DIVERSITY OF *SACCHAROMYCES CEREVISIAE* YEASTS ASSOCIATED TO SORGHUM BEER AND PALM WINES REVEALED BY INTERDELTA SEQUENCE TYPING

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Abstract
The aim of the present work was to assess the genetic diversity of fermenting Saccharomyces cerevisiae strains found in the natural niches from Côte d'Ivoire in order to constitute a strain collection representing the Ivorian's fermented food products biodiversity wealth as a basis for future strain selection and domestication programs. For this purpose, a total of 274 yeast strains isolated from three traditional alcoholic beverages (raffia wine, palm oil wine and tchapalo) through 13 production areas were identified with NTS2 PCR-RFLP, sequencing of the D1/D2 domain of the LSU rDNA and typed with interdelta PCR analysis. A total of 21 different interdelta profiles, corresponding to 21 different biotypes were found among the 115 isolates of raffia wine, 30 biotypes among the 92 isolates of palm wine and 20 biotypes among the 67 isolates of tchapalo. Most of these strains were specific to a region under study for each beverage. Saccharomyces cerevisiae diversity in tchapalo was the same for all the regions under study (an average of 4 profiles for 10 isolates). For raffia and palm oil wines, S. cerevisiae diversity was low at Adzopé and Bingerville respectively compared to the other sampling areas. The strains of biotypes Riii (20%) and Rii (18.26) in the raffia wines, Pviii (14.1%) and Pv (10.8%) in palm oil wines and those of biotype Txvi (14%), Tviii (10.5%) and Txii (10.5%) in the tchapalo beers were the dominant strains in the three beverages.

Keywords: Interdelta,palm wine, *Saccharomyces cerevisiae,* biotype, tchapalo, typing

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1. INTRODUCTION

Saccharomyces cerevisiae is yeast belonging to the phylum Ascomycota and to the family Saccharomycetaceae. It used by humans for thousands of years and is the main actor involved in various fields of biotechnology. Thus, it plays a very important role in the food industry as fermentation agent to produce bread, wine, beer, etc. In the pharmaceutical and medical field, it is used for the production of vaccines, probiotics or proteins such as insulin (Roberts and Olivier, 2011). In the chemical industry, it also plays a key role in the synthesis of commodity products such as lactic acid for the production of plastics. It is also used in the field of renewable energies for the production of biofuels (Aldiguier *et al*., 2004; Cot, 2006).

In Côte d'Ivoire, as everywhere in Africa, *S. cerevisiae* is also the species most encountered in the wild state in traditional fermentations (Jespersen, 2003). It takes part with other yeasts in the alcoholic fermentation of a variety of traditional fermented beverages such as palm oil wine, raffia wine and tchapalo beer, three traditional beverages that play an important role in traditional customs and population diet (Jespersen, 2003). These Ivoirian traditional fermented beverages constitute then an important genetic reserve of wild strains of baker's yeast whose diversity remains little explored.

However, the interest this yeast stir up today is due to its large genotypic diversity. Thus, in

winery, the distinctive character of many wines can be linked to particular strains of *S. cerevisiae* involved in the fermentation (Romano *et al*., 2008). Consequently, differentiation of yeasts at the subspecies level is an important requirement. Indeed, differentiation at the subspecies level is a fundamental step to investigate the biodiversity of this yeast and to examine population dynamics during the fermentative process (Lopes *et al*., 2002; Cappello *et al*., 2004; Lopandic *et al*., 2008). Intraspecies classification of *S. cerevisiae* is also essential for the selection and monitoring of a fermentation starter (Lopes *et al*., 2007) and for unequivocally distinguishing clinical isolates responsible for infections in immunodepressed patients (McCullough *et al*., 1998).

Several molecular methods have been developed for this purpose to identify and discriminate *S. cerevisiae* at the species and/or subspecies. Those methodologies include restriction fragment length polymorphisms (RFLP) of genomic and mitochondrial DNA (Querol *et al*., 1992; Torriani *et al*., 1999), chromosome karyotyping (Schuller *et al*., 2004), randomly amplified polymorphic DNA (RAPD) (Ness *et al*., 1993; de Barros Lopes *et al*., 1998), amplified fragment length polymorphism (AFLP) (de Barros Lopes *et al*., 1999), application of microsatellite markers (Techera *et al*., 2001; Legras *et al*., 2005), the amplification of genomic sequence blocks flanked by delta elements (Ness *et al*., 1993; Legras and Karst, 2003) and, recently, single nucleotide polymorphisms (SNPs) (Jubany *et al*., 2008).

