analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed with 999 permutations using the GenAlex software (Peakall and Smouse, 2006).

The presence/absence matrix were built for the multivariate analyses, using the unweighted pair group method with arithmetic mean and the Jaccard similarity index using the vegan package (Oksanen et al. 2013) in R 3.0.2 (R Development Core Team 2008).

3 RESULTS

From the six distilleries studied, 358 yeast strains were isolated and identified based on physiological tests; 330 were identified as *S. cerevisiae*, and 28 isolates were designated as *non-Saccharomyces* yeasts and then not included in this work. The mtDNA profiles of all 330 *S. cerevisiae* isolates were characterized. The distilleries showed the presence of at least one dominant molecular pattern throughout the fermentation cycle. A total of 30 different molecular patterns were found (Figure 2), and varying over time during the fermentation process, highlighting distillery 6 and 3 (Figure 3, Table 1). The molecular patterns were exclusive to each distillery. The greatest richness was found in distilleries 3 and 6, which exhibited eight and seven mtDNA patterns, respectively (Figure 2 and 4). The concentrations of soluble solids (° Brix) was very similar among distilleries, but with higher initial values in distillery 6 than the general average. In addition, the temperatures (°C) in the distillery 5 were above that observed for other distilleries at all time intervals, and the smallest variation was observed in distillery 4 (Figure 4). Our comparison between the study distilleries, performed with a presence/absence matrix, revealed no molecular similarities (Figure 3). Distilleries 1 and 4 showed five different molecular patterns, and the lowest diversity was found in distilleries 2 and 5 with three and two molecular patterns, respectively. Table 1 shows the frequencies of the molecular mtDNA patterns obtained for the *S. cerevisiae* isolates from the six distilleries studied. A total of 40 bands, of which only three were exclusive, were identified from all of the *S. cerevisiae* strains. Distilleries 3 (Ilhéus) and 6 (Rio de Contas) showed the greatest number of bands (34 and 33, respectively), representing 77.5 % of polymorphic bands, followed by distillery 1 (Ibirataia) with 28 bands and 62.5 % of polymorphic bands. The yeast population from distillery 2 showed 24 bands (47.5 % of polymorphic bands), and distilleries 4 and 5 showed 18 and 19 bands, respectively, representing 35 % of bands polymorphism.

Figure 2. Different mtDNA-RFLP patterns of the *Saccharomyces cerevisiae* strains present in spontaneous fermentative cycles from the six *cachaça* distilleries studied. Lanes 1 and 32: 1 kb ladder; the numbers on the right indicate the molecular weights (bp) of the DNA fragments. The codes on the top represent the mtDNA patterns found in each distillery. The dominant molecular patterns were 1A, 1E, 2A, 2C, 3A, 3B, 4A, 5A, 6A, 6C and 6D.

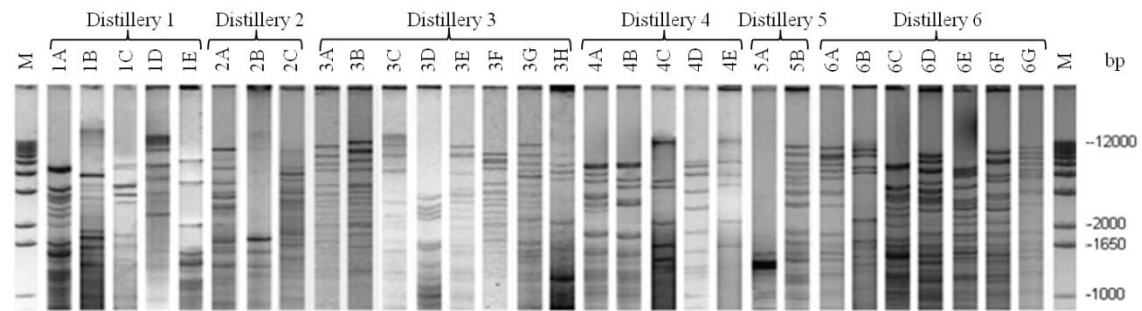


Table 1. Frequencies of the molecular patterns obtained from the mtDNA-RFLP analysis of the *S. cerevisiae* strains isolated from six *cachaça* distilleries in northeastern Brazil at different stages of the fermentation cycle. Concentrations of soluble solids ($^{\circ}$ Brix), times, temperatures of the sugar cane must and number of isolates are indicated.

