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ORIGINAL ARTICLE

Yeast species associated with spontaneous fermentation of *taberna*, a traditional palm wine from the southeast of Mexico

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Abstract The aim of this study was to isolate and identify the yeasts present in *taberna*, a traditional palm wine from Mexico, which is produced by natural fermentation of the palm sap obtained from *Acrocomia aculeata*. A total of 450 yeast isolates were obtained from 45 *taberna* samples collected over 15 days of tapping at the end of fed-batch fermentation (12 hours of feeding and fermentation of the sap in the *canoe*) from three coyol palm trees. The yeast isolates were identified using restriction pattern analysis of the internal transcribed spacer region 5.8S-ITS and by sequence analysis of the D1/D2 divergent domain of the 26S rRNA gene. Nine different yeast species were identified in the *taberna* samples tested, namely, *Saccharomyces cerevisiae*, *Hanseniaspora guilliermondii*, *Candida tropicalis*, *Candida intermedia*, *Kazachstania unispora*, *Kazachstania exigua*, *Meyerozyma guilliermondii*, *Pichia kudriavzevii* (*Issatchenkia orientalis*) and *Pichia kluyveri*. The non-*Saccharomyces* yeasts *H. guilliermondii* and *C. tropicalis* were detected in samples from all three palm trees, while *S. cerevisiae* was detected in samples from only two of the palm trees. The frequency and distribution of the yeast species were different in the samples of each palm tree, which indicated that the inoculum in the palm sap may be deposited randomly by different vectors. This study is the first to characterize the yeast population associated with the palm wine named *taberna*.

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Introduction

Taberna is a palm wine produced by the natural fermentation of the sap obtained by tapping the coyol palm tree (*Acrocomia aculeata* (Jacq.) Lodd. ex Mart.). This palm wine is consumed as a refreshing sour and alcoholic beverage in the southeast of Mexico and Central America (Balick 1990; Alcántara-Hernández et al. 2010). The process of *taberna* production has been reported previously by Santiago-Urbina et al (2013). The palm sap undergoes a natural fermentation in which the inoculum may come from the tools, the natural microbiota present in the trunk, and/or the insects that are constantly attracted to the sweetness of the sap (Karamoko et al. 2012; Santiago-Urbina et al. 2013). *Taberna* production is carried out via a fed-batch fermentation process, wherein every 12 hours the sap in the *canoe* is collected (drained) and then the new sap flow is used to begin a new batch of fermentation (Santiago-Urbina et al. 2013). The *taberna* product is collected twice a day, in the morning and in the afternoon. After the morning collection of the sap, the producer scrapes the *canoe*, cutting a thin slice of the walls to expose a fresh layer of the palm tissue; this process physically removes the microbiota that colonized the walls of the *canoe*, thus reducing the microbial load for the next batch. The unfermented sap contains mainly sucrose, about 11.36 % (w/v), and has a pH of 7.25 (Santiago-Urbina et al. 2013). In contrast, the fermented sap contains from 0.21 to 4.78 % (w/v) ethanol, 0.05 to 0.48 % (w/v) lactic acid, 0.01 to 0.24 % (w/v) acetic acid, and has pH values from 6 to 4. The composition of the sap depends on the stage of fermentation at which the *taberna* is consumed. *Taberna* has similar physicochemical and microbiological characteristics as other palm wines such as Bandji, Lagmi,

Toddy, Tuak, Mimbo, and Mnazi (Atputharajah et al. 1986; Jepersen 2003; Kadere et al. 2008; Ben Thabet et al. 2009; Ouoba et al. 2012). During tapping of *Acrocomia aculeata*, the sap develops alcoholic, lactic, and acetic acid fermentations similar to those in the palm wine from *Elaeis guineensis* (Amoa-Awua et al. 2007; Stringini et al. 2009) and Toddy from *Cocos nucifera* (Atputharajah et al. 1986). Therefore, yeasts, and lactic and acetic acid bacteria have been found in *taberna* in concentrations of about 10^3 and 10^7 colony forming units (CFU)/mL for yeast, and 10^7 to 10^8 CFU/mL for lactic and acetic acid bacteria (Santiago-Urbina et al. 2013). A previous study of the microbial community in *taberna*, which was focused on the identification of the bacteria, showed that *Zymomonas mobilis*, *Fructobacillus durionis*, *Fructobacillus fructosus*, *Pantoea agglomerans*, and other Gammaproteobacteria, lactic acid bacteria such as *Lactobacillus nagelli*, *Lactobacillus sucicola*, *Lactobacillus* sp., and acetic acid bacteria such as *Acetobacter pasteurianus* were present at some stages of the in vitro fermentation of *taberna* (Alcántara-Hernández et al. 2010). However, until now, no studies have reported the identification of yeasts from *taberna*. Yeast species such as *Saccharomyces cerevisiae* are important for alcoholic fermentation, and they have been found in the palm wine of *E. guineensis* in Ghana and Cameroon, as well in Bandji from Burkina Faso (Amoa-Awua et al. 2007; Stringini et al. 2009; Ouoba et al. 2012). The major total volatiles and alcohols are produced by *S. cerevisiae* and *Saccharomyces chevalieri* (Uzochukwu et al. 1999; Nur Aimi et al. 2013). However, other yeast species are also capable of influencing, in a positive way, the aromatic profile of palm wines.

