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MOLECULAR IDENTIFICATION OF YEAST OF THE PULQUE BY PCR-DGGE, A TRADITIONAL MEXICAN BEVERAGE

Marcial-Quino J.[1, 3], Garcia-Ocón B.[2], Mendoza-Espinoza J.A.[2], Gómez-Manzo S.[3], Enríquez-Flores S.[3] and Sierra-Palacios E[2*]
[1] Cátedras CONACyT, Instituto Nacional de Pediatría, Secretaría de Salud. D.F., México.
[2] Colegio de Ciencias y Humanidades. Plantel Casa Libertad. Universidad Autónoma de la Ciudad de México. D.F., México.
[3] Laboratorio de Bioquímica Genética. Instituto Nacional de Pediatría, Secretaria de Salud. D. F. México.
*Correspondence Author: edgar.sierra@uacm.edu.mx

Abstract:
Currently it is well known that yeasts play an essential role in the production of different beverages. In this paper, were identified some of the yeasts involved in the fermentation process of the pulque, a Mexican traditional beverage. Samples were collected from different regions of Mexico and yeasts were detected directly from samples without cultivation. Identifying the yeasts was obtained using amplification the D1/D2 domain of the 26S rRNA gene and Denaturing Gradient Gel Electrophoresis (DGGE). The results of DGGE showed different profiles of bands in each of the analyzed samples, indicating the presence of several species of yeast, which was also confirmed by sequencing of the bands corresponding to the domain D1/D2, succeeded in identifying five species of yeasts. The results obtained in this work demonstrated that the technique used for identification of yeasts of pulque was efficient. Besides, the optimization of this method could also allow rapid identification of yeasts and help understand the role of these in the fermentation process of this beverage, as well as the isolation of strains of interest for biotechnological purposes such as production of ethanol or metabolites with nutraceutical activity.

Keywords:
Pulque, PCR, DGGE, Yeast, 26S rRNA.

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1. INTRODUCTION

In Mexico, there are a vast variety of alcoholic traditional beverages elaborated with different processes, some beverages are distillated (hard liquor, mezcal, tequila), other are only fermented (wine, cider, beer and pulque), which are produced and consumed in different regions of this country [7; 35]. The production and consumption of these beverages have an important effect in the daily life of the inhabitants, mainly in the socioeconomic aspects. The most typical and

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traditional pre-hispanic non-distilled beverage with a high consumption in the center of this country is the pulque [37]. Possibly considered as the most antique Mexican alcoholic beverage, it is obtained by an artisan process, that involves the fermentation of a substrate known as 'savia' or 'aguamiel' which is extracted from different Agave species, such as *Agave salmiana*, *A. atrovirens* y *A. mapisaga* [22; 27; 39]. The fermentation product of this craft process has around the 6% of alcoholic content, shows white color, viscose and slightly acid [36].

Besides to historical, religious and economic importance also has been reported that consumption may produce health benefits, because it contains vitamin C (48%), iron (26%), riboflavin (24%), niacin (23%) and thiamine (10%), respectively [3; 4]. For the latter reasons the pulque has been turned in one model of study of beverages since different points of view. Several researchers have conducted different studies centered in the fermentation process, the biochemical changes and the microorganisms implicated. To know the microbial diversity that intervenes in the pulque fermentation is of special interest because this microbiota is responsible of the production of various chemical compounds which confers the essential characteristics of the final product [2]. Among the present and important microorganisms in the pulque are the yeasts, these organism seem to play a fundamental role in the fermentative process due to the ethanol production from substrates such as glucose, fructose and sucrose [1; 7]. Besides of generate distinct volatile chemical compounds that confer the aromatic profile of the beverage [2; 10; 33; 37]. Some of the yeast implicated in the pulque production is considered important at a nutritional level, because the yeasts contain essential amino acids, proteins and vitamins of microbial origin [36].

