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Full Length Research Paper

Identification and typing of the yeast strains isolated from bili bili, a traditional sorghum beer of Chad

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Seventy six yeast strains isolated form bili bili and others sample were identified and typed in purpose of selecting appropriate starter culture. Identification techniques included conventional phenetic method, PCR/RFLP of NTS2 rDNA region, partial sequencing of the D1/D2 region of 26S rDNA and karyotyping using contour clamped homogenous electric field (CHEF) technique. The *Saccharomyces cereviseiae* strains were also compared to industrial strains according to their fermentation profiles on maltose in the presence of 2-deoxy- D-glucose and to their karyotypes. We observed that the fermentation of bili bili was carried out by an indigenous natural flora predominantly represented by highly polymorphic *S. cerevisiae* strains whereas early steps in the process were carried out mainly by *Kluyveromyces maxianus* strains. All of the *S. cerevisiae* strains which were used in trial fermentation gave a good rate of fermentation suggesting that they may be used as starter cultures.

Key words: Sorghum beer, bili bili, yeast flora, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, PCR/RFLP of the NTS2-rDNA; karyotyping, fermentation.

INTRODUCTION

In Africa, various kinds of traditional alcoholic beverages commonly named sorghum beers or opaque beers, have been recorded and described (Dirar, 1978; Dirar, 1979; Faparusi et al., 1973; Novellie and De Chaepdrijver, 1986; Odunfa, 1985). Bili bili is one of these beers that results from the fermentation of sorghum and millet in Chad (Djanan et al., 2002; Nanadoum, 2001). Its production has led to a thriving cottage industry exclusively managed by women in both rural and urban areas. The brewing of bili bili (Nanadoum, 2001) involves malting, mashing, souring, straining, boiling and fermentation. Bili bili production involves two successive steps of fermentation which occur at ambient conditions. A primary lactic fermentation, or souring stage, confers the souring taste and storage longevity (Haggblade and Holzapfel, 1989). Then, the alcoholic fermentation is usually initiated by pitching wort with a portion of previous brew or dried yeast harvested from bili bili (Nanadoum, 2001). Since the bili bili fermentation process is run by uncontrolled inoculation under no standard hygienic conditions, producers have a major problem to make a good brew with a long shelf-life (more than 24 h).

Phenotypic characterizations of yeasts isolated from sorghum beers in many areas of Africa were reported in literature (Demunyakorand Ohta, 1991; Sefa-dehed et al., 1999) but reports dealing with genotypic studies of these flora is limited (Kühle and al., 2000). The bili bili process itself has not been investigated scientifically in great detail. In order to achieve a better control of these fermentations, it is important to know the exact

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composition of the microflora involved and to be able to characterize it at subspecies level. A second prerequisite is then to describe their technological properties. In this study, we used conventional and molecular methods to characterize the yeasts involved in bili bili and to provide some information for the development of starter culture for bili bili production in Chad.

MATERIALS AND METHODS

Yeast strains

The yeast strains used in this study were isolated from 20 samples collected during souring and 20 others, during fermentation stages of 4 successive preparations of bili bili by one producer in N'Djamena (Chad).

Conventional identification

Yeast identification was carried out following morphological, physiological and cultural characters as described by (Barnett et al., 1983; Chu et al., 1986) using the Yeast Identification software of Barnett (Barnett et al., 1996).

Molecular identification and karyotyping

Isolation of genomic DNA: Yeast cells were grown overnight in YPD broth with shaking at 28°C. Then the genomic DNA were extracted from 2 ml of the culture as described by Hoffman and Winston (1987).

PCR/RFLP condition for NTS2 region: The amplification procedure of the rDNA region NTS2 (Ribosomal Non Transcribed Spacer 2) was carried out using a published method (Kurtzman and Robnett, 1991; Nguyen et al., 2000a,b) . Enzymatic digestions were carried out on 6 µl of amplified DNA in a final volume of 20 µl with *Alu* I and *Ban* I (GibcoBRL) for 1 h at 37°C. Restriction fragments were separated for 2 h in 2% agarose gel (Nusieve 3:1) in 0.5X TBE buffer at 120 V. Gels were stained with ethidium bromide (2 ng ml^{-1} in water) for 30 min and distained in sterile water. Photographs were taken with a Bio prob print 5.08 Camera system (Vilber Lourmat), and then scanned using Adobe photoshop 5.0 software.

Partial sequencing of 26S of rDNA and sequences analysis: DNA for sequencing was amplified using a 9600 Perkin-Elmer Cetus Thermal Cycler apparatus as described by Kurtzman and Robnett (1991) with forward and reverse primers (NL1-NL4). The amplified D1/D2 fragments were purified with QIAquik PCR purification kit (Quiagen). Sequences obtained were analyzed and compared using the Staden Pakage (Genetic Computer Group Madison, Wi,USA) and sequences deposited in Genbank.

DNA preparation and pulsed field electrophoresis: Conditions for preparation of plugs were as described before (Vezinet et al., 1990) with a few modifications (Ngyen et al., 2000b). Chromosome separation was carried out using contour clamped homogenous electric field (CHEF) MAPER apparatus (Biorad). To separate chromosome of strains identified as *S. cerevisiae*, plugs were inserted in wells formed in 1.1% agarose gel (Seakem GTG, FMC bio product) in 0.5X TBE buffer and electrophoresed for 24 h at 14°C in sterile 0.5X TBE buffer using following ramping condition at 6 V cm^{-1}; 40 s initial pulse time for 18 h and 120 s final pulse time for 6 h. For the karyotyping of strains identified as *K. marxianus*, 0.8% agarose gel (Seakem GTG,FMC bioproduct) in 0.5X TBE buffer was used for plugs and electrophoresed following linear ramping conditions (Nguyen et al., 2000b): block 1, 90 -120 s for 8h at 5.2 V cm^{-1} ; block 2, 120 - 360 s for 24 h at 4 V cm^{-1} and block 3, 360 - 1200 s for 8 h at 3 V cm^{-1}. The including angle for all of three blocks is 120°. Gels obtained were treated and photographed as described earlier.