Variations in the chemical composition of *taberna* through the tapping process, the source and microbial inoculum in the natural fermentation, as well as the environmental conditions such as the temperature in the place where *taberna* is produced, which ranges from 35 to 38 °C (Serrano-Macias, pers. comm.), make this traditional beverage an interesting source of microorganisms with potential biotechnological applications. Until now, most studies have focused on the bacterial content of *taberna*, and no studies on the yeast population have been reported so far. Hence, the aim of the present study was to identify the yeast population in samples collected during *Taberna* production from 15 fed-batches during 15 days of tapping.

Materials and methods

Samples

In March 2012, a total of 45 *taberna* samples were obtained during 15 days of tapping from three coyol palms in Benito Juárez in the state of Chiapas, México, where the ambient temperature varies from 30 to 38 °C. Each day during tapping, approximately 50 mL of palm sap was collected directly from the *canoe* at the end of the fed-batch fermentation. The samples were collected into pre-sterilized 50-mL Falcon tubes in the morning (6:00 am), before the *canoe* scraping was performed. The samples were transported in an ice box (4 °C) to the laboratory at the Universidad de Ciencias y Artes de Chiapas (UNICACH) within 2 h of their collection. The palm trees were identified as I, II, and III. The samples were collected in triplicate from each palm tree.

Chemical and statistical analyses

The sugar (sucrose, glucose, and fructose) and ethanol concentrations in the *taberna* samples from each coyol palm (three) were determined using a liquid chromatography system (Waters Corporation, Milford, MA, USA) following the methodology described by Santiago-Urbina et al (2013). The pH was determined using an Accumet AB15 pH meter (Cole-Palmer, IL, USA). All the samples from each palm tree were analyzed in duplicate. Data obtained were subjected to an analysis of variance (ANOVA) using the Fisher's least significant difference (LSD) test to determine significant differences between each palm tree sample ($P \leq 0.05$). The analysis was performed using Statgraphics Centurion XV software (Statpoint Technologies Inc., Warrenton, VA, USA).

Yeast isolation

The samples were processed 3 h after their collection. The Falcon tubes containing the *taberna* samples were shaken gently by hand and ten-fold serial dilutions were prepared in 0.1 % (w/v) of sterile peptone water (Bioxon, Becton, Dickinson and Company, New Jersey, USA). Next, 0.1 mL of the dilutions were spread-plated in duplicate on plates of Wallerstein Laboratories nutrient agar (WL, Difco^{tm}, Le Pont-de-Claix, France), supplemented with 0.01 % w/v chloramphenicol (Sigma-Aldrich, MO, USA). The plates were incubated at 30 °C for 3–5 days (Cocolin et al. 2000; Verdugo Valdez et al. 2011). After the incubation period, the colony-forming units (CFU) were counted. Then, 10 colonies from each sample were picked up from the countable plates on the basis of different color and colony morphology. Selected colonies were sub-cultured on YEPD agar plates (yeast extract 10 g/L; peptone 20 g/L; dextrose 20 g/L; agar 20 g/L) by streaking. The purified isolates were suspended in YEPD broth containing 30 % (v/v) glycerol and stored at -20 °C until identification.

DNA isolation

A total of 450 isolated yeasts from *taberna* samples were analyzed. Yeasts were grown in 5 mL of YEPD broth at

30 °C with shaking at 200 rpm for 18 h. Approximately 1.5 mL of culture was centrifuged at 20,000 x *g* for 5 min at 4 °C three times (for more biomass recovery). The pellet was used for DNA extraction using a modified method reported by Cocolin et al. (2000). The cells were resuspended in 200 μL of breaking buffer (2 % triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA pH 8), 0.3 g of glass beads (diameter 0.42–0.6 mm), and 200 μL of buffered phenol, chloroform, and isoamyl alcohol (25:24:1, v/v) (Sigma-Aldrich, Saint Louis, MO, USA) were added to the mixture. After vortexing for 1 min and centrifuging at 20,000 x *g* for 10 min, 200 μL of TE buffer (10 mM Tris, 1 mM EDTA pH 7.6) were added. The mixture was centrifuged at 20,000 x *g* at 4 °C for 10 min. The upper phase was pipetted into a 1.5-mL Eppendorf tube. The DNA was precipitated with 1 mL of ice-cold 96 % ethanol and maintained in a freezer at -20 °C for 20 min, then centrifuged at 20,000 x *g* at 4 °C for 10 min. The pellet was washed with ice-cold 70 % ethanol, and dried overnight. The DNA pellet was resuspended in 100 μL of sterile deionized water for 1.5 h at room temperature and stored at -20 °C until use.

Yeast molecular identification

The 5.8S-ITS (internal transcribed spacer region) rDNA of the isolated yeasts was amplified by polymerase chain reaction (PCR) assay with the forward primer ITS1 (5'-TCCGTAGG TGAACCTGCGG-3') and reverse primer ITS4 (5'-TCCTCC GCTTATTGATATGC-3'). PCRs were performed in a 50-μL reaction volume containing: 1x PCR buffer, 2.25 mM $MgCl_2$, 100 μM dNTP mix, 0.5 μM each of forward and reverse primers, 1.25 U of Taq DNA polymerase (Invitrogen, CA, USA), and 2 μL of DNA solution. The amplification was performed with a PIKO 24 thermal cycler (Thermo Scientific, Vantaa, Finland) under the PCR conditions described by Esteve-Zarzoso et al (1999). The PCR products were separated by applying 10 μL of each amplicon with 1.5 μL of 6x loading buffer (Invitrogen) to a 1.5 % (w/v) agarose (Invitrogen) gel containing 0.7 μg/mL of ethidium bromide (Bio-Rad, CA, USA). The approximate sizes of the amplicons were determined using a standard molecular weight marker (100-bp DNA ladder; Invitrogen). The gel was run in 0.5 x TBE buffer (44.5 mM Tris (Sigma-Aldrich, MO, USA), 44.5 mM boric acid (Sigma-Aldrich), 1 mM Na_2-EDTA (Sigma-Aldrich)) for 90 min at 100 V and photographed under transilluminated ultraviolet (UV) light. Band sizes were estimated by comparison against the DNA ladders using Kodak Molecular Imaging Software version 5.0 (Carestream Health, Inc, Rochester, NY, USA).