Several studies of the pulque microorganisms has been conducted in the last years; however, the complex microbial diversity of the beverage has not been well defined, the majority of these studies give only qualitative descriptions of the yeasts isolated and they do not study the influential factors of the occurrence and predominance of the yeasts in the beverages [27]. The methods for the isolation and identification of the yeasts in the pulque, has been conducted by conventional microbiological techniques, but the usage of this techniques limit the characterization of these microorganisms. The conventional microbiological techniques cannot differentiate between some of the yeasts species presents in the fermentative process such as *Saccharomyces cerevisiae*, *S. bayanus*, *S. paradoxus* y *S. pastorianus* [20; 28], therefore the molecular biology techniques could be an alternative for the characterization of these yeasts strains. The use of the polymerase chain reaction (PCR) technique will allow the amplification of nuclear, mitochondrial or ribosomal genes.

These methods of identification particularly based in specific DNA and rRNA sequences, have been applied for the characterization of yeast species, including the analysis of the ribosomal 18S gene [19], internal transcribed spacers (ITS), intergenic spacer (IGS), 5.8S and the 26S subunits, these latter are evolutionary conserved regions [15; 34]. Some studies have utilized the 26S subunit domain [8; 9; 21; 22; 23; 24; 29], this region is universally accepted as a taxonomic tool for the yeast identification. Another regions recently used for the identification of species of

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different genera of yeasts, are nuclear genes as actin, the translation elongation factor-1, RNA pol II, also mitochondrial genes as the small subunit of rDNA and the COX II gene [23].

Other molecular biology technique employed in the yeast identification is the denaturing gradient gel (DGGE), which permits to differentiate DNA molecules with small changes in the nucleotide sequences [30], this technique is regularly employed in ecology research to distinguish DNA fragments obtained by PCR from different environments [30; 31; 32]. In this context, the aim of this work was to implement the PCR-DGGE method using the ribosomal region D1/D2 of the 26S gene with the finality to report for the first time the identification of the yeasts species involved in the fermentation process of pulque.

In this manner we demonstrated using the technique of DGGE, differences in banding pattern of each of the samples collected from different regions, in addition to obtaining sequences of some bands that showed similarity with yeasts, indicating the presence of these microorganisms in the traditional Mexican beverage (pulque), which only has conducted with conventional microbiological techniques. Moreover, the results obtained in this study allow to isolate strains of biotechnological interest, which can be used for the production of ethanol with better efficiency from different carbohydrates and characterization of some metabolites of nutraceutical interest.

2. MATERIALS AND METHODS

2.1. OBTAINING SAMPLES FROM PULQUE

The samples were collected in three regions of the central area of Mexico being the regions with most traditional elaboration of this beverage (Table 1). The samples were placed in sterile plastic containers and were transported on ice to the "Laboratorio de Productos Naturales de la Universidad Autónoma de la Ciudad de México" for its subsequent analysis. All samples and DNA extracted of pulque were frozen at -20°C until required.

2.2. DNA EXTRACTION FROM PULQUE

The DNA extraction was performed using 1 mL of pulque, the biomass was concentrated by centrifugation at 1500 x*g*. The pellet was suspended into 350 µL of buffer phosphate (PBS) for the DNA extraction, which was performed with the AxyPrep Multisource Genomic DNA Miniprep kit (Axygen Biosciences, Union City, CA, USA), following the procedure indicated by the supplier. The DNA integrity was observed in agarose gels 1% (w/v) and the concentration was quantified on an equipment Multiskan™ Microplate Spectrophotometer (Thermo Scientific, USA).

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2.3. DNA AMPLIFICATION FOR PCR-DGGE

The total DNA extracted from the pulque sample were used to amplified a fragment of approximately 230 bp, this latter belongs to a near region of the 5' end of the 26S rRNA gene, using as forward primer NL1-GC, 5´-cgc ccg ccg cgc gcg gcg ggc ggg gcg ggg gcc ata tca ata agc gga gga aaa g-3´ and the reverse primer LS2, 5´-att ccc aaa caa ctc gac tc-3´ previously reported [8]. The PCR-amplified was performed with the Dream Taq DNA polymerase enzyme (Thermo Scientific, USA) and 100 ng of extracted DNA solution. The PCR conditions was conducted along 30 cycles: denaturing at 95°C for 1 minute, hybridization at 52°C for 2 minutes and elongation at 72°C for 1 minute. The reactions were incubated initially for 5 minutes for its denaturalization at 94°C and finally 5 minutes at 72°C for polymerization. The PCR products of the DNA were analyzed in 2% (w/v) agarose gel, visualized with GelRed® (Biotium, USA) and were recorded in a Multi-Doc imaging system (UVP, UK).