Distillery 1	T0	T1	T2	T3	T4	T5	T6	T7	T8
Brix ($^{\circ}$)	13	11	9.5	7	5	3.5	2	0	-
Time (h)	0	0.58	1.40	2.40	4.00	5.10	5.56	8.00	
Temperature ($^{\circ}$ C)	21	24	27	28	30	34	35	34	-
Number of isolates	7	6	7	8	7	4	7	10	
Pattern 1A ¹	4	3.7	9.2	7.4	5.5	5.5	7.4	12.9	-
Pattern 1B ¹	0	1.8	0	0	0	0	0	0	-
Pattern 1C ¹	0	1.8	0	0	0	0	0	0	-
Pattern 1D ¹	0	0	0	1.8	0	0	0	0	-
Pattern 1E ²	5	3.7	3.7	5.5	7.4	1.8	3.7	5.5	-
Distillery 2	T0	T1	T2	T3	T4	T5	T6	T7	T8
Brix ($^{\circ}$)	14	11	9	8	6	5	2	0	-
		10.0	20.0	30.0	40.0	50.0	68.0	82.2	
Time (h)	0	0	0	0	0	0	5	5	
Temperature ($^{\circ}$ C)	27	28	28	30	30	31	30	31	-

Number of isolates	5	8	4	7	6	7	5	10	
5.									
Pattern 2A ¹	7	9.6	5.7	5.7	5.7	7.6	5.7	13.4	-
Pattern 2B ¹	0	0	0	0	0	1.9	0	0	-
3.									
Pattern 2C ²	8	5.7	1.9	7.6	5.7	3.8	3.8	5.7	-
Distillery 3	T0	T1	T2	T3	T4	T5	T6	T7	T8
Brix (°)	13	11	9	7	5	3	1.6	0	-
						10.4	12.5	15.2	
Time (h)	0	1.30	4.00	5.50	8.15	4	0	5	-
Temperature (°C)	24	26	27	29	31	33	33	34	-
Number of isolates	3	9	8	4	4	7	4	10	
2.									
Pattern 3A ^{1,2}	5	12.5	7.5	5	5	12.5	5	15	-
Pattern 3B ^{1,2}	5	0	2.5	5	2.5	0	0	2.5	-
Pattern 3C ¹	0	0	0	0	0	2.5	0	0	-
Pattern 3D ¹	0	0	0	0	0	0	2.5	0	-
Pattern 3E ¹	0	0	0	0	0	0	0	2.5	-
Pattern 3F ²	0	2.5	0	0	0	0	0	0	-
Pattern 3G ²	0	0	5	0	0	0	0	0	-
Pattern 3H ²	0	0	0	0	2.5	0	0	0	-
Distillery 4	T0	T1	T2	T3	T4	T5	T6	T7	T8
Brix (°)	10	8	6	4	2	0	-	-	-
		12.0	22.4	33.1	47.5	73.1			
Time (h)	0	0	8	6	6	1	-	-	-
Temperature (°C)	25	27	28	29	29	28	-	-	-
Number of isolates	7	8	10	7	5	18			
Pattern 4A ^{1,2}	13	13.2	18.8	13.2	5.6	28.3	-	-	-
Pattern 4B ¹	0	1.8	0	0	0	0	-	-	-

