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Isolation and molecular identification of yeast strains from "*Rabilé*" a starter of local fermented drink

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"*Rabilé*" is dried yeast harvested from Sorghum beer, used as a traditional starter culture but more especially as ingredient in sauce and food cooking in Burkina Faso. The present study aimed to isolate and identify indigenous yeast flora of "*Rabilé*". Standard microbiological process was carried out to value and isolate yeast in different samples of "*Rabilé*" coming from four localities of Burkina Faso. Phenotypical method and molecular method (PCR and RFLP) were used for yeast strains characterization and identification. The results showed that yeast counts ranged from 9.49 to 10.35 log cfu/g of "*Rabilé*". A total of twenty yeast strains were isolated. Based on phenotypical characters three genera were detected: *Candida* (40%), *Saccharomyces* (35%) and *Rhodotorula* (25%). Molecular identification revealed two specific strains among yeasts isolated as *S. cerevisiae* with a frequency of 35% and *R. mucilaginosa* with a frequency of 25%. This data highlights the diversity of indigenous yeast flora of "*Rabilé*".

Key words: *Rabilé*, yeast, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), traditional starter culture.

INTRODUCTION

Sorghum beer is a popular alcoholic beverage from African countries where sorghum is produced (Maoura et al., 2005; N'Guessan et al., 2016). The beers are consumed at various festivals and African ceremonies and constitute a source of incomes for beer women producers (Lyumugabe et al., 2012; Djêgui et al., 2015). It is known as Tchapalo in Côte d'Ivoire, Tchoukoutou in benin (N'Guessan et al., 2016). Sorghum beer is commonly called *"Dolo"* in Burkina Faso (Sawadogo-Lingani et al., 2007; Abdoul-latif et al., 2013) where 60%

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of population are consumers (Bationo et al., 2015). Local fermented drink as "*dolo*" results from the fermentation of *Sorghum bicolor.* It is mainly produced by women (Maoura et al., 2005) using various processes depending on the geographic location. The manufacturing process consists of three phases: Malting, mashing and fermentation (Kayodé et al., 2012). The fermentation step is the most important step of the process (Djegui et al., 2014). However, this fermentation is uncontrolled and takes place in poor hygienic conditions (Benjamin et al., 2015) and its success depends on the accurate knowledge of the processor in terms of the starter handling (Kayodé et al., 2012). In Burkina Faso, "*Rabilé*" is used as traditional starter culture for the production of sorghum beer ("*dolo*"). This starter is obtained by drying the pellet from the fermentation of "*dolo*" at room temperature. It was reported that "*Rabilé*" is also largely used as ingredient in sauce and food cooking (Konlani et al., 1996b). "*Rabilé*" brings to those people a wide range of nutritional benefits and contributes to their dietary need, as it is mainly constituted of yeast, lactic acid bacteria and various metabolites resulting from fermentation process. Indeed, brewer's yeast is an important source of group B vitamins and minerals such as Ca, P, K, Mg, Cu, Fe, Zn, Mn and Cr, in addition to its profile balanced in amino acids (Bekatorou et al., 2006; Feldmann, 2012). Despite its common use in diet, very limited information exists on microbiological and nutritional characteristics of "*Rabilé*" in particular its yeast diversity. The present study was focused on isolation and identification of yeast strains from "*Rabilé*" using conventional and molecular methods.

MATERIALS AND METHODS

Sampling

Samples of dried yeast harvested "*Rabilé*" from sorghum beer were collected from commercial sites of four localities of Burkina Faso (Banfora, Fada N'Gourma, Ouagadougou and Tenkodogo) as indicate in Figure 1. In each locality, 25 g of ferment were purchased from five local beer producers. Once at laboratory, the 20 samples collected were stored at 4°C for yeasts isolation.

Yeast strains enumeration and isolation

An amount of 10 g from each sample was crushed in blender suspended and mixed in 90 ml of sterile diluents (physiological water). Serial 10-fold dilution was carried out and yeast was isolated on Sabouraud Agar (Biomerieux) with addition of chloramphenicol at 30°C. Chloramphenicol is an antibiotic used to inhibit growth of other microorganisms and allow only the growth of yeast. Colony counts were carried out using a colony counter (IUL Instruments). Total counts of yeasts population were expressed as log colony forming units per gram (log cfu/g) of "*Rabilé*". The representative colony forming units were recorded and purified twice on MYGP Agar (Malt extract, yeast extract, glucose and peptone). A total of 20 selected yeasts was grown on the YEPD liquid media and stored 4°C.