2.4. DGGE CONDITIONS

The separation PCR amplified bands of each pulque sample was carried out using electrophoretic technique with the Dcode Universal Mutation detection System (Bio-Rad, USA). An 8% acrylamide gel with two chemical denaturing gradients was employed with the denaturing conditions of 40-60% and 25-45%, and acrylamide/bis-acrylamide ratios of 37.5:1 and 19:1, respectively. A load of 10 μL acrylamide reactions per gel well was separated by electrophoresis in a buffer containing Tris-HCl/acetic acid/ethylenediaminetetraacetic acid (TAE 0.5X). The samples were submitted to a first run at 200 Volts during 10 minutes, afterwards the voltage was reduced to 85 Volts during 16 hours. Once the electrophoresis ended, the gel was stained with silver solution for band observation accordingly to the previous studies reported [38].

2.5. REAMPLIFICATION OF DNA OF THE BANDS SEPARATED FROM THE DGGE GEL.

The selected bands were cut off from the DGGE gel with a sterile scalpel and were placed in 1.5 mL tubes, then 50 μL of sterile distilled water was added and stored at 4°C overnight to elute the DNA. The re-amplification of each band was performed with the same PCR mix and conditions mentioned before. The PCR products fragments were observed once more in a 1.5% (w/v) agarose gel. The PCR products of each selected band were purified with the Qiaex II Gel Extraction Kit (Qiagen, NL), later the purified bands were sent to sequencing at the "Unidad de Síntesis y Secuenciación del Instituto de Biotecnología de la UNAM" facilities.

3. RESULTS AND DISCUSSIONS

3.1. DNA FRAGMENT AMPLIFICATION OF THE PULQUE YEASTS FOR IDENTIFICATION

The molecular identification of the pulque yeasts has been performed with diverse conserved nuclear regions or with a low nucleotide variability, as ITS, IGS or 26S rRNA. In this work we

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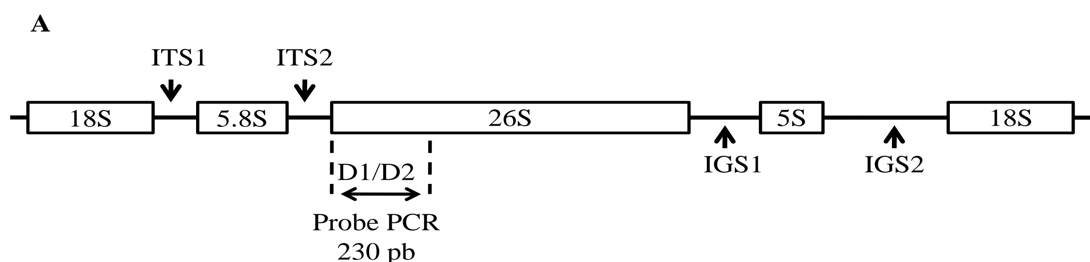
used the D1/D2 domain of the 26S rRNA gene (Fig. 1A) for the identification of the yeast strains involved in the fermentation process of pulque beverage; this nucleotide region has been used in other previous works for identify to level the yeast genera and species [28]. As first result we observed that despite the viscosity and the complexity of the pulque samples, the primers NL1-GC y LS2 were highly specific for the obtention of amplicons of the yeasts presents in each pulque sample as mentioned in Table 1. The obtained PCR product was approximately 230 bp which correspond with the expected fragment size, and this latter was amplified in every pulque sample, as is shown in the Figure 1B. All the PCR amplified products were used for the DGGE analysis, this last permitted to identify by molecular biology techniques the yeasts presents in the pulque processing fermentation.

Table 1. Collection regions of the pulque samples for molecular identification of the yeasts by PCR-DGGE.