For restriction reactions of the amplified DNA (5.8S-ITS rDNA), 11.5 μL of the PCR products were digested without further purification, in 15 μL reaction volume (1.5 μL of 10 x buffer, 0.2 μL of restriction enzyme (2 U) and 1.8 μL sterile deionized water). The restriction enzymes used for all amplified DNA from the yeast isolates were *Hae*III, *Hinf*I, and *Hha*I (Invitrogen). The digestion reaction was incubated for 3.8 h at 37 °C. Restriction fragments were separated by gel electrophoresis (Bio-rad) on a 2 % (w/v) agarose (Invitrogen) gel containing 0.7 μg/mL of ethidium bromide (Bio-Rad), in 0.5x TBE buffer. The gel was run in 0.5 x TBE for 90 min at 90 V and photographed under transilluminated UV light. The fragment sizes were estimated by comparison against the standard DNA markers (100-bp DNA Ladder, Invitrogen) using Kodak Molecular Imaging Software, version 5.0 (Carestream Health, Inc). The restriction patterns were compared with previously published studies by Esteve-Zarzoso et al. (1999), Granchi et al. (1999), Las Heras-Vazquez et al. (2003), Osorio-Cadavid et al. (2008), Stringini et al. (2008), Pham et al. (2011), and Wang and Liu (2013).

Isolates were grouped based on their restriction fragment length polymorphism (RFLP) patterns after identification of unique common restriction profiles using unweighted pair group average (UPGMA) cluster analysis based on the Jaccard similarity index using the PAST software, version 2.17c (Jeyaram et al. 2008).

Sequence analysis of the 26S rDNA D1/D2 domain

Sequencing of the D1/D2 divergent domain of the large subunit (26S) rDNA was performed on representative strains of each created group by PCR-RFLP. The NL1 (5'-GCATATCA ATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTG TTTCAAGACGG-3') primers (Kurtzman and Robnett 1998) were used to amplify the D1/D2 domain. The reactions were performed in a PIKO 24 thermal cycler (Thermo Scientific) under the following conditions: initial denaturation at 95 °C for 5 min; 35 cycles of denaturing at 94 °C for 1 min, annealing at 55.5 °C for 2 min, and extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. The amplified fragments where then sequenced by Laragen Sequencing and Genotyping (Culver, CA, USA). The sequences were edited using Mega 5.1 (Tamura et al. 2011) and compared with the sequences in the GenBank database at http://www.ncbi.nlm.nih.gov/nucleotide using the basic local alignment search tool (BLAST).

Nucleotide sequence accession number

The D1/D2 26S rRNA sequences have been deposited in GenBank under accession numbers KF241558, KF241559, KF241560, KF241561, KF241562, KF241563, KF241564, KF241565, and KF241567.

Frequency percentage analysis

To study the species distribution in our samples, the method proposed by Solieri et al. (2006) was used. This method evaluates of the number of times each species is detected in a sample, without considering the number of strains that belong to the species. In this way, the number of positive samples for each species and the corresponding frequency, defined as the number of positive samples for a species divided by the total number of samples expressed as a percentage, was estimated.

Results

Chemical composition and yeast counts of the *taberna* samples

The physicochemical characteristics of *taberna* are listed in Table 1. The initial total sugar content (sucrose, glucose, and fructose) of fresh palm wine was 11.63±0.54 % (w/v) in the samples from all three palm trees (I, II, and III), and this concentration decreased similarly over time in all the samples. However, from day 11 onwards the lowest sugar concentrations were found in the samples from coyol palm III (Table 1). The initial pH values in the first samples from the three palms were almost neutral (7.26±0.02), and then decreased to values of approximately 4 in all samples. On the other hand, in the samples taken on the first 4 days, no ethanol content was detected (Table 1), then, from sample 5 onwards, the ethanol content was found to range from approximately 0.6 to 4.8 % (w/v). The yeast count was 3.67 $\log_{10}$ CFU/mL in the first samples from all three palms, and then increased to approximately 7 $\log_{10}$ CFU/mL in the samples taken on day 4. After that, the yeast count remained close to 6 $\log_{10}$ CFU/mL until day 11 (Table 1), when a 2.32, 2.96, and 2.8 log reduction was observed in the samples from palm trees I, II, and III, respectively (Table 1).

Isolation and identification of yeast isolates

To identify the dominant yeasts present during *taberna* production, a total of 450 yeasts were isolated from *taberna* samples obtained from three different coyol palm trees over the 15-day tapping process. Different ITS-PCR product sizes (390–850 bp long) were detected in the isolates. When the PCR products were digested with *Hae*III, *Hinf*I, and *Hha*I restriction enzymes and then analyzed, nine different restriction patterns were obtained (Fig. 1 and Table 2). The nine pattern groups were identified directly after comparing their restriction fragment profiles with the data from Esteve-Zarzoso et al. (1999), Granchi et al. (1999), Las Heras-Vazquez et al. (2003), Osorio-Cadavid et al. (2008), Stringini et al. (2008), Pham et al. (2011), and Wang and Liu (2013) as: *Saccharomyces cerevisiae* (I), *Pichia kluyveri* (II), *Kazachstania unispora* (III), *Meyerozyma guilliermondii* (IV), *Candida tropicalis* (V), *Candida intermedia* (VI), *Hanseniaspora guilliermondii* (VII), *Kazachstania exigua* (VIII), and *Pichia kudriavzevii* (*Issatchenkia orientalis*) (IX) (Table 2).