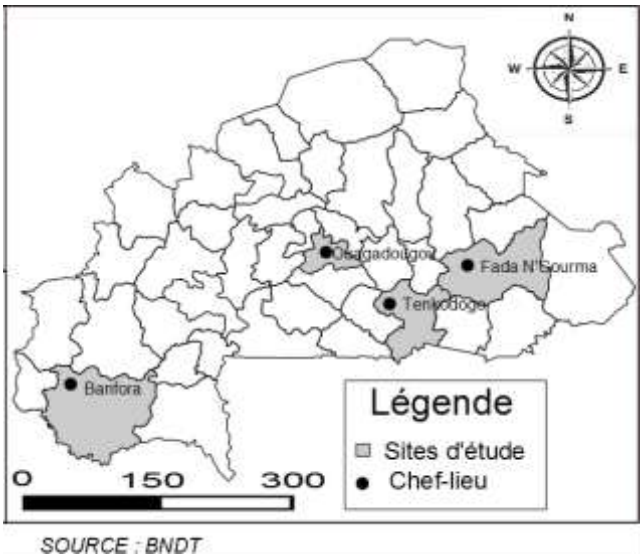

Figure 1. Map of Burkina Faso. Colored parts = sites of sample collection (Banfora, Fada N'Gourma, Ouagadougou and Tenkodogo).

Morphological, physiological and biochemical identification

Morphological and physiological characteristics determined, were color of colonies on solid media. Cell shape and mode of vegetative growth were determined by microscopic observation (Bonciu et al., 2010; Stoicescu et al., 2011). Ascospores were highlighted on the media of sporulation Mac Clary (Nishida et al., 2004). Fermentation and assimilation tests of carbon compounds, nitrate, and sodium acetate were carried out according to Guiraud et al. (1984).

Molecular characterization

Microorganism preparation and genomic DNA extraction

The 20 representative isolated yeast strains were grown on MYPD agar at 30°C for 72 h. For each strain culture a loop full was collected for DNA extraction. Genomic DNA was extracted and purified according to CTAB extraction method used by Kumar et al. (2014).

Specific-PCR

DNA amplification was performed in a reaction volume of 25 µl, containing 0.4 µM of each primer (MWG Operon Eurofins, USA), 2 ng/µl of genomic DNA, 0.8 mM deoxynucleotides (dATP, dCTP, dGTP, dTTP), 4 mM of $MgCl_2$, 0.04 U/µl Taq polymerase and buffer 1 X (Invitrogen, China). Sequences of the primers pairs used separately are shown in Table 1. The amplification was performed with an automatic thermocycler (MJ Research PTC-200) using program with an initial denaturation step at 94°C for 5 min, amplification reaction was performed in 35 cycles of denaturing at 95°C for 1 min, annealing at 60°C for 1 min, elongation at 72°C for 2 min and a final extension step at 72°C for 10 min. Amplified DNA fragments were separated on 3% agarose gel. After electrophoresis, gels were scanned under ultraviolet ray. All fragments were analyzed with Kodak 1D 3.5. software to determine the size of the bands obtained.

Table 1. Primers used for specific-PCR.

Target species	Primer	Sequence (5' – 3')	Size (bp)	Reference
Rhodotorula mucilaginosa	RM5-fw RM3-bw	GCGCTTTGTGATACATTTTC CCATTATCCATCCCGGAAAA	280	
Candida tropicalis	CTR1 CTR2	CAATCCTACCGCCAGAGGTTAT TGGCCACTAGCAAAATAAGCGT	357	HSU et al., 2003
Saccharomyces cerevisiae	SC-5fw SC-3bw	AGGAGTGCGGTTCTTTCTAAAG TGAAATGCGAGATTCCCCCA	215	Diaz et al., 2013
Hanseniaspora uvarum	HU-5fw HU-3bw	GGCGAGGGATACCTTTTCTCTG GAGGCGAGTGCATGCAA	172	Diaz et al., 2013

Table 2. Yeasts content of "Rabilé" and distribution of isolated yeast strains.