Sample	State	Municipality
M1	México	Coatepec, Ixtapaluca
M2	México	San Francisco, Ixtapaluca
M3	México	Loma Alta, Ixtapaluca*
M4	México	Loma Alta, Ixtapaluca*
T1	Tlaxcala	Españita
T2	Tlaxcala	Nanacamilpa
P1	Puebla	Huejotzingo

*These samples were obtained in two different establishments due to its popularity on sale

A



B

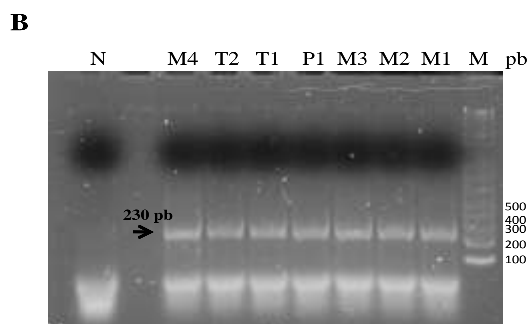


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Figure. 1. A) Schematics of the fungal ribosomal genes. ITS, internal transcribed spacer; D1/D2, domains 1 and 2; IGS, intergenic spacer.
B) Agarose electrophoresis gel of PCR products of the D1/D2 subunit of the studied pulque samples and the molecular weight marker (M, 100 bp DNA Ladder, Invitrogen).

3.2. PCR-DGGE ANALYSIS

The analysis performed to obtain the profile of the bands yeast in pulque samples was carried by DGGE technique, for this purpose two chemical denaturing gradient conditions were tested in acrylamide gels (8%) with an acrylamide/bis-acrylamide ratio of 37.5:1, and a denaturing gradient of 40-60% and a second gel condition with an acrylamide/bis-acrylamide ratio of 19:1, and a denaturing gradient of 25-45%. These conditions allowed us to observe different separation patterns of bands for each analyzed sample. Under the first DGGE denaturing condition a different bands profile was observed between samples (Fig. 2A), which pointed us in the presence of several species of yeast. Furthermore, as can be observed in the figure 2A, nine properly defined bands were visualized in the pulque beverages elaborated in the 'Loma Alta' region in the Mexico State (named M3 and M4), while the samples T1, T2 y P2, corresponding to Tlaxcala and Puebla regions, only seven bands were observed. It is important to note that the profile of the identified bands is not the same in all pulque beverages, although the bands numbered 8 y 9 (Fig. 2A) are present in all samples. The intensity and presence of these bands could indicate that these yeasts may be important in the fermentation process of pulque, however this does not guarantees that relate to the same species because there may be variability in the nucleotide sequence of the D1/D2 domain of the 26S rRNA gene. Furthermore, the intensity and thickness of the bands may indicate the presence of more than one species of yeast, for these reasons, we decided to use a second DGGE gel (25-45%) with other denaturing conditions, with the purpose of achieve a better DNA bands resolution. The obtained results under this conditions demonstrated that the initially observed bands turned diffuse (the numbered en figure 2A, 1 to 7), thus the latter DGGE condition did not permitted a good band separation, interestingly, new bands were separated in the samples of the regions of Puebla (P1) and the State of Mexico (M1-M3), these bands are pointed with arrows in the Fig. 2B. The appearance of new bands indicate the presence of other yeasts as expected and possibly these strains are very close phylogenetically.

3.3. SEQUENCE COMPARISON

Although the second condition of denaturation for DGGE gel showed better separation of the samples M1-M3, the reamplification could not be achieved, possibly due to the low DNA concentration present in the gel section. The bands 1 to 9 in the Fig. 2A were cut and extracted from the gel prior to its molecular characterization and sequencing, the obtained sequences were analyzed with three different databases: 1) National Center for Biotechnology Information (NCBI), 2) European Molecular Biology Laboratory (EMBL) and 3) DNA Data Bank of Japan (DDBJ); the results of the analysis conducted of sequences in all databases mentioned are shown in Table 2, which also shows the percent identity and E-value of each of the bands. The data