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Pattern 4C ¹	0	0	0	0	1.8	0	-	-	-
Pattern 4D ¹	0	0	0	0	1.8	0	-	-	-
Pattern 4E ¹	0	0	0	0	0	1.8	-	-	-
Distillery 5	T0	T1	T2	T3	T4	T5	T6	T7	T8
Brix (°)	16	14	12	10	8	6	4	2	0
Time (h)	0	1.43	2.47	3.03	4.00	4.43	5.02	5.53	6.20
Temperature (°C)	24	31	33	35	37	38	38	39	38
Number of isolates	7	6	5	7	6	5	6	6	10
Pattern 5A ^{1,2}	12	10.3	8.6	12	10.3	6.8	8.6	29.3	10
Pattern 5B ¹	0	0	0	0	0	0	1.7	0	0
Distillery 6	T0	T1	T2	T3	T4	T5	T6	T7	T8
Brix (°)	23	20	17	14	11	8	5	2	0
			10.5	14.0	19.4	26.0	35.2	53.2	73.1
Time (h)	0	6.55	5	5	5	5	8	5	5
Temperature (°C)	24	29	31	32	33	33	33	30	28
Number of isolates	7	6	7	5	7	6	7	6	9
Pattern 6A ^{1,2}	6	6.6	6.6	5	8.3	1.6	3.3	10	1.6
Pattern 6B ¹	6	0	0	0	0	0	0	0	1.6
Pattern 6C ^{1,2}	6	3.3	1.6	1.6	3.3	3.3	1.6	0	0
Pattern 6D ^{1,2}	0	0	1.6	1.6	0	1.6	1.6	0	0
Pattern 6E ¹	0	0	0	0	0	3.3	0	0	0
Pattern 6F ^{1,2}	0	0	1.6	0	0	0	5	0	0
Pattern 6G ²	6	0	0	0	0	0	0	0	0

¹ mtDNA patterns obtained from colonies with different morphotypes.

² mtDNA patterns obtained from colonies with common morphotypes

The genetic divergence among and within populations was also analyzed using the AMOVA. We observed greater genetic variation ($P < 0.001$) among populations from different distilleries (71 %) rather than within them (29 %).

Figure 3. Dendrogram of cluster analysis using the Jaccard similarity index and the unweighted pair group method with arithmetic mean of the *Saccharomyces cerevisiae* strains isolated of the spontaneous fermentation of cachaça from northeastern Brazil.