Differentiation of *S.cerevisiae strains* isolated from bili bili from industrial strains

Discrimination according to the ability to ferment maltose and glucose: Fermentation of maltose in the presence of 2-deoxy-D-glucoe (2DOG) was tested in Durham tubes. 4 ml of 0.5% (w/v) yeast extract (Difco) in deionized water were pipetted into sterile plugged Durham tubes and autoclaved for 15 min. 1 ml of 10% (w/v) maltose (Sigma) solution in deionized water and sterilized by passing through 0.22 µ microfilter (Millex GS Bed Ford Massachessettes, USA) was added aseptically to the tubes. Using a 2DOG stock solution (1 mg ml^{-1}), 0, 5, 10 and 15 µg of 2DOG respectively was added to 4 Durham tubes. Then 0.1 ml of a suspension of strain made by suspending the growth of 48 h YPD agar culture in 4.5 ml sterile water. Tubes were incubated at 28°C then regularly shaken and observed for the accumulation of gas in inserts over a period of 5 days.

Discrimination by comparison of karyotype: For this comparison, six strains producing carbon dioxide in Durham tube containing maltose and 2-DOG (1 µg ml^{-1}) were karyotyped according to the methods described earlier (Nguyen end Gaillardin, 1997; Nguyen et al., 2000b; Vezinet et al., 1990).

Trial fermentation test on sorghum wort

The sorghum wort was prepared from coarse malted sorghum flour according to bili bili process described by Nanadoum (2001) at laboratory scale. For starter culture preparation, each indigenous *S. cerevisiae* strain selected according to its aptitude to ferment glucose in Durham tube, were streaked on YPD agar and incubated at 28°C for 48 h. The culture was harvested with a loop to prepare a dense suspension in 4 ml of sterile deionized water. The suspension was added to 40 ml of sterile sorghum wort and incubated at 28°C for 24 h to obtain the starter. Erlenmeyer flasks containing 400 ml of sterile sorghum wort were inoculated with each starter culture to obtain $2x10^7$ cell.ml^{-1} and incubated for 20 h at 28°C. The concentrations of glucose, maltose, maltotriose and ethanol in the fermented wort were determined by HPLC on Aminex Ion Exclusion Colum (Biorad Hercules, California, USA) and eluted with degased 6 mM H_2SO_4 in deionized water using a flow rate of 0.5 ml min^{-1} at room temperature. The HPLC apparatus consisted of an isocratic pump (model 501, Water Milford, Massachusetts, USA), an R401 refractive detector (Water) and a Rheodine 7125 injector (Rheodine Incorporated Cotati, California, USA) with a 25 µl loop. Concentrations were determined from standard curves relating peak areas to concentrations.

RESULTS AND DISCUSSION

Classical identification of bili-bili yeast strains

Seventy six yeast strains were isolated from the bili-bili samples and results of classical identification of these strains is shown in Table 1. Minor species identified

Table 1. Proportion of strains classically identified and their origin.

Origin	*S. cervisiae /S. paradoxus*	*K. marxianus*	Others species	Total	%
Bili bili	36	3	8	47	61.8
Others samples	1	19	9	29	38.2
Total	37	22	17	76	100
%	48.7	28.9	22.4	100	

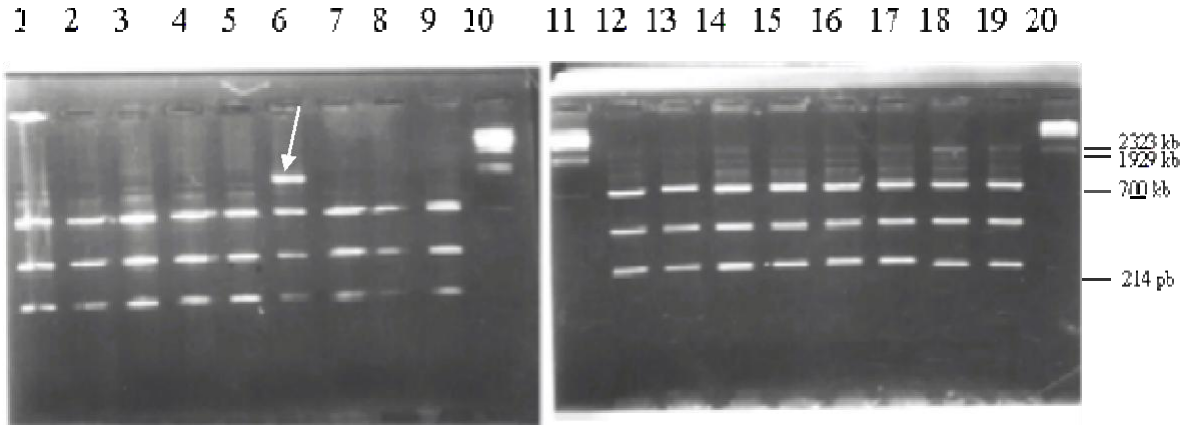

Figure 1. Digestion patterns on 1% Nusieve agarose of PCR NTS2-rDNA products generated by *Ban*I of the strains conventionally identified as *S. cerevisiae/S. paradoxus* . λBSTII is used as a size marker. All strains showed *S. cerevisiae Ban*I pattern except strain ST2G2. Legend: 1, C2G1; 2, ST1G1; 3, ST1G2; 4, ST1G3; 5, ST1G4; 6, ST2G2; 7, ST2G4; 8, ST4G2; 9, ST7G1; 10 and 11, BSTE II; 12, ST11G2; 13, ST11G3; 14, ST14G4; 15, ST16G2; 16, ST16G3; 17, ST16G4; 18, ST18G2; 19, ST18G4; 20, BSTE II.

(number) included *Cryptococcus albidius* var *albidius* (4), *Candida melibiosica* (1), *Debaryomyces hansenii* var *hansenii* (2), *Dekkera bruxelensis* (1), *Rodotorula mucilaginosa* (4) and *Torulaspora delbrueckii* (5). The program used for this identification cannot differentiate *S. cerevisiae* and *S. paradoxus* species, so they appear classified as one group.