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found with database of Reference Genomic Sequences (refseq_genomic) of NCBI, did not displayed the differences between each sequences, all sequences presented the same result indicating similitude with the yeast *Saccharomyces cerevisiae* S288 (Acc. NC_001144). On the other hand, the analysis with the EMBL database showed no difference between species, only with this database were discerned two yeast species denoted as clone dlvpf7 and dlvpf6 with the accession numbers KC_8200423 and KC_820042, respectively. Finally, we also conducted an analysis of the sequences using the DDBJ database, with which five of the extracted gel bands showed similarity with sequences of different yeasts of the genus *Saccharomyces*., which are involved in the fermentation process (Table 2). Such species were, band 1: *Saccharomyces cerevisiae* strain VI2 (EU441887); band 2: *Saccharomyces ellipsoideus* (AF005708); band 4 *Saccharomyces cerevisiae* IMAU:V3015 (GU138490); band 7: *Saccharomyces cerevisiae* strain Y4-6 (HQ711331) and band 8 *Saccharomyces cerevisiae* strain T140-NL1 (KF214443). This results demonstrated that DGGE technique was effectively to examine the yeasts presents in beverage of pulque and where sequences even allowed to differentiate species of the same genus.

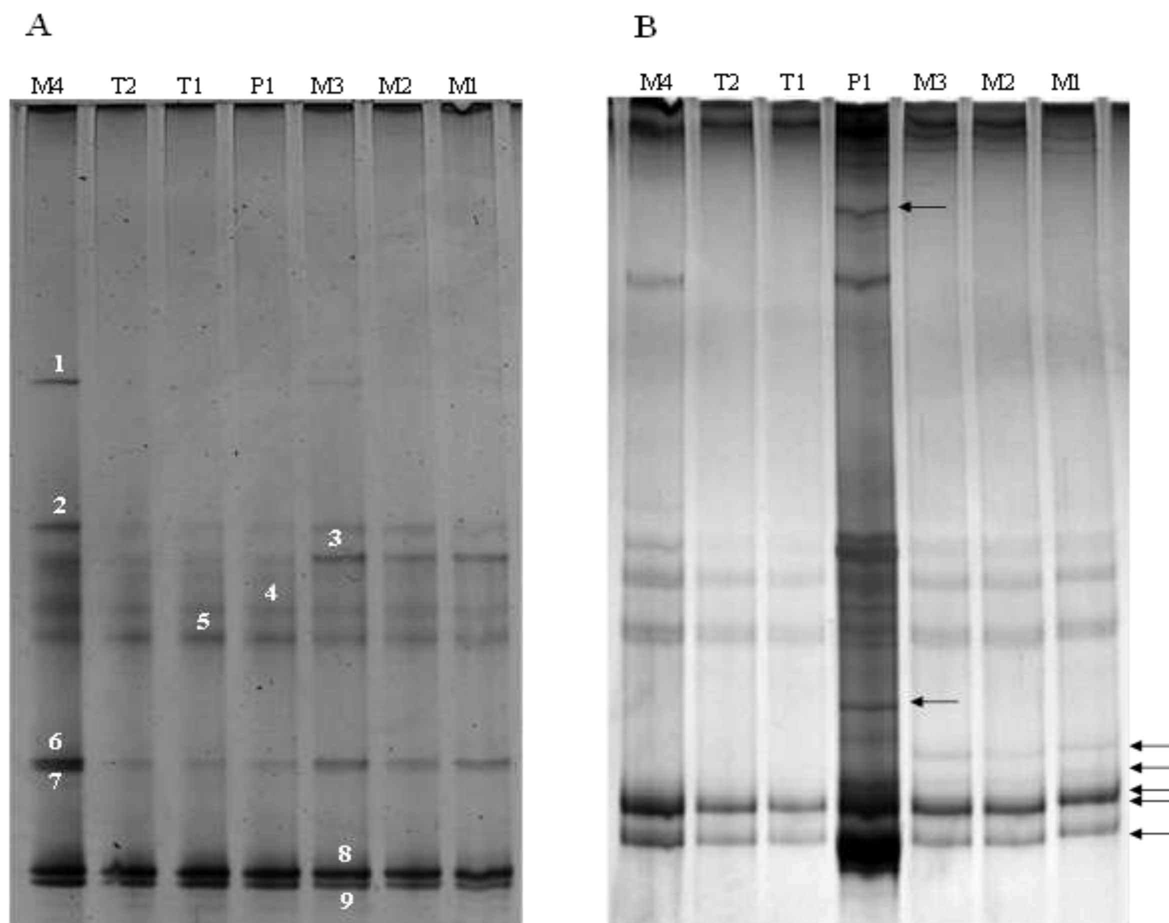

Figure. 2. PCR-DGGE profile from the analyzed yeasts in the pulque samples.