To confirm the identity of each of the groups, strains representing each profile were identified by sequencing of the D1/D2 domain of their 26S rDNA. The sizes of the sequences that were obtained ranged from 508–598 bp (Table 2). When the D1/D2 sequences were compared with the sequences in GenBank, they were found to share sequence homologies of 98–100 % with known sequences (Table 2). The D1/D2 domain of each representative strain of groups I, II, III, IV, V, VI, and VIII shared 100 % identity with sequences from *S. cerevisiae*, *P. kluyveri*, *K. unispora*, *M. guilliermondii*, *C. tropicalis*, *C. intermedia* and *K. exigua*, respectively. The group VII strain shared 99 % identity with *H. guilliermondii*, while the group IX strain shared 98 % identity with *P. kudriavzevii* (*I. orientalis*). All the isolates identified by the sequences of the D1/D2 domain of the 26S rDNA genes coincided with the results obtained by the RFLP analysis (Table 2).

Frequency percentage analysis

The frequency of positive samples of different species identified for each palm tree is shown in Table 3. *Candida tropicalis* strains (93.33 %) were the most common species in the *taberna* samples from palm tree I, followed by *H. guilliermondii* (86.67 %) and *C. intermedia* (40 %). Other yeast species detected in the samples were *M. guilliermondii*, *S. cerevisiae*, and *P. kluyveri*. In the samples from palm tree II, *H. guilliermondii* (100 %) was the dominant species, followed by *C. tropicalis* (80 %), and *K. unispora* (73.33 %). Low percentages of *C. intermedia*, *P. kluyveri*, and *K. exigua* were detected, and these strains could be considered as sporadic strain in the fermentation process. In the samples from palm tree III, *H. guilliermondii* (73.33 %) was the dominant species, followed by *S. cerevisiae* (53.33 %) and *C. tropicalis* (53.33 %). The most common and abundant yeast species in the *taberna* samples from the three palm trees were *C. tropicalis* and *H. guilliermondii*. *S. cerevisiae* was found in samples from palm trees I and III. *K. unispora* was detected only in the samples from palm tree II, while *P. kudriavzevii* was detected only in the samples from palm tree III.

Yeast distribution in the *taberna* samples

The distributions of the different yeast species in the samples of *taberna* taken from each palm tree are shown in the Fig. 2. The data for the first sample for all three palm trees are

Table 1 Physicochemical analysis and yeast count of the *taberna* samples obtained from three coyol palms during the 15-day tapping process

Taberna samples	Palm tree I				Palm tree II				Palm tree III			
	Total sugar[a] (% w/v)	pH	Ethanol (% w/v)	Yeast count[b]	Total sugar[a] (% w/v)	pH	Ethanol (% w/v)	Yeast count[b]	Total sugar[a] (% w/v)	pH	Ethanol (% w/v)	Yeast count[b]
1[c]	11.63±0.54*	7.26±0.02*	nd	3.67±0.03*	11.63±0.54*	7.25±0.02*	nd	3.67±0.03*	11.63±0.54*	7.25±0.02*	nd	3.67±0.04*
2	8.34±0.33*	5.40±0.01*	nd	4.81±0.02*	8.37±0.31*	6.07±0.05**	nd	4.99±0.01**	8.86±0.40*	6.63±0.03***	nd	4.94±0.03**
3	9.45±0.18*	5±0.02*	nd	5.98±0.04*	7.82±0.01**	4.62±0.14**	nd	6.08±0.01**	7.80±0.00**	5.24±0.08***	nd	6.05±0.11*,**
4	7.31±0.02*	4.83±0.01*	nd	6.86±0.03*	7.31±0.01*	4.36±0.03**	nd	7.02±0.06**	7.11±0.31*	4.46±0.07***	nd	6.98±0.03**
5	5.30±0.00*	4.7±0.00*	0.69±0.00*	6.56±0.02*	4.49±0.24**	4.37±0.04**	0.35±0.03**	6.74±0.03**	5.27±0.00*	4.42±0.01**	0.69±0.01*	6.65±0.03***
6	1.91±0.05*	4.73±0.01*	0.24±0.01*	6.10±0.04*	1.91±0.05*	4.34±0.03**	0.24±0.01*	6.43±0.04**	1.90±0.05*	4.33±0.01**	0.24±0.01*	6.34±0.18*
7	3.44±0.30*	4.80±0.02*	0.19±0.01*	6.19±0.04*	3.28±0.03*	4.45±0.03**	0.19±0.01*	5.67±0.05**	5.79±0.03**	4.68±0.07***	0.26±0.00**	5.80±0.04***
8	5.87±0.17*	4.52±0.06*	0.27±0.00*	6.19±0.01*	4.73±0.11**	4.57±0.06*	0.25±0.03*	5.75±0.07**	6.51±0.09***	4.57±0.01*	0.25±0.01*	5.86±0.06***
9	7.17±0.03*	4.38±0.02*	0.71±0.01a*	6.23±0.02*	8.17±0.20**	4.18±0.02**	0.66±0.00**	6.45±0.03**	8.64±0.08***	4.48±0.01***	0.41±0.02***	6.39±0.07**
10	5.31±0.01*	4.24±0.01*	1.14±0.01*	5.81±0.02*	7.71±0.16**	4.25±0.00*	1.02±0.02*	6.14±0.03**	5.61±0.03*	4.41±0.04**	1.50±0.18*	6.06±0.10**
11	1.91±0.03*	4.15±0.00*	4.33±0.12*,**	5.15±0.02*	1.42±0.06**	4.14±0.09*	4.37±0.02**	6.47±0.03**	1.16±0.01***	4.26±0.01**	4.06±0.09*	6.14±0.04***
12	1.90±0.04*	4.07±0.01*	4.40±0.34*	4.15±0.08*	1.39±0.01**	4.05±0.03*	4.80±0.09*	4.57±0.09**	0.84±0.00***	4.5±0.01**	4.49±0.02*	4.47±0.05**
13	0.61±0.04*	4.35±0.02*	4.57±0.02*	3.19±0.02*	1.23±0.02**	4.07±0.03**	4.85±0.05**	3.82±0.01**	0.73±0.01***	4.29±0.03***	4.58±0.02*	3.66±0.04***
14	1.81±0.15*	3.96±0.01*	5.26±0.03*	3.28±0.02*	1.35±0.09**	4.08±0.07**	4.84±0.03**	3.50±0.07*	0.70±0.01***	4.17±0.01***	3.59±0.01***	3.44±0.31*
15	1.80±0.06*	4.00±0.04*	4.89±0.15*	2.83±0.13*	1.81±0.13*	4.02±0.05*	4.81±0.05*	3.51±0.06**	0.88±0.03***	4.11±0.01***	4.64±0.05*	3.34±0.11***