Origin	Banfora	Ouagadougou	Tenkodogo	Fada N'Gourma
Yeasts (log cfu/g)	**9.68**	**9.94**	**9.49**	**10.35**
Yeast strains	$CRSBANYB_1$	$CRSBANYO_1$	$CRSBANYT_1$	$CRSBANYF_1$
	$CRSBANYB_2$	$CRSBANYO_2$	$CRSBANYT_2$	$CRSBANYF_2$
	$CRSBANYB_3$	$CRSBANYO_3$	$CRSBANYT_3$	$CRSBANYF_3$
	$CRSBANYB_4$	$CRSBANYO_4$	$CRSBANYT_4$	$CRSBANYF_4$
	$CRSBANYB_5$	$CRSBANYO_5$	$CRSBANYT_5$	$CRSBANYF_5$

ITS amplification

Primers (MWG Operon Eurofins, USA) ITS1 (5'-TCCGTAGGTGA ACCTGCGG-3 ') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify ITS-5.8S-DNAr region. Amplification and electrophoresis conditions are mentioned above.

RFLP analysis

An aliquot (10 µl) of PCR product was digested separately with four restriction endonucleases *EcoR* I, *Hind* III, *Apa* I and *BamH* I (Invitrogen, China) to generate restriction fragments. Reaction mixture consisted of 1 µl enzyme, 2 µl buffer, 10 µl amplicon and 7 µl pure water, to a total volume of 20 µl. Digestion was carried out at 37°C for 1 h according to the manufacturer's instructions (Invitrogen, China). Restriction fragments were visualized by ethidium bromide staining and UV transillumination. Identification was carried out using specific standards. CECT database (http://cectvirt11.uv.es/searchdb/) was used to verify suitable restriction enzymes as *Hind* III for selection and for identification of *S. cerevisiae.*

RESULTS

Yeast amount and distribution of isolated yeast

Table 2 shows the yeast counts obtained for "Rabilé" samples from four localities of Burkina Faso. Yeast counts ranged from 9.49 to 10.35 log cfu/g respectively for samples from Tenkodogo and Fada N'Gourma. A total of 20 strains were isolated from collected samples. The origin of these strains is given in Table 2.

Morphological, physiological and biochemical identification

Strains in solid and liquid allowed to notice morphological, physiological and biochemical characteristics as described in the Table 3. The phenotypical yeast identification was based on criteria of Guiraud et al. (1984). In solid media, all strains appear white colonies except strains CRSBANYF1, $CRSBANYF_4$, $CRSBANYO_3$, $CRSBANYT_3$ and $CRSBANYT_4$ which presented red coloring. Under microscope, vegetative cells had an oval form and divided by budding. An asexual reproduction leading to the formation of asque (4 ascospores) was observed in strains $CRSBANYB_3$, $CRSBANYF_3$, $CRSBANYB_1$, $CRSBANYB_4$, $CRSBANYF_2$, $CRSBANYT_2$ and $CRSBANYB_5$.

Results of fermentation tests showed that strains $CRSBANYF_1$, $CRSBANYF_4$, $CRSBANYO_3$, $CRSBANYT_3$ and $CRSBANYT_4$ are deprived of fermentative ability while all the others were able to make ferment glucose, sucrose, maltose and fructose. In addition, only strains $CRSBANYO_1$, $CRSBANYT_1$, $CRSBANYT_5$, $CRSBANYO_2$, $CRSBANYO_4$, $CRSBANYF_5$, $CRSBANYB_2$ and $CRSBANYO_5$ are able to assimilate starch, nitrate and acetate of sodium. These phenotypical characters

Table 3. Morphological, physiological and biochemical identification of isolated yeast strains.