Among these methodologies, inter-delta PCR is one of the most used to differentiate *S. cerevisiae* isolates from fermented foods and beverages. Indeed, the *S. cerevisiae* genome contains repetitive DNA sequences, such as delta sequences, that are frequently associated with the Ty1 and Ty2 transposons. The number and location of these elements have a certain intraspecific variability that can be used as a genetic fingerprint to identify strains of *S. cerevisiae*. This method developed by Ness *et al*. (1993) is suitable for the characterization of high numbers of strains because it is easy to perform, cheap and rapid. Furthermore, due to its high reproducibility, discriminatory power and good time-requirement/price relationship, this method compares favourably with other techniques such as AFLP, MSP-PCR, RFLPmt, RAPD and PFGE that have also been used to differentiate strains during wine fermentations (Xufre *et al*., 2011). Recently, alternative primers (d12 and d21) have been designed by Legras and Karst (2003) to improve this method. The combination of these primers (d12/d21 or d12/d2) increased the discriminatory power of the method (Legras *et al*., 2005).

In the present study, interdelta PCR was used to differentiate and regroup *S. cerevisiae* isolates in biotype according to their profile generated on electrophoresis gel after amplification of regions included between two LTRs using two specific primers (delta 12 and delta 21) during spontaneous fermentations of three traditional fermented beverages collected in south-eastern of Côte d'Ivoire. The first step in yeast selection involved knowledge of the diversity of wild yeasts in each production area. The principal aim of the present work was to evaluate their genetic diversity and to know which strains are dominant in the fermentation process in order to constitute a strain collection representing the Ivorian's fermented food products biodiversity wealth as a basis for future strain selection and domestication programs.

2. MATERIAL AND METHODS

2.1. Sample collection and yeast isolation

Samples of raffia wine (16), palm oil wine (20) and sorghum beer or tchapalo (16) were collected with local producers in 26 different sites in Côte d'Ivoire. The sampling sites for raffia wine were located at Grand-Lahou, Alépé, Adzopé and Abengourou; those for palm oil wine were located at Bingerville, Bonoua, Grand-Lahou, Alépé and Attinguié while those for tchapalo were located at Adjamé, Yopougon, Abobo and Bingerville.

Samples were collected at weekly intervals into pre-sterilized 250 ml Plexiglas containers which were immediately immersed in an isothermal box and brought to the laboratory. The yeast colonies were isolated on Yeast Extract Peptone Dextrose (YPD) agar and purified by streaking two times on the same medium. The *Saccharomyces* yeasts were presumptively isolated by cultivation of purified colonies on lysine agar medium (Sigma-Aldrich, France). All isolates that were not able to grow on this medium but grew on YPD agar were considered as *Saccharomyces* strains and selected for molecular identification.

2.2. Molecular identification of *Saccharomyces* species

The presumptive *Saccharomyces* isolates were identified to species level by non-transcribed spacer PCR-restriction fragment length polymorphism (NTS2 PCR-RFLP) analysis using Alu I enzyme in comparison with reference strains (McCullough *et al*., 1998). Further, the identification was confirmed by D1/D2 region sequence similarity analysis with primers pair of NL-1 (5-GCATATCAATAAGCGGAGGAAAAG-3) and NL-4 (5-GGTCCGTGTTTCAAGACGG-3) (Kurtzman and Robnett, 1998). DNA sequence alignments were carried out using the pairwise alignment method for the forward and reverse complement sequences of each strain in order to obtain a consensus sequence. Identification queries were fulfilled by a BLAST search of the National Centre for Biotechnology Information database (NCBI, Bethesda, USA) and the YeastIP databases (http://genome.jouy.inra.fr/yeastip/).