Differentiation between *S. cerevisiae* and *S. paradoxus* species by the PCR/RFLP of NTS2 region of rDNA

To distinguish between *S. cerevisiae* and *S. paradoxus*, PCR/RFLP profiles of one portion of rDNA unit, the species specific NTS2 variable region were obtained (Woolford and Warner, 1991) . This was carried out using only two restriction enzymes, *Alu*I and *Ban*I as described before (Nguyen and Gaillardin, 1997). This technique seems more informative and reliable than the classic identification hampered by a large number of variable physological characters (Barnett et al., 1983). The PCR product is in the range of 1.3 kb for all species of the complex *Saccharomyce sensu stricto* (Barnett, 1992; Vaugham-Martini et al., 1985). There are two *Ban*I sites in the PCR product of *S. cerevisiae* (3 bands: 620, 420 and 220 pb) and none in the case of *S. paradoxus*, *S. bayanus*, *S. pastorianus* and *S. uvarum*. With *Alu*I, three types of patterns are expected: one is common to *S. bayanus* and *S. pastorianus* with 4 sites (5 bands: 470, 370, 240, 170 and 76 bp), the second is common to *S. cerevisiae* and *S. paradoxus* with 1 site, the third is specific to *S. uvarum* with 2 sites (Nguyen and Gaillardin, 1997) (3 bands: 620, 520 and 170 pb). We examined the NTS2 PCR/RFLP patterns of 40 strains conventionally indentified as *S. cerevisiae/S. paradoxus.* All exhibited patterns of amplification and digestion by *Ban*I and *Alu*I characteristic of *S. cerevisiae*, with the exception of one strain (ST2G2). This particular strain showed an amplification profile with a second, lighterband (result not shown). The *Ban* I digestion profile showed for this strain, in addition to the three bands characteristic of *S. cerevisiae* (Figure 1, lane 6), a fourth heavier band. The digestion of this PCR product by *Alu* I generated, in

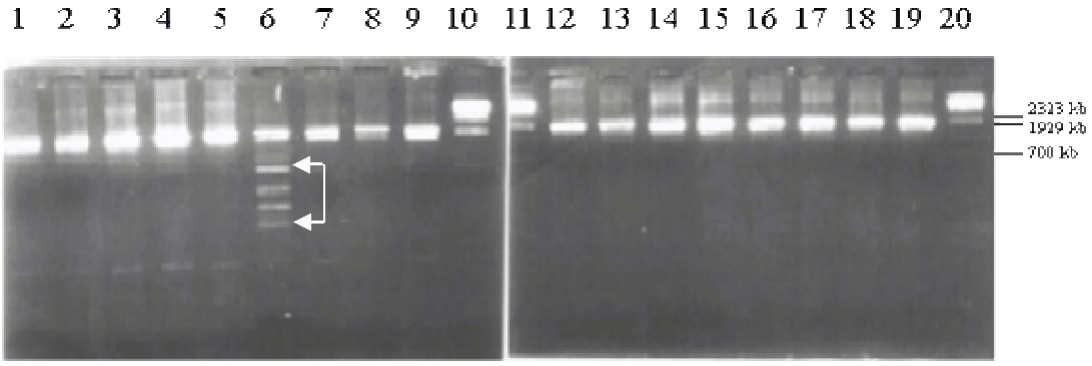

Figure 2. Digestion patterns on 1% Nusieve agarose of PCR NTS2-rDNA products generated by *Alu*I of the strains conventionally identified as *S. cerevisiae/S. paradoxus*. λBSTII is used as a size marker. All strains showed *S. cerevisiae Alu*I pattern except strain ST2G2. Legend: 1, C2G1; 2, ST1G1; 3, ST1G2; 4, ST1G3; 5, ST1G4; 6, ST2G2; 7, ST2G4; 8, ST4G2; 9, ST7G1; 10 and 11, BSTE II; 12, ST11G2; 13, ST11G3; 14, ST14G4; 15, ST16G2; 16, ST16G3; 17, ST16G4; 18, ST18G2; 19, ST18G4; 20, BSTE II.

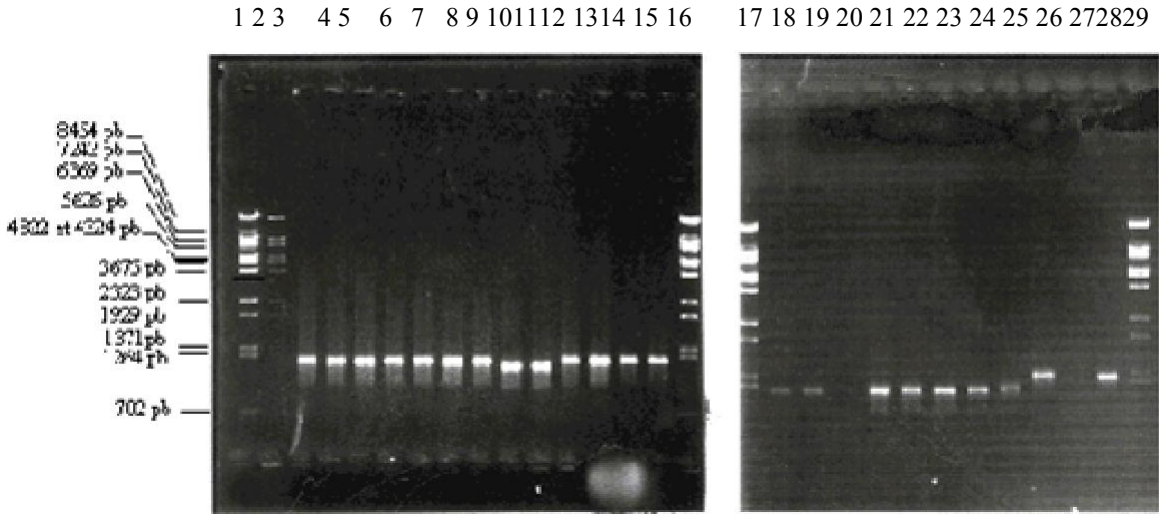

Figure 3. Electrophoretic profiles on 0.7% standard agarose gel 29 of PCR NTS 2-rDNA products from strains identified as *K. marxianus* and other species. The size marker (left) used is BSTE II. Legend**:** 1-2, BSTE II; 3, A1G1; 4, A1G2; 5, A1G3; 6, A2G1; 7, A2G2; 8, A2G3; 9, A2G4; 10, B2G3; 11, B2G4; 12, B5G1; 13, B5G2; 14, B5G3; 15, B5G4; 16 - 17, BSTE II; 18, ST2G2; 19, ST2G4; 20, empty; 21, E1G1; 22, E1G2; 23, E2G1; 24, E2G2; 25, ST2G3; 26, ST7G3; 27, nothing; 28, ST18G1; 29, BSTE II.

addition to the two bands characteristic of *S. cerevisiae,* several additional bands (Figure 2, lane 6).