Strains	Fermentation/assimilation							Assimilation				Colony shape	Cell shape	Vegetative growth	Ascospores
	Glu	Suc	Mal	Fru	Gal	Lac	Mel	Ara	Nit	Sta	Ace				
$CRSBANYB_1$	+/+	+/+	+/+	+/+	-/+	-/+	-/-	-/-	-	-	-	White	Ovoid	Budding	+
$CRSBANYB_2$	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	+	+	+	White	Ovoid	Budding	-
$CRSBANYB_3$	+/+	+/+	+/+	+/+	-/+	-/+	-/-	-/-	-	-	-	White	Ovoid	Budding	+
$CRSBANYB_4$	+/+	+/+	+/+	+/+	-/+	-/+	-/-	-/-	-	-	-	White	Ovoid	Budding	+
$CRSBANYB_5$	+/+	+/+	+/+	+/+	-/+	-/+	-/-	-/-	-	-	-	White	Ovoid	Budding	+
$CRSBANYO_1$	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	+	+	+	White	Ovoid	Budding	-
$CRSBANYO_2$	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	+	+	+	White	Ovoid	Budding	-
$CRSBANYO_3$	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/-	-	-	-	Red	Ovoid	Budding	-
$CRSBANYO_4$	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	+	+	+	White	Ovoid	Budding	-
$CRSBANYO_5$	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	+	+	+	White	Ovoid	Budding	-
$CRSBANYF_1$	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/-	-	-	-	Red	Ovoid	Budding	-
$CRSBANYF_2$	+/+	+/+	+/+	+/+	-/+	-/+	-/-	-/-	-	-	-	White	Ovoid	Budding	+
$CRSBANYF_3$	+/+	+/+	+/+	+/+	-/+	-/+	-/-	-/-	-	-	-	White	Ovoid	Budding	+
$CRSBANYF_4$	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/-	-	-	-	Red	Ovoid	Budding	-
$CRSBANYF_5$	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	+	+	+	White	Ovoid	Budding	-
$CRSBANYT_1$	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	+	+	+	White	Ovoid	Budding	-
$CRSBANYT_2$	+/+	+/+	+/+	+/+	-/+	-/+	-/-	-/-	-	-	-	White	Ovoid	Budding	+
$CRSBANYT_3$	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/-	-	-	-	Red	Ovoid	Budding	-
$CRSBANYT_4$	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/-	-	-	-	Red	Ovoid	Budding	-
$CRSBANYT_5$	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	+	+	+	White	Ovoid	Budding	-

Glu = Glucose; Suc = Sucrose; Mal = Maltose; Gal = Galactose; Mel = Melibiose; Ara = Arabinose; Nit = Nitrate; Sta = Starch; Ace: sodium acetate; + = positive; - = negative

obtained (Table 3) show that strains $CRSBANYB_3$, $CRSBANYF_3$, $CRSBANYB_1$, $CRSBANYB_4$, $CRSBANYF_2$, $CRSBANYT_2$ and $CRSBANYB_5$ could belong to the genera *Saccharomyces* (35%), strains $CRSBANYO_1$, $CRSBANYT_1$, $CRSBANYT_5$, $CRSBANYO_2$, $CRSBANYO_4$, $CRSBANYF_5$, $CRSBANYB_2$ and $CRSBANYO_5$ to *Candida* (40%) and strains CRSBANYF1, $CRSBANYF_4$, $CRSBANYO_3$, $CRSBANYT_3$ and $CRSBANYT_4$ to *Rhodotorula* (25%).

Yeast characterization by specific-PCR

Specific primers were used to check if yeast strains belong to species *Candida tropicalis*, *Hanseniaspora uvarum*, *Rhodotorula mucilaginosa* or *Saccharomyces cerevisiae*. After amplification, the profile of isolates $CRSBANYB_3$, $CRSBANYF_3$, $CRSBANYB_1$, $CRSBANYB_4$, $CRSBANYF_2$, $CRSBANYT_2$ and $CRSBANYB_5$ revealed the presence of specific band 215 bp, and were identical of the profile of *Saccharomyces cerevisiae* (35 %). Amplicons of strains $CRSBANYF_1$, $CRSBANYF_4$, $CRSBANYO_3$, $CRSBANYT_3$ and $CRSBANYT_4$ gave bands of 280 bp, specific to *Rhodotorula mucilaginosa* (25 %) as indicated in Figure 2. No match profile was found to *C. tropicalis* and *H. uvarum* (Table 4) and which could notify the absence of these two species among 20 yeast strains.

Yeast PCR-RFLP characterization

In order to verify profile difference between isolated yeast strains, PCR-RFLP was used. ITS-5.8S-rDNA region was amplified with primers ITS1 and ITS4 then digested with restriction enzymes (*EcoR* I, *Hind* III, *Apa* I and *BamH* I). The amplicons obtained (Figure 3) and the restriction fragments (Table 5) indicated three profiles relating three specific species of strains. PCR-RFLP indicated existence of a large polymorphism between the three groups of yeasts isolated as well by the size of amplicons and then their restriction profile. The first group of species, initially identified was *S. cerevisiae* yielded amplicons of 880 bp and presented on their ITS-5.8S-DNAr region one restriction site for *EcoR* I and *Apa* I enzymes. The second group of species identified was *R. mucilaginosa* with amplicons of 630 bp and had two and one restriction sites respectively for *EcoR* I and *Hind* III enzymes. The third group of species gave 500 bp amplicons and had only one restriction site for the enzyme *EcoR* I. The use of CECT database (http://cectvirt11.uv.es/searchdb/) has shown the amplicons of 850 bp only for *S. cerevisiae* with restriction enzyme as *Hind* III.