2.3. Interdelta PCR analysis

DNA from *Saccharomyces cerevisiae* isolates was extracted and purified using FTA paper Whatman classic (Whatman FTA® Card Technology,) method as described by Borman *et al*. (2010). Primers used for amplification were delta 12 (5'-TCAACAATGGAATCCCAAC-3') and delta 21 (5'-CATCTTAACACCGTATATGA-3') designed by Legras and Karst (2003). Amplification reactions were performed in 50 µl mixtures containing 5 µl of DreamTaq buffer 10 X (Thermo Scientific, Lithuanie), 0.4 µl of DreamTaq DNa Polymerase (5 U/µl; Thermo Scientific, Lithuanie), 200 µM of each dNTP, 5 µl of each oligonucleotide primer (5 pmol/µl) and a piece of FTA paper containing DNA. A Gene Amp® PCR System 2700 thermal cycler (Applied Biosystems, Singapour) was used and the following program applied: initial denaturation for 3 min at 95ºC, then 35 amplification cycles (95ºC for 30s, 45ºC for 40s, and 72ºC for 90s), final extension step of 5 min at 72ºC. The amplification products were separated on 2% agarose gels in 0.5x TBE buffer during 2 h 20 min. Gels were stained with ethidium bromide, visualized and photographed under UV light.

2.4. Data analysis

In order to understand the genetic relationships between the strains from each biotype in the three beverages, the size (pb) of the bands of each profile was determined using the UvibandMAX software version 15. 06 a (Uvitec Cambridge, Cambridge, United Kingdom). This includes a representative of each biotype from the 71 different profiles obtained by interdelta PCR. Only consistent and reproducible bands were considered to perform the corresponding statistical analyses. Polymorphic DNA bands were recorded as discrete variables by considering the presence of "1" and the absence of "0" to construct a binary data matrix. Later, the data were processed in Free Tree statistics software version 0.9.1.5 (Pavlicek *et al*., 1999) to produce a genetic distance matrix using the Dice coefficient, also known as similarity coefficients (Nei and Li, 1979). The resulting matrix was analysed using the unweighted pair method with the arithmetic mean (UPGMA), to construct a dendrogram with 1000 bootstrap repeats. The MEGA 7.0 software (Kumar *et al*., 2016) was used to visualize the obtained dendrogram.

3. RESULTS AND DISCUSSION

The 274 natural *S. cerevisiae* from three traditional alcoholic beverages (raffia wine, palm oil wine and tchapalo) collected in 13 production areas were investigated to evaluate their genetic diversity and to know which strains are dominant in each beverage. Thus, analysis of the 115 *S. cerevisiae* isolates from raffia wine in the four production areas revealed the existence of 21 different profiles, that is 21 biotypes, named with Roman numerals from Ri to Rxxi (Figure 1a). Out of these 21 biotypes, five were observed in four or three production areas with different isolation frequencies. These are biotypes Riii (20%), Rii (18.6%), Ri (12.17%), Rv (7.82%) and Rxi (6.95%). These biotypes presented a wide geographical distribution compared to other biotypes. So, they could be considered as specific to this alcoholic beverage. For strains isolated from palm oil wine, 30 different profiles (Pi to Pxxx) were found among the 92 isolates, indicating the existence of 30 biotypes. As shown in Figure 1b, each population of *S. cerevisiae* was also characterized by the composition and frequency of the constituent biotypes in each production area. Among the 30 biotypes, only strains of the Pviii biotype showed a wide geographical distribution with its detection in four (Attinguié, Bonoua, Grand-Lahou and Bingerville) of the five production areas. The diversity of tchapalo strains evaluated with 67 isolates revealed the existence of 20 different biotypes (Ti to Txx). Contrary to biotypes of raffia palm and palm oil wines, each biotype of sorghum beer was specific to a production area. Only strains of biotypes Tx, Txii, Txviii and Txx were each isolated in two different production areas (Figure 1c).