All of these informations lead us to conclude that all strains were *S. cerevisiae* except for strain ST2G2 which was later shown to belong to *K. maxianus*.

Validation of the strains conventionally identified as *K. marxianus*

According to previous work (Vezinet et al., 1990; Pulverirenti et al., 2000), the NTS2 region of the rDNA of *K marxianus* species is also amplified by the NL1-NL4 primer couple described earlier (Nguyen and Gaillardin, 1997) and gives a fragment of about 1.3 kb, whose digestion by *Alu*I generates three bands characteristic of *K. marxianus*. This technique was applied to validate 22 stains identified as *K. marxianus* species. All of these strains showed identical amplification profiles with a single band of about 1.3 kb, except for two strains (Figure 3, lanes 26 and 28). These last strains presented a heavier band and were identified as *S. cerevisiae* after digestions of the PCR product by *Alu*I and *Ban*I. All remaining strains exhibited the expected *K. marxianus* profile after *Alu* I digestion (Figure 4), with three bands of (520, 370 and 300 pb), except for two strains (Figure 4, lanes 9 and 10) which yielded a three band profile but with a band at 440 pb instead of 520 pb as expected.

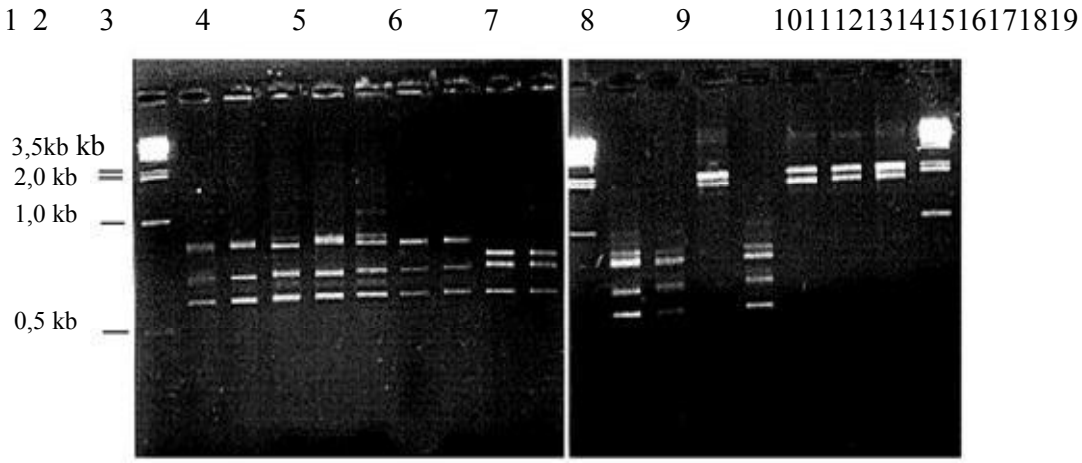

Figure 4. Digestion patterns on 2% Nusieve agarose of PCR NTS2-rDNA products generated by *Alu*I from strains identified as *K. marxianus* and others. The size marker (left) used is 1 kb ladder. Legend**:** 1, 1kb ladder; 2, A1G1; 3, A1G2; 4, A1G3; 5, A2G1; 6, A2G2; 7, A2G3; 8, A2G4; 9, B2G3; 10, B2G4; 11, 1 kb ladder; 12, B2G2; 13, B7G2; 14, B7G3; 15, ST13G4; 16, ST7G3; 17, ST16G1; 18, ST18G1; 19, 1 kb ladder.

Table 2. Identity percent of nucleotides of the D1/D2 rDNA region of strains compared to the sequences in GenBank.

Tested strains	Corresponding GenBank Code	Size of nucleotides compared	Number of nucleotide divergents	% Identity
E2G2	KM U94924	493	0	100
B2G4	-"-	490	1	99
ST2G3	-"-	496	1	99
ST2G2	-"-	510	4	99
B2G1	FN U94941	360	45	87
B7P1	-"-	541	77	85

These two strains had chromosomal patterns characteristic of *K. marxianus* likely represent a rare case of polymorphism (deletion of 80 pb or most probably apparition of an additional *Alu*I site).

The PCR/RFLP of the NTS2 was also applied to detect *S. cerevisiae* or *K. marxianus* abnormally classified among the other species group. Three strains (ST15G1, ST16G1 and B7G3) showed amplification and digestion by *Alu*I and *Ban*I patterns characteristic of *S. cerevisiae* and one strain (Figure 4, lane 1) presented the amplification and digestion by *Alu* I patterns characteristic of *K. marxianus*. The other species group is now limited to 13 strains.

PCR of D1/D2 domain and sequencing of the amplification products

As control for six strains whose identity was not clarified by the PCR/RFLP of the NTS2-rDNA, we analysed the nucleotide sequences from 5'end of the 26S rDNA (D1/D2 region) that has been demonstrated to be instrumental for the identification of ascomycetous yeast. After amplifying and sequencing the D1/D2 domain of the large (26S) rDNA sub-unit, sequences were compared to D1/D2 sequences from currently recognised ascomycetous species in GenBank (result shown on Table 2). KM U94924 corresponds to a standard *K. marxianus* strain CBS 712, and FN U94941 to a standard *Filobasidiella neoformans* strain CBS 882 (Kurzman and Robnett, 1998). Hundred percent of identity was found between strain E2G2 and the CBS 712 strain of *K marxianus* over 489 nucleotides compared. Three strains (B2G4, ST2G2 and ST2G3) were found to be different from *K. marxianus* CBS 712 only by one nucleotide (99% identity): a substitution of a G by A. A low level of similarity was observed when the D1/D2 sequence of two strains (B2G1 and B7P1) was compared to that of *F. neoformans* CBS882, which suggests that these strains are different from all recongnised species deposited in