DISCUSSION

The yeast concentration of “Rabilé” obtained (Table 2),

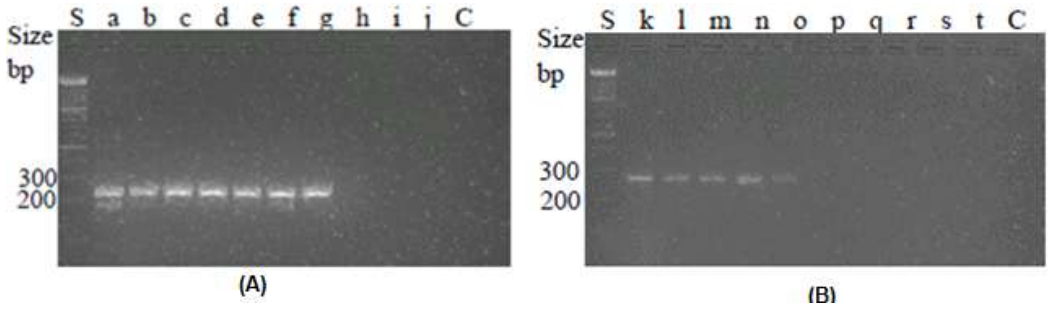

Figure 2. Bands obtained after specific species PCR. (A) Amplification with primers specific to *S. cerevisiae*. (B) Amplification with primers specific to *R. mucilaginosa.* Lanes a-j: $CRSBANYB_3$, $CRSBANYF_3$, $CRSBANYB_1$, $CRSBANYB_4$, $CRSBANYF_2$, $CRSBANYT_2$, $CRSBANYB_5$, $CRSBANYB_2$, $CRSBANYO_5$, $CRSBANYT_4$; S = Standard; C= Control negative. Lanes k-t: $CRSBANYF_1$, $CRSBANYF_4$, $CRSBANYO_3$, $CRSBANYT_3$, $CRSBANYT_4$ $CRSBANYB_3$, $CRSBANYF_3$, $CRSBANYO_1$, $CRSBANYT_1$, $CRSBANYT_5$.

Table 4. Molecular identification of strains.

Target species	Band size (bp)	Presence	Strains
C. tropicalis	375	0/20	None
H. uvarum	172	0/20	None
R. mucilaginosa	280	5/20	$CRSBANYF_1$, $CRSBANYF_4$, $CRSBANYO_3$, $CRSBANYT_3$, $CRSBANYET_4$
S. cerevisiae	215	7/20	$CRSBANYB_3$, CRSBANY F_3, $CRSBANYB_1$, CRSBANY B_4, $CRSBANYF_2$, $CRSBANYT_2$, $CRSBANYB_5$