The data are the mean of two repetitions for each sample taken on different days±standard deviation. The comparison of means was made between the same parameter among palm trees; mean values followed by different number of superscript asterisks are statistically different (Fisher's LSD at $P \leq 0.05$). [a] Total sugar indicates the combined sucrose, glucose, and fructose content of a sample. [b] Yeast count is given as Log_{10} colony forming units (CFU)/mL. [c] The data for sample 1 is identical for all three palm trees, because the sap from each palm tree was mixed (for more volume) and only the one mixed sample was analyzed. w/v: weight/volume; nd: none detected

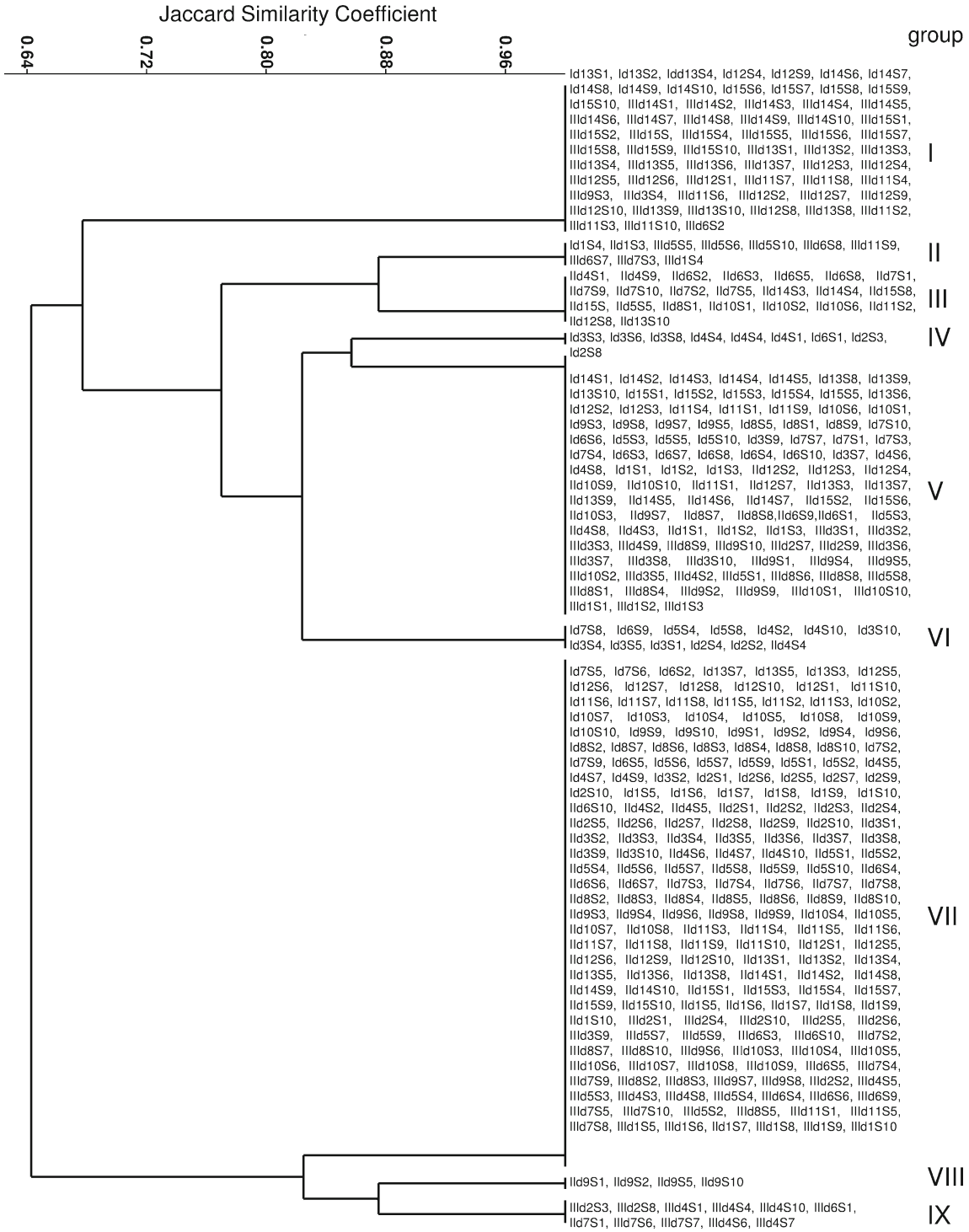

Fig. 1 Cluster analysis of the RFLP patterns of the yeasts isolated from *taberna* samples. The numbers I, II, and III represent to the palm tree I, palm tree II, and palm tree III, respectively, the letter d represents the day (1 to 15), and the letter S represents the strain (1 to 10)

identical because, at this point, the sap from each palm tree was mixed (for more volume) and only the one mixed sample was analyzed. For palm tree I, non-*Saccharomyces* yeasts were predominant in samples 1–11 (Fig. 2a), in which the most abundant species were *C. tropicalis* and *H. guilliermondii*. However, from samples 12–15, *S. cerevisiae* was also identified, and in samples 14 and 15 the yeast population was composed of 50 % *C. tropicalis* and 50 % *S. cerevisiae*.

Non-*Saccharomyces* yeasts were detected in all the samples from palm tree II (Fig. 2b); *H. guilliermondii* was the most abundant yeast species, followed by *K. unispora* and

Table 2 Identification of yeast isolates by 5.8S-ITS PCR product size with three restriction endonucleases and sequencing of the D1/D2 domain of the 26S rDNA of representative strains

ITS-RFLP group	AP[a] (bp)	Restriction fragment length (bp)			Sequence length[b] (bp)	Species identification[c]	Identity[d] (%)	Accession no.
		*Hae*III	*Hinf*I	*Hha*I				
I	850	310+230+175+135	360+360+130	370+340+140	559	*S. cerevisiae*	100	KF241567
II	470	365+90	255+215	170+100+60	563	*P. kluyveri*	100	KF241565
III	740	500+125	370+360	310+190+150+80	556	*K. unispora*	100	KF241561
IV	620	390+130+100	320+300	310+260	550	*M. guilliermondii*	100	KF241563
V	520	440+80	260+260	280+250	558	*C. tropicalis*	100	KF241559
VI	390	390	200+190	210+180	508	*C.intermedia*	100	KF241558