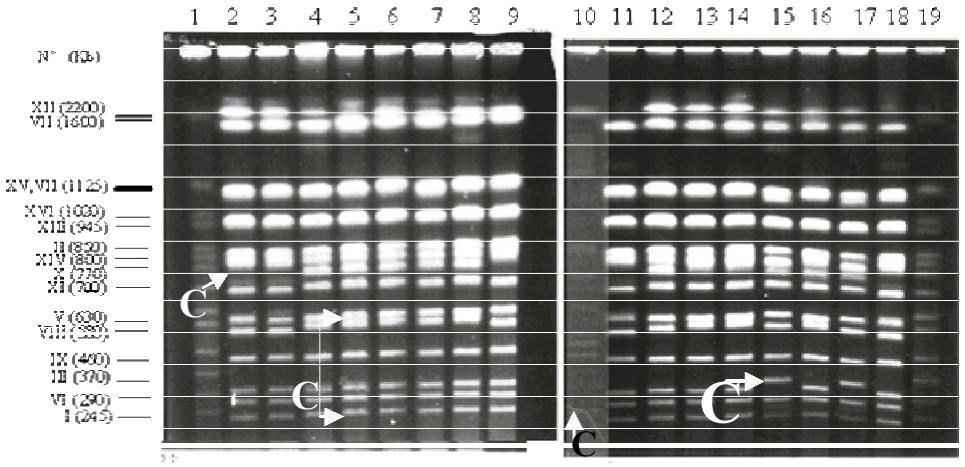

Figure 5a. Contour clamped homogenous electric field (CHEF) banding patterns of chromosomal DNA of indigenous strains *S. cerevisiae* isolated from bili bili; with the size in Kb (left) of the chromosome from reference strain *S. cerevisiae* YNN 295. Legend: 1, YNN 295; 2, ST18G2**; 3, ST7G4**; 4, ST4G1*; 5, ST14G4*; 6, ST8G3*; 7, ST10G1*; 8, ST12G1*; 9, ST1G4**; 10, B7G3****; 11, C2G1**; 12, ST10G2*; 13, ST12G2*; 14, ST12G4*; 15, ST15G1***; 16, ST16G3*; 17, ST18G3***; 18, ST20G1**; 19, YNN 295. * = C1 = Group 1 (62.5%), ** = C2 = Group 2 (35%), *** = C3 = Group 3 (9.4%) and **** = C4 = Group 4 (3.1%).

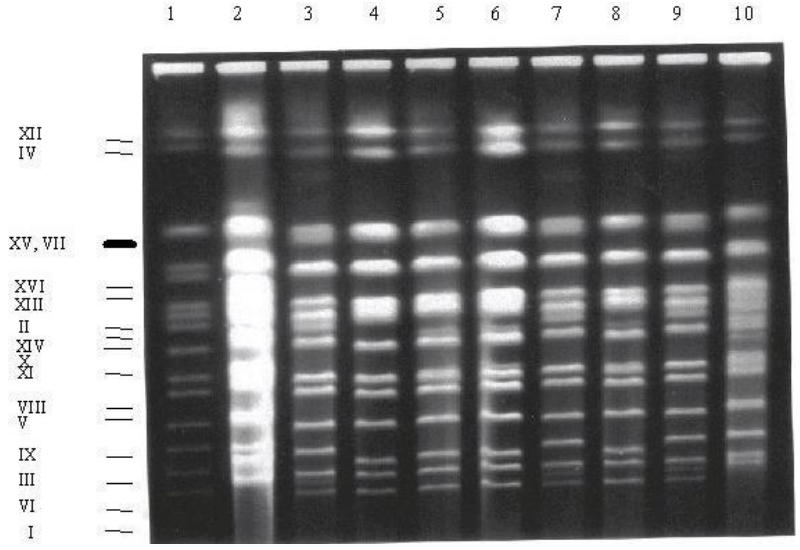

Figure 5b. CHEF banding patterns of chromosomal DNA from bili-bili. Legend: 1, Standard YNN 295; 2, Fermipan rouge; 3, ST1G1; 4, ST1G3; 5, ST7G3; 6, ST7G4, 7, ST16G1; 8, ST18G2; 9, ST18G3; 10, Fala 2.

GenBank. Based on classical criteria, they were considered as *Candida melibiosica* and *Rodotorula mucilaginosa*, respectively.

Karyotypic analysis of isolates identified as *S. cerevisiae*

Karyotypic analysis has been widely used to characterize yeasts isolate from various kinds of fermented foods and beverages (Bakalinsky and Snow, 1999; Vaugham-Martini et al., 1985; Zimmerman and Fournier, 1996) . To analyse the diversity of bili-bili strains, the CHEF gel elctrophoresis technique (Chu et al., 1986) was used to separate the intact chromosomal DNA of 32 isolate identified as *S. cerevisiae*. All chromosome profiles obtained from these strains were similar to, but but not completely identical to that of the staandard *S. cerevisiae* strain YNN295. Chromosome patterns exhibited by the 32 isolates can be divided into four groups (see example of these karyoptypes Figure 5a). The dominant karyotype