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A) 8% acrylamide, with an acrylamide/bis-acrylamide ratio of 37.5:1 and denaturing gradient of 40-60%.
B) 8% acrylamide, with an acrylamide/bis-acrylamide ratio of 19:1 and denaturing gradient of 25-45%. Each lane was loaded with 10 µl of the PCR products. The DGGE was ran in buffer TAE (0.5X), the samples initially were run 10 minutes at 200 Volts and then the samples were run again for 16 hours at 85 Volts. M1 (Mexico, Coatepec, Ixtapaluca); M2 (Mexico, San Francisco, Ixtapaluca); M3 (Mexico, Loma Alta, Ixtapaluca); M4 (Mexico, Loma Alta, Ixtapaluca); T1 (Tlaxcala, Españita); T2 (Tlaxcala, Nanacamilpa); P1 (Puebla, Huejotzingo).

Table 2. Sequence analysis obtained of three different databases (NCBI, EMBL and DDBJ) for the identification of the yeasts contained in the pulque beverage samples.

	NCBI				ENA				DDBJ			
Band	Organism	Identity (%)	E-value	Accession	Organism	Identity (%)	E-value	Accession	Organism	Identity (%)	E-value	Accession
1	*S. cerevisiae* S288c	100	3e-123	NC_001144	Unculture fungus clone dlvpf7	100	3E-129	KC820043	*S. cerevisiae* strain VI2	100	e-145	EU441887
2	*S. cerevisiae* S288c	97	4e-106	NC_001144	Unculture fungus clone dlvpf6	96	4E-108	KC820042	*S. ellipsoideus*	96	e-108	AF005708
3	*S. cerevisiae* S288c	99	8e-119	NC_001144	Unculture fungus clone dlvpf6	98	7E-122	KC820042	*S. cerevisiae* strain VI2	98	e-124	EU441887
4	*S. cerevisiae* S288c	99	8e-103	NC_001144	Unculture fungus clone dlvpf6	99	7E-106	KC820042	*S. cerevisiae* IMAU:V3 015	100 %	e-124	GU138490
5	*S. cerevisiae* S288c	99	3e-118	NC_001144	Unculture fungus clone dlvpf6	98	2E-122	KC820042	*S. cerevisiae* strain VI2	98	e-126	EU441887
6	*S. cerevisiae* S288c	99	1e-121	NC_001144	Unculture fungus clone dlvpf6	98	7E-126	KC820042	*S. cerevisiae* strain VI2	99	e-130	EU441887
7	*S. cerevisiae* S288c	100	1e-122	NC_001144	Unculture fungus clone dlvpf6	100	5E-127	KC820042	*S. cerevisiae* strain Y4-6	100	e-133	HQ711331
8	*S. cerevisiae* S288c	98	2e-94	NC_001144	Unculture fungus clone dlvpf7	95	8E-106	KC820043	*S. cerevisiae* strain T140 -NL1	98	e-133	KF214443
9	*S. cerevisiae* S288c	99	9e-113	NC_001144	Unculture fungus clone dlvpf7	99	5E-123	KC820043	*Saccharomyces cerevisiae* strain Y4-6	98	e-126	HQ711331

In summary, with this technique could be determined the sequences by amplification of the nucleotide D1/D2 region of the ribosomal 26S rRNA gene in each pulque sample, the sequences fit with known yeast species found in the GenBank and in the other databases utilized in this paper, being *S. cerevisiae* the better identified as prevalent in all the samples, these strains is well known to be involved in the fermentation processes. Despite the low identification percentage, the adaptation of the PCR-DGGE method allowed the segregation and identification of the different yeast types present in diverse pulque beverage.