The presence of different *S. cerevisiae* interdelta profiles in the different regions indicates that several strains are responsible for the fermentative process of palm oil wines, raffia wines and sorghum beers produced in south and southeast of the Côte d'Ivoire. Despite the biotypic diversity of *S. cerevisiae* isolates, the biotypes were specific to each beverage. They were different from one beverage to another. This biotypic specificity would be linked to an adaptation of these strains to the beverage from which the yeasts originate. Besides the high interdelta profiles diversity, the biotypes with a dominant strain were found in each beverage and each production area. This is the case of strains of biotypes Riii (20%) and Rii (18.26) in the raffia wines, strains of biotype Pviii (14.1%) and Pv (10.8%) in palm oil wines and those of biotype Txvi (14%), Tviii (10.5%) and Txii (10.5%) in sorghum beers. Strains of these dominant biotypes could be represented the principal responsible of beverages fermentation. They can be considered the most adapted to conditions of each sampling area and/or each beverage production. The data obtained from these particular ecological niches appear to be in agreement with data from several other studies that have been carried out on the microbial ecology of wine fermentation where the *S. cerevisiae* population was composed of multiple strains, with often one dominant strain (Agnolucci *et al.*, 2007; Mannazzu *et al.*, 2007). Most of the strains found in each beverage were specific to a production area with two or three strains representing more than 50% of the biomass associated with a higher number of minority strains. Although in this study, a control of population dynamic was not carried out, it could be assumed that those strains present at low frequency were present at initial stages and they were substituted by the dominant strain during the fermentation process. These findings may explain the variability observed in organoleptic quality of traditional fermented beverages between regions and within a region. Indeed, different strains of yeast may interact with one another, by the sharing of metabolites and co-modification of flavour-producing metabolic pathways. The capacity of different strains to withstand the stress imposed by increasing ethanol concentrations may account for dominance towards the final stages of fermentation (Carrasco *et al.*, 2001).

Considering ratio (R) between the number of biotypes encountered and the number of

isolates tested for each sampling area as a rough estimate of biodiversity, *S. cerevisiae* diversity in the raffia wine was low at Adzopé where only five profiles out of 32 isolates were put in evidence, giving an average of 1.6 profiles for ten isolates in this population. In the other sampling areas, an average of four profiles for ten isolates was revealed in the different populations as shown in Table 1. For palm oil wine strains, Alépé and Grand-Lahou were genetically the most diversified with an average of six and five biotypes detected in ten isolates analyzed and those of Bingerville with an average of two biotypes detected in ten isolates analyzed, the least diversified (Table 1). With an average of four biotypes per ten isolates, the diversity of *S. cerevisiae* in tchapalo was genetically the same for all production areas under this study. This R value evidences a very high intraspecies variability, higher than that reported for *S. cerevisiae* populations from several wine-growing regions (Sabate *et al*., 1998; Torija *et al*., 2002). This biodiversity is however lower than the reports of Ezeronye and Legras (2009) and Mercado *et al*.(2010) who found respectively, 20 genotypes among 23 palm wine strains from Nigeria and 29 profiles among 35 *S. cerevisiae* strains involved in winemaking from Argentina. These differences could be related to ecological and/or geographical factors (Goddard *et al*., 2010). Indeed, we found that Alépé and Grand-Lahou with R values of 0.6 and 0.5 respectively, showed the highest biodiversity regardless the beverage. The similar genetic diversity of *S. cerevisiae* in tchapalo beers would reflect the history of the inoculum used by the traditional brewers. In Côte d'Ivoire, tchapalo beer was initially produced by populations living in the North and North-East of the country (Djè *et al*., 2008; N'guessan *et al*., 2010). Inocula found in Southern and Eastern Côte d'Ivoire may have been introduced by the traditional brewers from North and North East of Côte d'Ivoire and those of Burkina Faso, Mali and Ghana
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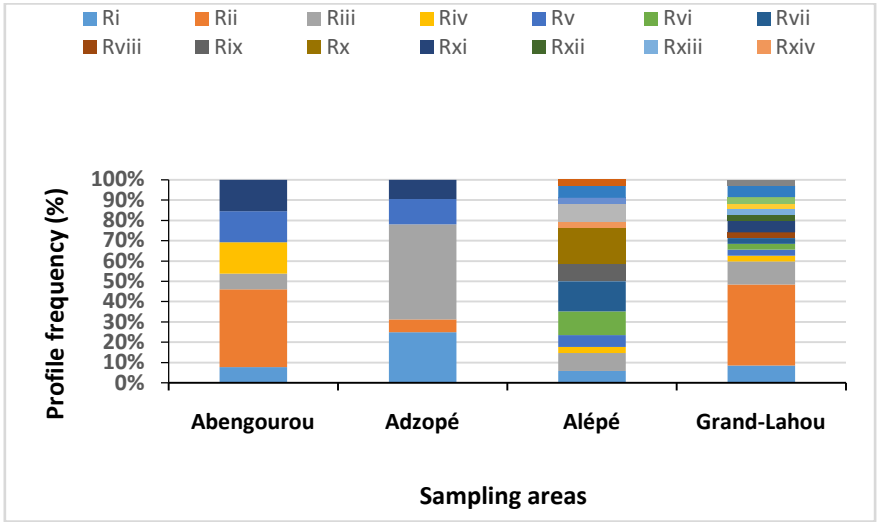
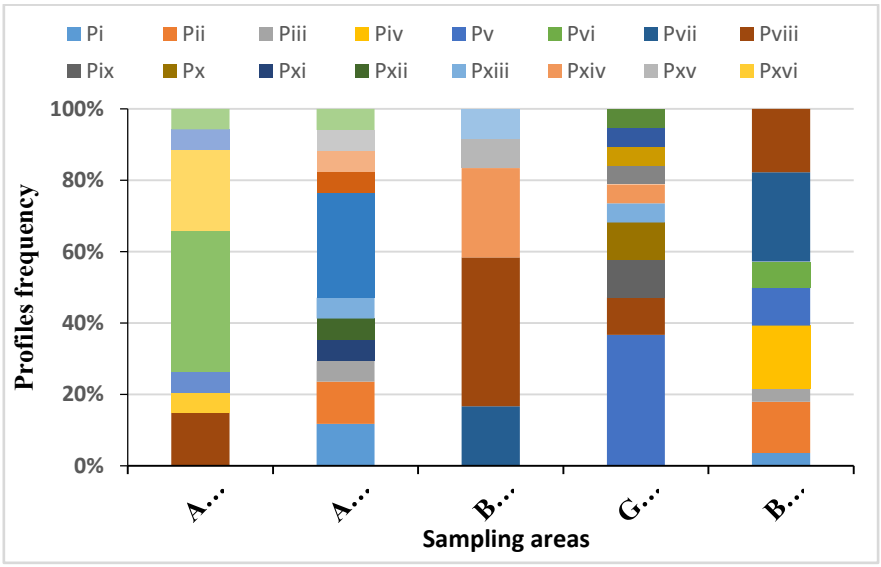
Table 1. Number of different profiles obtained by PCR interdela analysis for each sampled area.