VII	750	730	340+220+190	320+310+100	550	*H. guilliermondii*	99	KF241560
VIII	730	475+240	345+245+140	360+280+75	556	*K. exigua*	100	KF241562
IX	535	390+100	225+160+150	210+180+70	598	*P. kudriavzevii*	98	KF241564

[a] AP is the 5.8S-ITS amplified product size in base pairs (bp). [b] Sequence length is the size of the D1/D2 domains of the 26S rDNA of the strains amplified with universal primers NL1 and NL4. [c] Yeast species were identified by comparing the 5.8S-ITS PCR product size pattern obtained for each group with the pattern of the strains described by Esteve-Zarzoso et al. (1999), Granchi et al. (1999), Las Heras-Vazquez et al. (2003), Osorio-Cadavid et al. (2008), Stringini et al. (2008), Pham et al. (2011), and Wang and Liu (2013). [d] Identity is the percentage of identical nucleotides in the sequence of the D1/D2 domains of 26S rDNA and the sequence with the best hit in the GenBank database. The ITS-RFLP groups are *Saccharomyces cerevisiae* (I), *Pichia kluyveri* (II), *Kazachstania unispora* (III), *Meyerozyma guilliermondii* (IV), *Candida tropicalis* (V), *Candida intermedia* (VI), *Hanseniaspora guilliermondii* (VII), *Kazachstania exigua* (VIII), and *Pichia kudriavzevii* (*Issatchenkia orientalis*) (IX).

C. tropicalis. In samples 2 and 3, the yeast population was 100 % *H. guilliermondii*. From sample 4 onwards, *K. unispora* and *C. tropicalis* were also present. The yeast species identified in samples from palm tree II were different from the species found in palm tree I. *Kazachstania unispora* and *K. exigua*, which were not found in samples from palm tree I, were identified in samples from palm tree II. Furthermore, *S. cerevisiae* was not identified in the samples from palm tree II, although it was found in the samples from palm trees I and III.

In the palm tree III samples (Fig. 2c), non-*Saccharomyces* yeasts predominated in samples 1–10, in which *H. guilliermondii* and *C. tropicalis* were the most abundant. However, from samples 11–15, *S. cerevisiae* predominated. In samples 12–15, the yeast population was 100 % *S. cerevisiae*. Thus, in palm tree III, in the first 10 days of tapping, non-*Sacharomyces* species predominated, and after 11 days, *S. cerevisiae* dominated in the fermenting samples. These results are similar to those reported previously in winemaking from grapes (Zott et al. 2008).