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3.4. DISCUSSION

The pulque is one of the most ancient indigenous beverages and currently remains high consumption in the Mexican population [26]. Infants, pregnant women, young people and adults make this drink because they believe contain certain nutritional properties, which several studies have characterized and identified different metabolites considered beneficial to health [3; 4]. Many of this compounds can by generated by the microbiota (bacteria, yeast), present in the beverage [14; 39]. However, identification of these organisms are only performed using traditional methods, and most of them only has focused on the study of bacteria [14]. For these reasons, knowing the yeasts play an important role in fermentation processes, and produce different metabolites is of great interest to identify these organisms in drinking pulque. Nevertheless, studies for the identification and taxonomic classification of yeasts has not been easy, the morphology and physiology were the first identification parameters, but due to the several quantity of these methods (about 100) is hard [11]. After that, biochemical techniques and microscopy were used also as alternatives; all the same, these techniques only allow identification gender, the latter caused by the presence of anamorphs and teleomorphs stages in yeast, preventing accurate identification process [6]. Actually, the exploration with molecular biology tools has allowed to classify genus and the species of the strains accurately. Among the techniques that displayed better results can be find: the karyotype analysis, the study of restriction patterns and the DNA/RNA region sequencing [5; 21]. The usefulness of the analysis of RFLP of the 5.8S rRNA gene and the intergenic ribosomal regions for the identification of the yeast species has been demonstrated by different researches [13; 15; 16; 17; 34].

Other authors have amplified specific regions of the DNA/RNA ribosomal units as an alternative for the yeasts identification, due these regions contain the sequences with a high degree of conservation and have a relatively high variability between strains of different species [18]. In the majority of the cases, the PCR amplified products of strains of the same species and genus exhibit identical sizes in length and the species of the same genus show similar sizes. Although the same size, the sequences of these amplified regions can differ in the nucleotides depending of the species. With this context, in this work we amplified the region D1/D2 of the 26S rRNA gene to observe the diversity and identification of the yeasts contained in different pulque samples. One characteristic of this sequence of the D1/D2 subunit is the difference of 1% between species and less of 1% between strains of the same species [5; 21; 22; 23]. This latter ribosomal subunit has been utilized by different researchers for the yeasts identification [9; 12; 28; 29] despite only were applied in regions of strains previously isolated and not in raw samples, achieving the identification of species of *Saccharomyces cerevisiae*, *Pichia kluyveri, Pichia ohmeri, Kluyveromyces marxianus, Candida pseudointermedia, Issatchenkia orientalis, Torulaspora delbrueckii*, *Physalis ixocarpa*, [8; 20; 25; 29] involved directly in fermentative processes. In this paper, was achieved the amplification of PCR products of all the pulque sample of the subunit D1/D2 and with the complementation of the DGGE technique the characterization of the yeasts contained in the pulque samples was achieved. However, not all sequences extracted bands could be identified despite using three different databases (NCBI, EMBL and DDJB) allowing only the identification of five strains different of *S. cerevisiae*. Similar results were obtained by Kurtzman