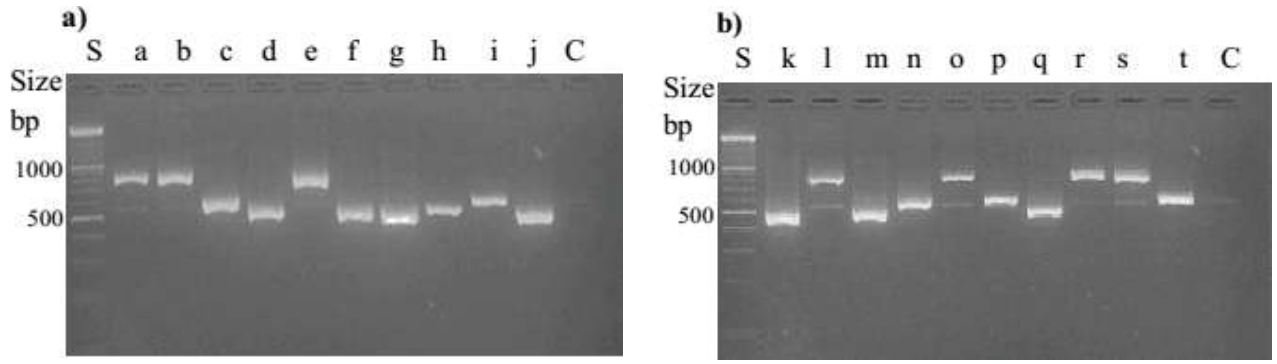

Figure 3. Size of fragments obtained by amplification of ITS-5.8S-DNAr region. Lanes a-j: $CRSBANYF_2$, $CRSBANYT_2$, $CRSBANYT_3$, $CRSBANYT_5$, $CRSBANYB_5$, $CRSBANYO_2$, $CRSBANYO_4$ $CRSBANYO_5$, $CRSBANYT_4$, $CRSBANYF_5$; Lanes k-t: $CRSBANYB_2$, $CRSBANYB_3$, $CRSBANYO_1$, $CRSBANYF_1$, $CRSBANYF_3$, $CRSBANYF_4$, $CRSBANYT_1$, $CRSBANYB_1$, $CRSBANYB_4$, $CRSBANYO_3$; S = Standard; C = Control negative.

was higher than those reported by Kayodé et al. (2012) on dried traditional starter of African opaque sorghum beers but closed to values reported in "*Kpètè kpètè*" by Djêgui et al. (2015) and "Otchè" by Djegui et al. (2014) which are liquid traditional starter of African sorghum beers. The number of viable yeasts obtained could justify the wild use of "*Rabilé*" as a traditional starter of sorghum beer in Burkina Faso. However, further investigation would be necessary to determine the long shelf life of this starter.

Phenotypical and molecular characterization revealed respectively three genera and two different species among isolated strains from "Rabilé". As indicated in Table 5, 35% of isolates belong to *S. cerevisiae* and 25% to *R. mucilaginosa*. The high prevalence of *S. cerevisiae* in "*Rabilé*" from Banfora, *Candida* from Ouagadougou and *R. mucilaginosa* from Fada N'Gourma and Tenkodogo demonstrates a biodiversity of yeast in the

Table 5. Size of ITS-5.8S-DNAr region and RFLP restriction pattern with enzymes *EcoR* I, *BamH* I, *Hind* III and *Apa* I.

Group	Strains	Size ITS (bp)	Restriction fragments size (bp)			
			***EcoR*I**	***BamH*I**	***Hind*III**	***Apa*I**
S. cerevisiae	$CRSBANYB_3$, $CRSBANYF_3$, $CRSBANYB_1$, $CRSBANYB_4$, $CRSBANYF_2$, $CRSBANYT_2$ et $CRSBANYB_5$	880	460 360	880	880	530 350
R. mucilaginosa	$CRSBANYF_1$, $CRSBANYF_4$, $CRSBANYO_3$, $CRSBANYT_3$ and $CRSBANYT_4$	630	280 250 160	630	490 140	630
-	$CRSBANYO_1$, $CRSBANYT_1$, $CRSBANYT_5$, $CRSBANYO_2$, $CRSBANYO_4$, $CRSBANYF_5$, $CRSBANYB_2$ et $CRSBANYO_5$	500	310 210	500	500	500

local starter "*Rabilé*" responsible to traditional fermentation. This is in agreement with the idea according to which the composition of the yeast population responsible for the spontaneous fermentation of sorghum beer could be related to the regional location (van der Aa Kühle et al., 2001). Due to the number of yeast selected strains their geographic repartition need to be complete by other investigation. In many studies, *S. cerevisiae* has been reported as responsible for the spontaneous fermentation of sorghum beer (Konlani et al., 1996a; Naumova et al., 2003; Lyumugabe et al., 2014). *S. cerevisiae* is fully accepted for human consumption and is the most common food grade yeast (Bekatorou et al., 2006). Contrary the presence of *R. mucilaginosa* in sorghum beer could be dangerous because it has been reported to cause Onychomycosis (Da Cunha et al., 2009; Jimoh et al., 2011).

It was reported that certain yeasts involved in sorghum beer production were phenotypically different from reference strains (van der Aa Kühle et al., 2001). PCR-RFLP using *EcoR* I, *Hind* III, *Apa* I and *BamH I*, did not allow to prove this difference at the molecular level and to discriminate at subspecies level. Nevertheless, it permitted to confirm the results of specific-PCR. The results obtained are in accordance with the studies of several authors in which RFLP method was found useful for differentiation of yeast at species level (Esteve-Zarzoso et al., 1999; Granchi et al., 1999). The high prevalence of *S. cerevisiae* would indicate a predictive characteristic of good brewer's starter.

Conflict of interests

The authors have not declared any conflict of interests.

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