Discussion

The results from the present study revealed that the composition of the yeast species in the samples varied among the different palm trees. Also, the yeast population in *taberna* was different from the yeast population in palm wines produced from *Elaeis guineensis* in Ghana and Cameroon (Amoa-Awua et al. 2007; Stringini et al. 2009). Different

Table 3 Number of positive samples and frequency of yeasts species present in *taberna* from three palm trees

Species	Palm tree I		Palm tree II		Palm tree III	
	Positive sample	Frequency (%)[a]	Positive sample	Frequency (%)[a]	Positive sample	Frequency (%)[a]
H. guilliermondii	13	86.67	15	100	11	73.33
K. unispora	nd	-	11	73.33	nd	-
C. tropicalis	14	93.33	12	80	8	53.33
C. intermedia	6	40	1	6.67	nd	-
S. cerevisiae	4	26.67	nd	-	8	53.33
M. guilliermondii	4	26.67	nd	-	nd	-
K. exigua	nd	-	1	6.67	nd	-
P. kudriavzevii	nd	-	nd	-	4	26.67
P. kluyveri	1	6.67	1	6.67	5	33.33

[a] Frequency was calculated based on the total number of samples from the tree. nd: none detected.

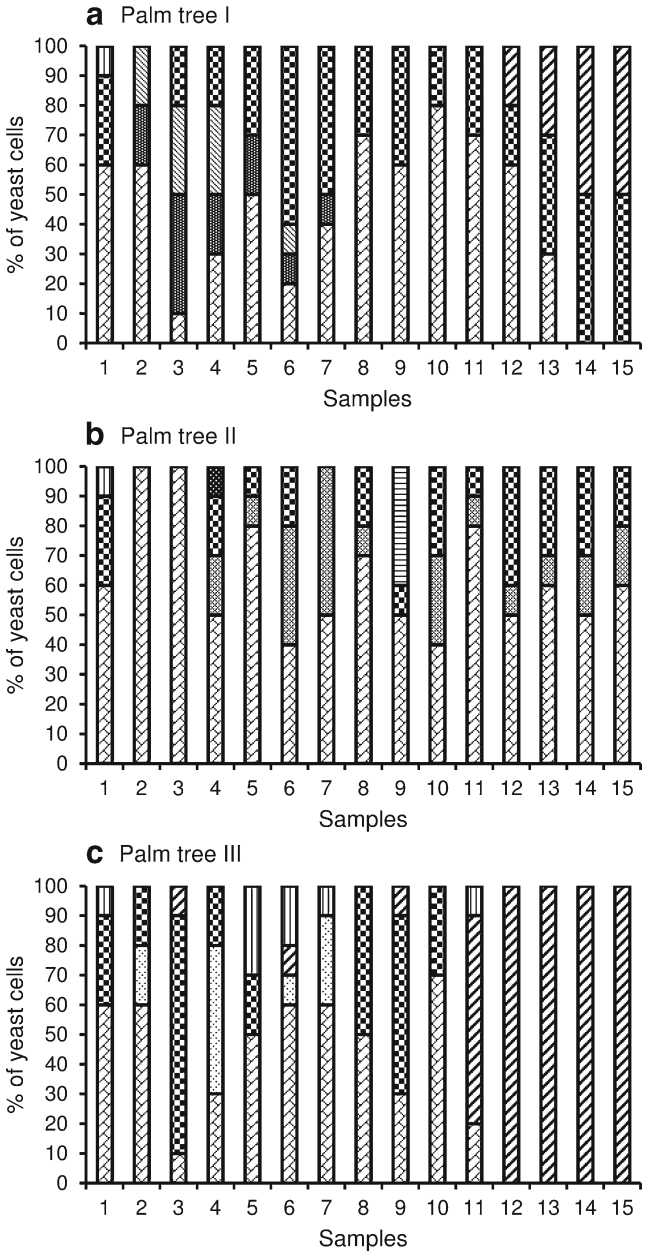

Fig. 2 Distribution of the yeast populations in the *taberna* samples from three palm trees. Samples were collected over the 15-day tapping process. Each sample was collected to the end of the fed-batch fermentation, approximately 12 h. The yeast cells are identified as: *Saccharomyces cerevisiae* (▨), *Candida tropicalis* (▚), *Hanseniaspora guilliermondii* (◪), *Kazachstania unispora* (▩), *Kazachstania exigua* (▤), *Pichia kudriavzevii* (⋰), *Pichia kluyveri* (◫), *Meyerozyma guilliermondii* (▧), and *Candida intermedia* (▦)

environmental conditions, different palm trees species, and differences in the production process (tapping) are likely causes of the observed variations in the yeast populations in palm wines from different countries. *Saccharomyces cerevisiae* and *C. tropicalis* have been reported in other palm wines such as those produced in Burkina Faso (Bandji), Nigeria (palm wine), and Sri Lanka (Toddy) (Atputharajah et al. 1986; Ezeronye and Okerentugba 2000; Ouoba et al. 2012). *Saccharomyces cerevisiae* has been described as being the yeast species responsible of the fermentation and aroma of palm wine (Amoa-Awua et al. 2007; Stringini et al. 2009). However, in the present study the non-*Saccharomyces* yeasts were found to predominate during the early days of *taberna* production, followed by *S. cerevisiae*. This finding suggests that both groups of yeast contribute to ethanol production. Indeed, the observed succession of yeast species in *taberna* production is similar to that reported previously for wine fermentation, where non-*Saccharomyces* yeasts dominated the early stages of the spontaneous fermentation process and showed lower fermentative activity than *S. cerevisiae* (Bauer and Pretorius 2000).