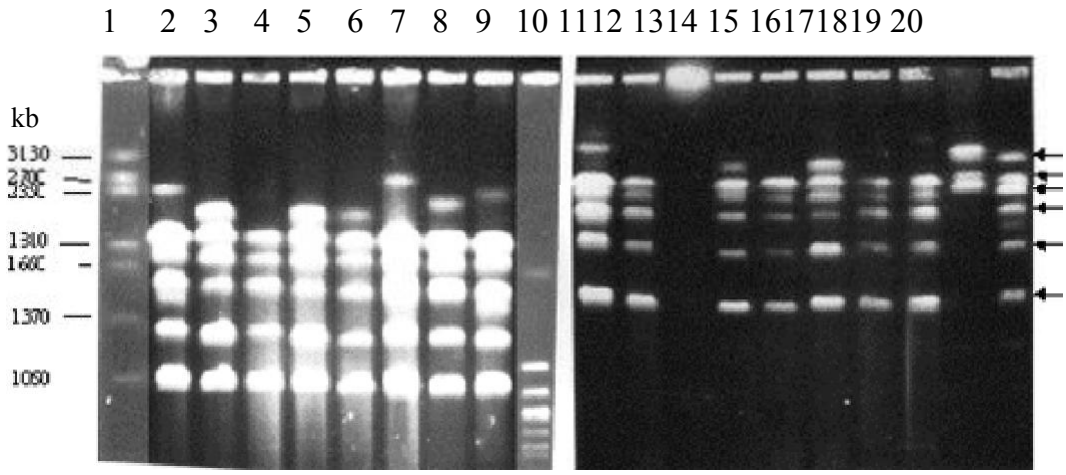

Figure 6. CHEF banding patterns of chromosomal DNA from indigenous strains *K. marxianus* isolated from bili bili with the size in Kb (left) of the chromosome from reference strain *H. wingei.* Legend**:** 1, *H. wingei*; 2, A1G1***; 3, E1G1**; 4, ST2G2*; 5, E2G2**; 6, B2G3**; 7, ST13G1*; 8, ST2G3*; 9, A2G2***; 10, YNN 295, *S. cerevisiae* reference strain; 11, A1G1***; 12, A2G3**; 13, v; 14, B2G3**; 15, B5G2*;16, E1G1**; 17, ST2G3*; 18, ST13G4*; 19, CBS 2105; 20, LsL7, *K. marxianus* reference strain. * = C1 = Group 1 (58.4%), ** = C2 = Group 2 (25%) and *** = C3 = Group 3 (16.6%).

(Goup 1, 65% of the strains) was caracterized by chromosomes I, VI, III, IX, V and VIII which appeared smaller than those of YNN295. This group is nonetheless heterogeneous in the region of chromosomes VIII to II.

Karyotypic analysis of isolates identified as *K. marxianus*

In order to analyse the genetic polymorphism among strains identified as *K. marxianus*, we first clustered these strains in 12 representative groups based on their physiological caracteristics and on their PCR/RFLP-NTS2 profiles. Karyotypes of strains representative of each group were obtained by CHEF gel elctrophoresis (Figure 6). The standard strain *K. marxianus* LsL7 presents a chromosomal pattern of 6 bands with an additional faint band in the range of 2 Mb (Figure 6, lane 20). All tested yeasts presented profiles compatible with their belonging to the *K. marxianus* species except for ST132G4 (Figure 6, lane 18). Variations in chromosomal size were however apparent, especially in the case of the upper, heavier band which was not reproducibly observed in all samples: it may correspond to a chromosome harbouring rDNA units and aberrantly migrating in the various samples.

Differentiation of *S. cerevisiae* isolated from"bili bili" to the industrial *S. cerevisiae*

The 2DOG test: Industrial baker yeasts have been selected for resistance to carbon catabolite repression of maltose assimilation. On a medium containing glucose and maltose as carbon source, they are able to ferment these two sugars simultaneously. On such a medium, wild yeasts will ferment glucose preferentially. By using a non metabolisable glucose analogue, 2 deoxy- D-glucose (2-DOG), which still may act as a repressor, it is possible to distinguish a wild yeast from an industrial strain by a fermentation test in a Durham's tube. A wild yeast will not ferment maltose in the presence of the 2-DOG and will not produce gas in the bell, whereas the industrial yeast will be resistant to 2-DOG repression and produce gas. We tested beforehand several strains isolated from commercial leavens and confirmed this principle. Specifically, Fermipan Rouge (Gist Brocades) and Fala 2 (Lesafre) fermented well maltose even at the highest 2-DOG concentration tested (5 µg ml^{-1}). Then we subjected 40 bili-bili isolates of *S. cervisiae* to this test. The majority of the strains tested (Table 3) did not ferment maltose in presence of 2-DOG, even at low concentration (1 µg ml^{-1}). Some rare strains however did ferment maltose in the presence (1 µg ml^{-1}) 2-DOG. All these strains were repressed by 2 µg ml^{-1} 2-DOG. . These results strongly suggest that wild strains of yeasts are responsible for the alcoholic fermentation of bili-bili.

Discrimination by karyotypic analysis: The chromosomal patterns of six strains able to ferment maltose in the presence of 1 µg ml^{-1} 2-DOG were compared to those of industrial strains (Figure 6b). None of the *S. cerevisiae* strains isolated from bili bili corresponded to these industrial strains that are usually characterized by the presence of supernumerary chromosomes. This further confirms that the bili-bili

Table 3. Result of maltose (2%) fermentation test in presence of different concentrations (0 to 3 µg ml^{-1}) of 2-deoxy-D-glucose (2-DOG).

No.	Strain	Glucose	2% Maltose + 2-deoxy-D-glucose (2DOG)			
			0	1 µg ml^{-1}	2 µg ml^{-1}	3 µg ml^{-1}
1	B7G3	+	-	-	-	-
2	C2G1	++	+++	-	-	-
3	ST1G1	+++	+++	+++	-	-
4	ST1G2	+++	+++	+++	-	-
5	ST1G3	+	+	+	-	-
6	ST1G4	++	++	+	-	-
7	ST3G3	+	+	-	-	-
8	ST4G1	+	+	-	-	-
9	ST4G2	+	+	-	-	-
10	ST7G1	+	+	-	-	-
11	ST7G3	++	++	++	-	-
12	ST7G4	++	++	++	-	-
13	ST8G1	+	-	-	-	-
14	ST8G2	+	-	-	-	-
15	ST8G4	+	+	-	-	-
16	ST9G3	+	+	-	-	-
17	ST10G1	+	+	-	-	-
18	ST10G2	++	++	-	-	-
19	ST10G3	++	++	-	-	-
20	ST10G4	++	++	-	-	-
21	ST11G2	+	+	-	-	-
22	ST11G4	+	-	-	-	-
23	ST12G1	+	+	-	-	-
24	ST12G2	+	+	-	-	-
25	ST12G3	+	+	-	-	-
26	ST12G4	+	+	-	-	-
27	ST14G4	+	+	-	-	-
28	ST15G1	++	++	-	-	-
29	ST16G1	++	++	++	-	-
30	ST16G2	+	+	-	-	-
31	ST16G3	+	+	-	-	-
32	ST16G4	+	+	-	-	-
33	ST17G1	+	+	-	-	-
34	ST18G1	++	++	++	-	-
35	ST18G2	+	+	+	-	-
36	ST18G3	++	++	++	-	-
37	ST20G1	+	+	-	-	-
38	ST20G2	+	+	-	-	-
39	Fermi rouge	++	++	++	++	++
40	Fala 2	+++	+++	+++	+++	+++
41	S288C	+	+	-	-	-

+: positive result
-: negative result

fermentative flora is not usually contaminated by industrial leavens marketed everywhere in the world.