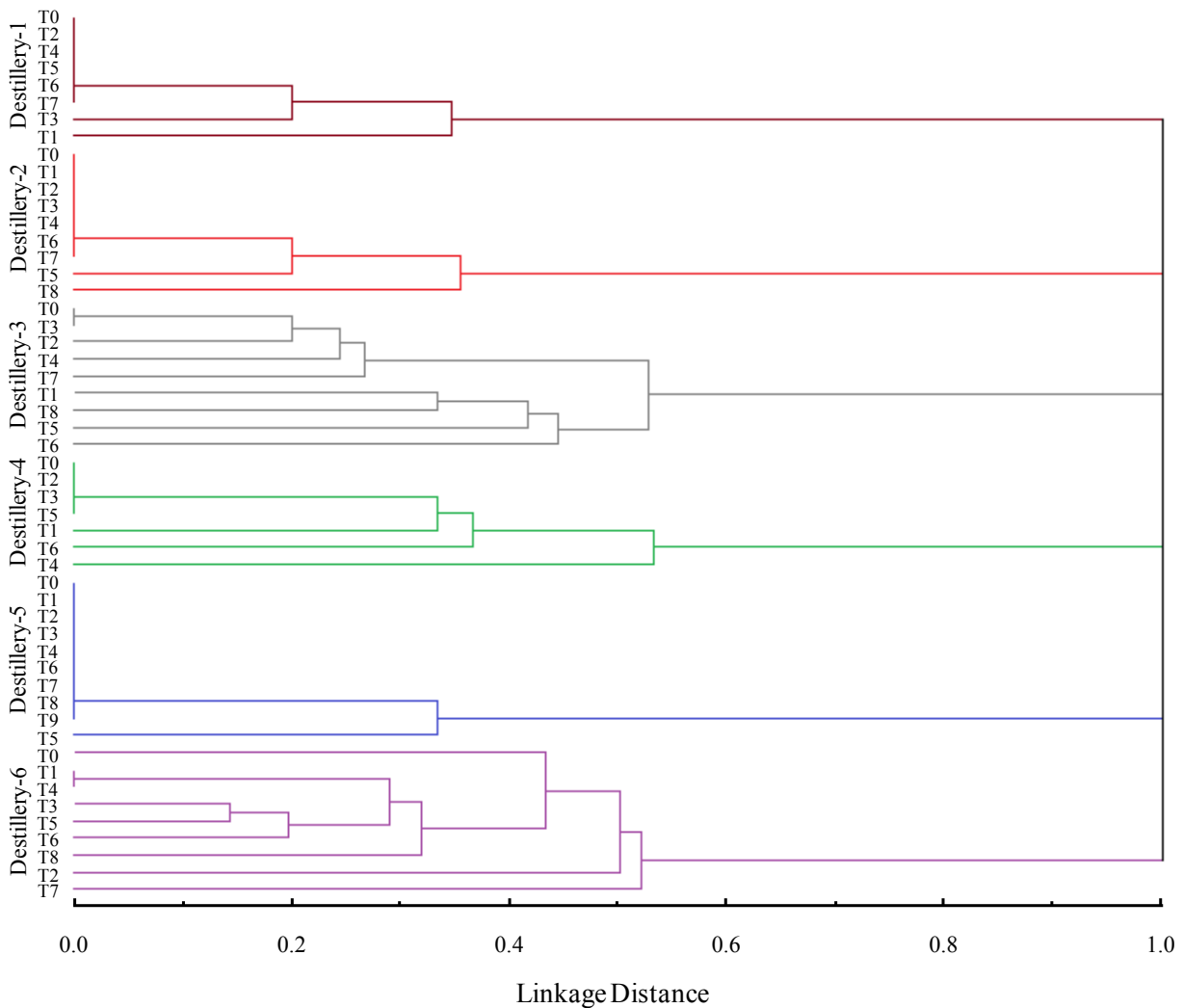
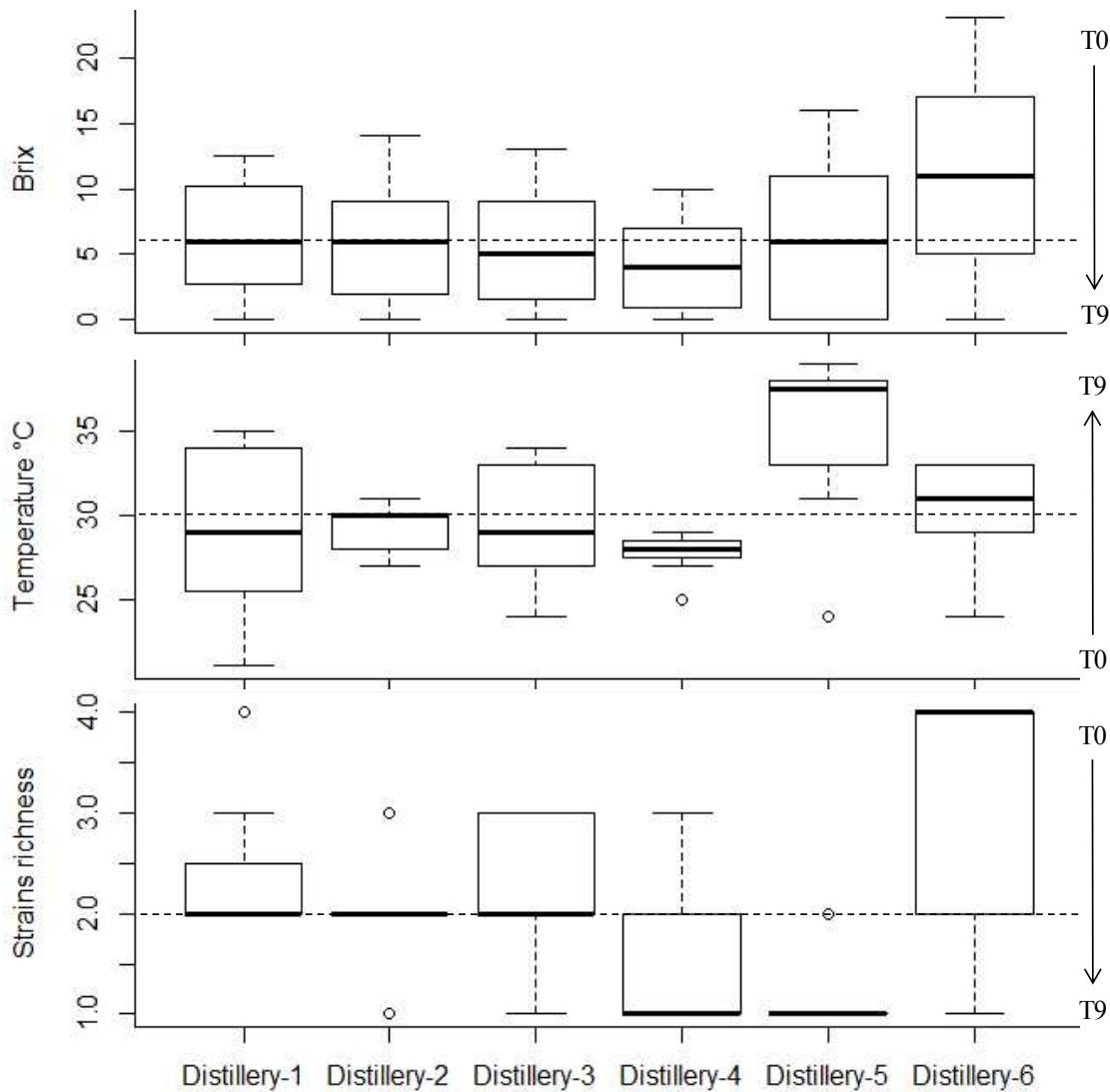