Beverages	Sampling areas	Number of *S. cerevisiae* isolates	Number of PCR interdela profiles	Profiles diversity by sampling area (Number of PCR interdela profiles / Number of *S. cerevisiae* isolates)	Profiles diversity by beverage
Raffia wine	Abengourou	13	6	0.46	0.18
	Adzopé	32	5	0.16	
	Grand-Lahou	35	15	0.43	
	Alépé	35	14	0.40	
Palm oil wine	Bingerville	28	8	0.28	0.33
	Attinguié	16	7	0.43	
	Bonoua	12	5	0.42	
	Grand-Lahou	19	10	0.53	
	Alépé	17	11	0.65	
Tchapalo beer	Adjamé	21	8	0.38	0.30
	Yopougon	13	5	0.38	
	Abobo	15	6	0.40	
	Bingerville	18	6	0.40	

a) c)



b)



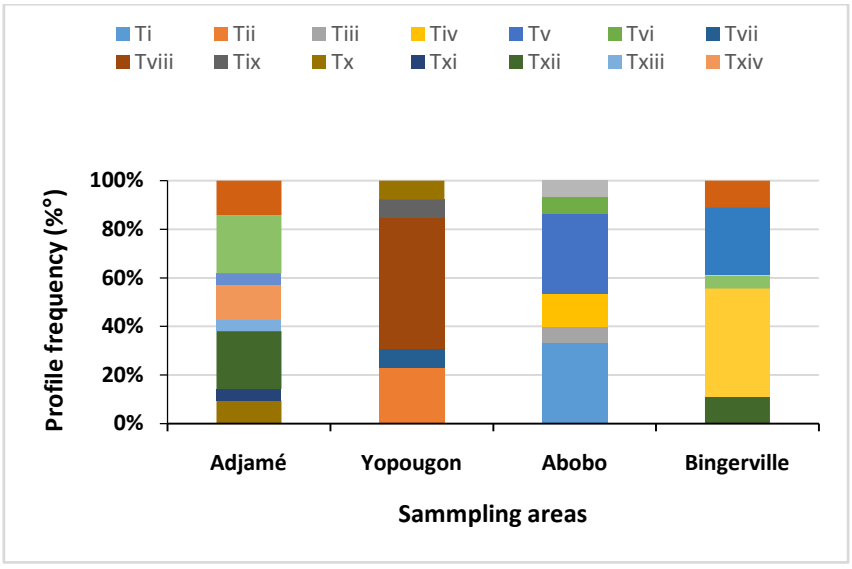


Fig. 1. Profiles frequency of *Saccharomyces cerevisiae*and their geographical distribution in raffia wines (a), palm oil wine (b) and tchapalo beers (c) according to production areas