Trial fermentation on sorghum wort: In order to select technologically active strains usable as inoculum for bili bili production, we carried out fermentation trials on sorghum wort in erlens with 10 indigenous *S. cerevisiae* strains. The percentage of fermentable sugars consumption (glucose, maltose and malotriose) and the concentation of ethanol produced after 20 h of fermentation are shown in Table 4. We observed that all strains fermented glucose completely (on average 94% of

Table 4. Result of trial fermentation on the sorghum wort. % of fermentable sugars (glucose, maltose, malotriose and total fermentescible sugars, TFS) and concentration (g) of ethanol produced after 20 h of fermentation. The initial wort contain: glucose, 21 g l^{-1}; maltose, 58.2 g l^{-1} ; maltotriose, 31.5 g l^{-1}; TFS, 110 g l^{-1} and ethanol, 0 g l^{-1}.

Tested strains	Glucose	Maltose	Maltotriose	TFS*	Ethanol
C2G1	94	62.5	49.9	64.9	19.4
ST1G1	94	82.4	55.1	76.8	30.9
ST1G3	94	63.1	52.4	65.9	20
ST1G4	98.9	38.2	55.6	54.7	23.8
ST7G3	94	69.8	54.2	70	25.6
ST7G4	94.5	85.3	69.8	82.6	31.9
ST16G1	94.5	85.7	58.7	79.7	29.4
ST18G2	94.5	64.2	53	66.8	18.1
ST18G3	94.9	87.3	59.7	80.8	30.6
Fala 2	94.5	72.4	46	69.1	25

*TFS = total fermentescible sugars

the initial content). Strains consuming maltose more efficiently were also those which produced more alcohol. On this basis, we ranked them in the following order: ST7G4, ST1G1, ST18G3 and ST16G1 according to their ability to produce alcohol yield. These strains may be useful for developing starters for bili bili productio.

In conclusion, the identifications reported here (Table 5) highlight a polymorphic complex flora which evolves during the process of bili-bili production. We have observed that the species *S. cerevisiae* was always present during the fermentative phase (76.6% of the strains). On the other hand, this species was barely detectable in samples taken prior to this step: during the souring phase, *K. marxianus* strains were predominant (65%). Other species such as *Cryptococcus albidius* var *albidius*, *Candida melibiosica*, *Debaryomyces hanseni* var *hanseni*, *Dekkera bruxelensis*, *Rodotorula mucilaginosa* and *Torulaspora delbrueckii* were occasionally found in samples of both phases. These results confirmed our preliminary work, and complete observations made on other African traditional beers (Dirar, 1978; Nout, 1980; Novellie and chaedrijver, 1986). These authors reported that *S. cerevisiae* was the main active species during fermentation of African's beers and was often associated with other species which may contribute to the organoleptic characters of beer. The presence of such accompanying flora is not tolerated in European beer (Moll, 1991). Ekundayo (1969) found that *Candida* yeasts were responsible of alcoholic fermentation of pito, a sorghum beer of Ghana, whereas *Geotrichum candidum* was the species responsible for the souring of beer. Kühle et al. (2000) identified exclusively *Saccharomyces* spp in pito samples and in tchacpalo's samples, the sorghum beer of Burkina Faso.

The PCR/RFLP of NTS2 sequences using *Alu*I and *Ban*I proved more practical, faster and more discriminating for the identification of the yeasts *S. cerevisiae* and *K. marxianus*. The CHEF technique, remains reliable to highlight polymorphism among *S. cerevisiae* and *K marxianus* species. Four different chromosomic karyotypes among *S. cerevisiae* and three among *K. marxianus* were observed. Kühle et al. (2000) and Haeford and Jesperson (1999) made similar observations respectively on samples of beers "tchacpalo" and "pito" showing that several strains of *S. cerevisiae* were implied in the fermentation of many kinds of beers and fermented foods in Africa.

We showed that the karyotypes and profiles of maltose fermentation in the presence of 2-DOG clearly differentiated bili-bili *S. cerevisiae* strains from industrial leavens marketed worldwide. These strains, in particular ST18G3, T7G4, ST16G1 and ST1G1, are probably indigenous wild strains which may constitue a good leaven for bili bili production.

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Table 5. Isolation, identification and typing results: BI-SP and BI -FP designate strains isolated from samples taken at the souring and fermentation stages of bili bili processing, respectively.