Figure 4. Comparison among the medians of the concentrations of soluble solids ($^{\circ}$ Brix), temperatures ($^{\circ}$ C), and richness of *Saccharomyces cerevisiae* strains isolated from sugar cane must, in six distilleries from northeastern Brazil. Dotted line represents the median of all distilleries. Right side indicates factor trend over time (T0 to T9).



4 DISCUSSION

In this study, the presence of 30 molecular patterns for *S. cerevisiae* strains was observed during the fermentation cycle in six distilleries using the mtDNA-RFLP technique. Colonies with different dominant morphotypes showed the same molecular patterns in most distilleries, similar to the observed by Araujo et al. (2007). These results demonstrate that criteria based only on morphological characterization are inadequate for distinguishing *S. cerevisiae* strains. Furthermore, the presence of different molecular patterns for strains of dominant morphotypes was observed in most of the distilleries (1, 2, 3 and 6), reinforcing that mtDNA-RFLP analysis is a useful technique to study the diversity of *S. cerevisiae* populations in *cachaça* fermentations.

The 30 molecular patterns detected for the *S. cerevisiae* populations revealed a high diversity among the strains in the microenvironments studied. Among the molecular patterns detected, one or two were found with high frequency in all of the distilleries, indicating that these strains are able to drive the fermentation process. Therefore, studies focused on the physiological and adaptive

characteristics of these strains could significantly contribute to improving the fermentation process of northeastern Brazil's *cachaça* producers. Badotti et al. (2010) obtained similar results in a study evaluating *S. cerevisiae* strains from different *cachaça*-producing areas in Brazil; in that study, specific *S. cerevisiae* population from each area presented mtDNA patterns that were not found in other regions.

Studies examining populations of *S. cerevisiae* in wine fermentation also demonstrated that certain molecular patterns dominate (Guillamón et al., 1996; Esteve-Zarzoso et al., 2000). These studies found that *S. cerevisiae* yeast strains are highly adapted to specific climate and substrate conditions. In *S. cerevisiae* populations from *cachaça*, stress factors, including high temperature, high sugar concentration at the beginning of fermentation and high ethanol concentration at the end of fermentation (which could induce mutagenic effects on mtDNA), seem to be the relevant factors determining the population structure (Pataro et al. 2000, Boulton and Quain, 2001, Badotti et al., 2010, Barbosa et al., 2016).

Finally, the dominant molecular patterns identified here could be used for further studies aimed at selecting specific *S. cerevisiae* strains that are well adapted to each *cachaça*-producing region. These selected strains could be used as starter strains to improve the quality of *cachaça* in their specific regions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no financial or commercial conflicts of interest.

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