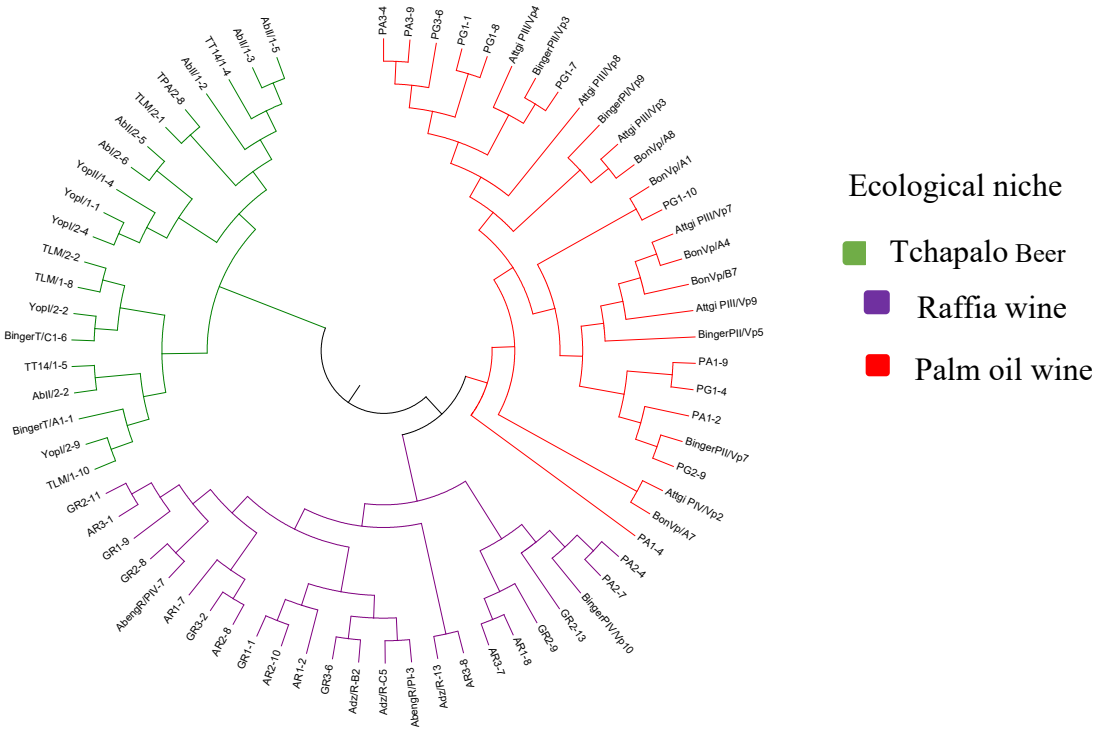


Fig. 2. UPGMA dendrogram showing genectic relationships based on interdelta region profile patterns of 71 *Saccharomyces cerevisiae* isolates from three Ivoirian traditional beverages collectedthroughout 13 prodcution aeeas

In order to understand the phylogenetic relationship between strains from each biotype, a neighbor consensus tree obtained from an individual distance matrix using the Dice coefficient was performed, including 71 strains of each biotype. According to the resulting dendrogram (Figure 2), the strains were distributed in three main groups. On this tree, strains of oil palm wine (in red), raffia palm wine (in blue) and tchapalo (in green) were genetically linked to the substrate from which the strains were isolated whatever their profile and origin of isolation. These results confirm the adaptation of the strains to their ecological niche as suggested above.

4. CONCLUSION

The results of the present study revealed that strains of *Saccharomyces cerevisiae* isolated from traditional fermented beverages produced in Côte d'Ivoire exhibit genotypic diversity in interdelta profiles. These strain profiles were specific to each beverage. Thus, the interdelta profiles constitute for these yeasts a kind of identity card via their genome that will not only recognize them in fermentation processes but also in traceability studies in the context of quality control. These strains with the dominant profiles are important genetic resources in which strains of biotechnological interest can be selected for use in many fermentation processes. Thus, physiological characterization is necessary to evaluate their biotechnological potential.

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