N°	Ref.	Origine of strain	Conventional method	PCR/RFLP of NTS2-rDNA patterns			Sequences	Karyotypes	Name of especie
				Amplification	*Alu* I digestion	*Ban* I digestion	D1/D2		
1	B7G3	BI-SP	*C.alb.var.albidi.*	1bande $^{cer.}$	2 bandes $^{cer.}$	3 bandes $^{cer.}$		Car $^{cer.}$ 4	*S.cerevisiae*
2	C2G1	BI-SP	*S. Cerev.padox.*	-"-	-"-	-"-		Car $^{cer.}$ 2	-"-
3	ST1G1	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 3	-"-
4	ST1G2	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 1	-"-
5	ST1G3	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 2	-"-
6	ST1G4	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 2	-"-
7	ST3G3	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 1	-"-
8	ST4G1	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 1	-"-
9	ST4G2	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 2	-"-
10	ST7G1	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 1	-"-
11	ST7G3	BI-FP	*T. delbrueckii*	-"-	-"-	-"-		Car $^{cer.}$ 2	-"-
12	ST7G4	BI-FP	*S. Cerev.padox.*	-"-	-"-	-"-		Car $^{cer.}$ 2	-"-
13	ST8G1	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 1	-"-
14	ST8G2	BI-FP	- "-	-"-	-"-	-"-		nd	-"-
15	ST8G3	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 1	-"-
16	ST8G4	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
17	ST9G3	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
18	ST10G1	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
19	ST10G2	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
20	ST10G3	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
21	ST10G4	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
22	ST11G2	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
23	ST11G3	BI-FP	- "-	-"-	-"-	-"-		nd	-"-
24	ST11G4	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 3	-"-
25	ST12G1	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 1	-"-
26	ST12G2	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
27	ST12G3	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
28	ST12G4	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
29	ST14G4	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
30	ST15G1	BI-FP	*T. Delbrueckii*	-"-	-"-	-"-		Car $^{cer.}$ 3	-"-
31	ST16G1	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
32	ST16G2	BI-FP	*S. Cerev.padox.*	-"-	-"-	-"-		nd	-"-
33	ST16G3	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 1	-"-
34	ST16G4	BI-FP	- "-	-"-	-"-	-"-		-"-	-"-
35	ST17G1	BI-FP	- "-	-"-	-"-	-"-		nd	-"-
36	ST18G1	BI-FP	*T. delbrueckii*	-"-	-"-	-"-		nd	-"-
37	ST18G2	BI-FP	*S. Cerev.padox.*	-"-	-"-	-"-		Car $^{cer.}$ 2	-"-
38	ST18G3	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 3	-"-
39	ST20G1	BI-FP	- "-	-"-	-"-	-"-		Car $^{cer.}$ 2	-"-
40	ST20G2	BI-FP	- "-	-"-	-"-	-"-		nd	-"-
41	A1G1	BI-SP	*K. maxianus*	1bande $^{max.}$	3bandes $^{max.}$	1bande $^{max.}$			*K. maxianus*
42	A1G2	BI-SP	- "-	-"-	-"-	-"-			-"-
43	A1G3	BI-SP	- "-	-"-	-"-	-"-			-"-
44	A1G4	BI-SP	- "-	-"-	-"-	-"-			-"-
45	A2G1	BI-SP	- "-	-"-	-"-	-"-			-"-
46	A2G2	BI-SP	- "-	-"-	-"-	-"-		Car $^{max.}$ 3	-"-
47	A2G3	BI-SP	- "-	-"-	-"-	-"-		Car $^{max.}$ 1	-"-
48	A2G4	BI-SP	- "-	-"-	-"-	-"-			-"-
49	A7G1	BI-SP	*D.han.var.hans*	-"-	-"-	-"-			-"-
50	B2G2	BI-SP	- "-	-"-	-"-	-"-			-"-
51	B2G3	BI-SP	*K. maxianus*	-"-	-"-	-"-		Car $^{max.}$ 2	-"-
52	B2G4	BI-SP	- "-	-"-	-"-	-"-	KM U94924		-"-
53	B5G1	BI-SP	- "-	-"-	-"-	-"-			-"-
54	B5G2	BI-SP	- "-	-"-	-"-	-"-		Car $^{max.}$ 1	-"-
55	B5G3	BI-SP	- "-	-"-	-"-	-"-			-"-
56	B5G4	BI-SP	- "-	-"-	-"-	-"-			-"-
57	E1G1	BI-SP	- "-	Others bandes	1 bande ^{other}	1 Other bande			-"-
58	E1G2	BI-SP	- "-	1bande $^{max.}$	3bandes $^{max.}$	1bande $^{max.}$		Car $^{max.}$ 2	-"-
59	E2G1	BI-SP	- "-	-"-	-"-	-"-			-"-
60	E2G2	BI-SP	- "-	-"-	-"-	-"-	KM U94924		-"-
61	ST2G1	BI-FP	- "-	-"-	-"-	-"-			-"-
62	ST2G2	BI-FP	*S. Cerev.padox.*	-"-	-"-	-"-	KM U94924		-"-
63	ST2G3	BI-FP	*K. maxianus*	-"-	-"-	-"-	KM U94924	Car $^{max.}$ 1	-"-
64	ST2G4	BI-FP	*S. Cerev.padox.*	-"-	-"-	-"-		Car $^{max.}$ 1	-"-
65	ST13G1	BI-FP	*K. maxianus*	-"-	-"-	-"-		Car $^{max.}$ 1	-"-
66	ST13G4	BI-FP	*T. Delbrueckii*	-"-	-"-	-"-		Car $^{max.}$ 1	-"-
67	B2G1	BI-SP	*C.melibiosica*	Others bandes	1 bande ^{other}	1 bande ^{other}	Other		*C.melibiosica*
68	B7G1	BI-SP	*C.alb.var.albidi.*	-"-	-"-	-"-	Other		*C.alb.var.albidi.*
69	B7G2	BI-SP	*C.alb.var.albidi.*	-"-	-"-	-"-			*C.alb.var.albidi.*
70	B7G3	BI-SP	*C.alb.var.albidi.*	-"-	-"-	-"-			*C.alb.var.albidi.*
71	B7G4	BI-SP	*De.Bruxellensis*	-"-	-"-	-"-			*De.Bruxellensis*
72	B7P1	BI-SP	*R. mucilaginosa*	-"-	-"-	-"-			*R. mucilaginosa*
73	B7P2	BI-SP	*R. mucilaginosa*	-"-	-"-	-"-			*R. mucilaginosa*
74	B7P4	BI-SP	*R. mucilaginosa*	-"-	-"-	-"-			*R. mucilaginosa*
75	ST13G2	BI-SP	*C.alb.var.albidi.*	-"-	-"-	-"-			*C.alb.var.albidi.*
76	ST13G3	BI-SP	*R. mucilaginosa*	-"-	-"-	-"-			*R. mucilaginosa*
77	ST14G1	BI-SP	*D.han.var.hans*	-"-	-"-	-"-			*D.han.var.hans*

Car $^{cer.}$ = Karyotype of *S.cerevisiae* | Car $^{max.}$ = Karyotype of *K. maxianus* | nd = no determined

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