In our analysis, *S. cerevisiae* and *C. tropicalis* dominated the fermentation of *taberna* in the later samples analyzed from palm trees I and III (Fig. 2), and this low diversity of species was reflected in the low yeast counts in these samples (Table 1). The results indicate the importance of these yeast species in the fermentation of palm sap. The predominant role of *S. cerevisiae* in the alcoholic fermentation process was also indicated by the relatively high ethanol content (4.06–4.89 % (w/v) for palm wines I and III) in the later samples (11–15) compared with the ethanol content (0.69–1.5 % (w/v) for palm wines I and III) in samples 5–10 (Table 1). However, in the samples from palm tree II, *S. cerevisiae* was not detected; therefore, the non-*Saccharomyces* yeasts, such as *C. tropicalis*, that were detected in the samples probably also play an important role in the alcoholic fermentation process. *Candida tropicalis* has been reported to have the ability to produce ethanol at high temperatures (Jamai et al. 2001) similar to those recorded in the areas where *taberna* is produced. Thus, the high environmental temperature may explain the dominance of the non-*Saccharomyces* yeasts in *taberna* production because, for instance, the local environmental conditions influence the composition of the microorganism community (Gonzalez et al. 2012). The yeast count was similar among the three palms, and their values decreased in the later samples where the ethanol content was high, pH values and sugar content were low (Table 1); the data indicates that, most likely, the microorganisms were inhibited by these conditions. The survival of microorganisms and their subsequent growth are related closely to the sum of various physical, chemical, and biotic factors such as temperature, humidity, presence of nutrients (such as the high level of sugars in the early samples of palm wine; Table 1), pH, which varied from 7 to 4 in the samples, and the presence of microorganisms that can inhibit the growth of other microorganisms. It has been reported that non-*Saccharomyces* yeasts can affect both the kinetics of growth and the metabolism of *S. cerevisiae* (Lema et al. 1996). These yeasts are capable of anaerobic and aerobic growth and can persist during fermentation, competing with

S. cerevisiae for nutrients (Romano et al. 2003). Importantly, the presence of *H. guilliermondii, K. unispora*, *K .exigua*, *M. guilliermondii*, *P. kudriavzevii*, *P. kluyveri*, and *C. intermedia* in palm wine has not been described previously. Consequently, differences in species diversity in *taberna* compared with the species diversity in other palm wines could contribute to the particular characteristics of *taberna*. The presence of non-*Saccharomyces* yeasts during fermentation has been associated with increased wine quality and complexity because these yeasts can have a significant effect on the higher levels of alcohols, esters, and fatty acids (Moreira et al. 2011; Zott et al. 2011). *Pichia kudriavzevii* has been shown to have a desirable profile for the higher alcohol levels and low acetaldehyde production during wine fermentation (Clemente-Jiménez et al. 2004). *Hanseniaspora guilliermondii*, an apiculate yeast, has been reported to produce higher alcohols such as 1-propanol, aliphatic higher alcohol, and 2-phenylethanol, as well as significant levels of esters such as 2-phenylethyl acetate and ethyl acetate, and high amounts of acetic acid (Moreira et al. 2011). On the other hand, *Zymomonas mobilis* has also been reported in *taberna* where it was considered to be responsible for the production of ethanol (Alcántara-Hernández et al. 2010). Although in the present study only the yeast populations were analyzed, bacteria could also be one of the ethanol producers in our palm wine samples.

The composition and frequency of yeast species differed among the *taberna* samples from the three coyol palm trees examined, which indicated that the fermentation of *taberna* is a heterogeneous process that depends on the types of vectors as the inoculum source, seasonal variations between days (Serrano-Macias personal communication), and the microbiota present. The yeast species in the palm sap are inoculated randomly by vectors such as insects (flies, ants, wasps, bees, dragonflies, mosquitoes, beetles, and others (Serrano-Macias, pers. comm.)), the material employed in scraping the *canoe* and in sap collection, the air and dust, and the microbiota present in the palm tree. The air acts as a support medium or carrier of microorganisms until they fall and are deposited on the substrate (Garijo et al. 2008). Therefore, the yeast species that were detected in the *taberna* samples were those deposited by any of the possible vectors and were those that could tolerate the conditions in the fermented palm sap, such as pH (which was almost neutral in the first sample and then, from the third day of tapping, had values between 4 and 5; Table 1), the presence of ethanol (0.24–4.89 % (w/v) approximately), the concentration of organic acids, oxygen availability, and temperature (Stringini et al. 2009). The yeast species that were common to all the samples probably were inoculated at the beginning of the tapping process, and were able to tolerate the conditions in the palm sap. They must have remained on the walls of the *canoe* in spite of the scraping, so that the species served as inoculum for the new fed-batch. Although *S. cerevisiae* was not detected in the samples from palm tree II, the ethanol concentrations in the samples from this tree were similar to the ethanol concentrations in the samples from palm trees I and III (Table 1). These results suggested that *C. tropicalis* and *S. cerevisiae* were probably the ethanol producers in *taberna* production. The results also confirm that inoculation in the palm sap was random; therefore, the yeast population in the samples from the different palm trees can be different.

Conclusions

The information obtained in the present study revealed the diversity of yeast species in the *taberna* fermentation process. Many yeast species, namely *H. guilliermondii*, *K. unispora*, *K. exigua*, *M. guilliermondii*, *P. kudriavzevii*, and *P. kluyveri*, have not been reported previously in palm wine prepared from other palm trees and may be specific to the sap from *Acrocomia aculeata*, thereby contributing to the typicity of *taberna*. The non-*Saccharomyces* yeasts *H. guilliermondii* and *C. tropicalis* were the most common species in the *taberna* samples, and other yeast species were different in the samples from the three palm trees analyzed. Further studies are necessary to obtain better knowledge about the participation of these yeast species in *taberna* fermentation and to investigate their contribution to the production and final quality of this palm wine.

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