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and Robnett [22], by means of molecular analysis with this ribosomal region finding difficult the differentiation between species, these authors mentioned the possibility of a phylogenetic close relationship between the yeast species or as in our instance, the length of the chosen fragment was not sufficient to achieve an adequate differentiation, due the analyzed sequence was only 230 bp and also the selected internal region of the fragment do not have enough nucleotide variability. This supposition is based in the alignment of the sequences of each sample, only are appreciated small differences in the nucleotide sequences (Fig. 3). A final analysis was conducted with the yeast-ID database (www.yeast-id.com/), the data showed that, the percentages of identity between species are very similar, with this information could be determined that the sequences obtained of the samples are related with yeasts that already have been previously identified in other fermented beverages [28; 29], such as the wine elaboration [8; 9; 24] and tequila [25]. This analysis showed the ratio of the sequences of samples with differents yeast genera, in addition, the percentage of similarity of the species of the different genus on average was, to *Saccharomyces* (93.3-99.5%), *Kazashstania* (91.1-96.6%), *Torulaspora* (93.7-91.8%), *Candida* (94%) and *Naumovozyma* (91.4%). Then, considering the species most frequently appeared when sequences were run in the blast and with higher similarity percentages, a phylogenetic tree was generated (Fig. 4), where proximity is shown with some strains of yeasts involved in fermentation processes, such as: *Saccharomyces cariocanis* (CBS 7975), *S. paradoxus* (CBS 472); *S. arboricolus* (10644), *S. bayanus* (CBS 380, 395), *S. pasturianus* (CBS 1538), *S. kudriavzevii* (CBS 88 40), *S. mikatae* (CBS 8665) and diverse fermentation yeasts as *Candida humilis* (CBS 5658), *Kazachtania baderi* (CBS 8638), *K. solicola* (CBS 6904), *K. servazzii* (4311), *K. aquatic* (CBS 10102), *Torulospora microellipsoides* (CBS 427), *T. delbrueckii* (CBS 1146), *T. pretoriensis* (CBS 2187), *T. franciscae* (CBS 2926), *T. maleeae* (CBS 10694) and *Naumovozyma darienensis* (CBS 421). Also, the constructed phylogenetic tree, showed a better relationship and closeness of the sequences of the bands with different yeast species deposited at the base of yeast-ID data, despite being a very short fragment under review. Finally, it is worth mentioning that the ribosomal region (D1/D2) was mainly used to identify yeasts isolated in cultures and not directly from the samples as was done in this study, possible reason why the molecular identification is difficult.

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Banda 1    ATATCAATAAGCGGAGGAAAAGAAACCAACCGGG.ATT.GCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAAT    82
Banda 2    ......ATAAGCGGAGGAAAAGAAACCAGA..GGCATTAGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAAT    76
Banda 3    ATATCAATAAGCGGAGGAAAAGAAACCAGACGGGCATTAGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAAT    84
Banda 4    ..............................CGGC.ATTAGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAAT    53
Banda 5    ATATCAATAAGCGGAGGAAAAGAAACCAACCGGG.ATTAGCCTCAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAAT    83
Banda 6    ATATCAATAAGCGGAGGAAAAGAAACCAACCGGG.ATTAGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAAT    83
Banda 7    ATATCAATAAGCGGAGGAAAAGAAACCAACCGGG.ATT.GCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAAT    82
Banda 8    ATATCAATAAGCGGAGGAAAAGACACCAACCGCATAAGCCTATAGATAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAAT    84
Banda 9    ATATCAATAAGCGGAGGAAAAGAAACCAACCGGG.ATTAGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAAT    83
Consensus                                     a       t   taacggcgagtgaagcggcaaaagctcaaatttgaaat
Banda 1    CTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTC   166
Banda 2    CTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTC   160
Banda 3    CTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGAAACTTTGGGGCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTC   168
Banda 4    CTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTC   137
Banda 5    CTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGAAACTTTGGGGCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTC   167
Banda 6    CTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTC   167
Banda 7    CTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTC   166
Banda 8    CTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTC   168
Banda 9    CTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGGTCCTTGTCTATGTTCCTTGGAACAGGACGTC   167
Consensus  ctggtaccttcggtgcccgagttgtaatttggagaggg aactttggggccg tccttgtctatgttccttggaacaggacgtc
Banda 1    ATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATAA       246
Banda 2    ATATAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAATATGCCTTCGAAGAGTCGAGTTGTTTGGGAATA.       239
Banda 3    ATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATA.       247
Banda 4    ATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATA.       216
Banda 5    ATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATA.       246
Banda 6    ATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATA.       246
Banda 7    ATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATA.       245
Banda 8    ATAGAGGGTGAGAATCCCGTGTGGCGAGGAGCCCGGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATAA       248
Banda 9    ATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATAA       247
Consensus  ata agggtgagaatcccgtgtggcgaggag  cggttctttgtaa  tgccttcgaagagtcgagttgtttgggaata
```

Figure. 3. Alignment of the obtained sequences of each pulque samples was realized and analyzed with the bioinformatics program DNAman

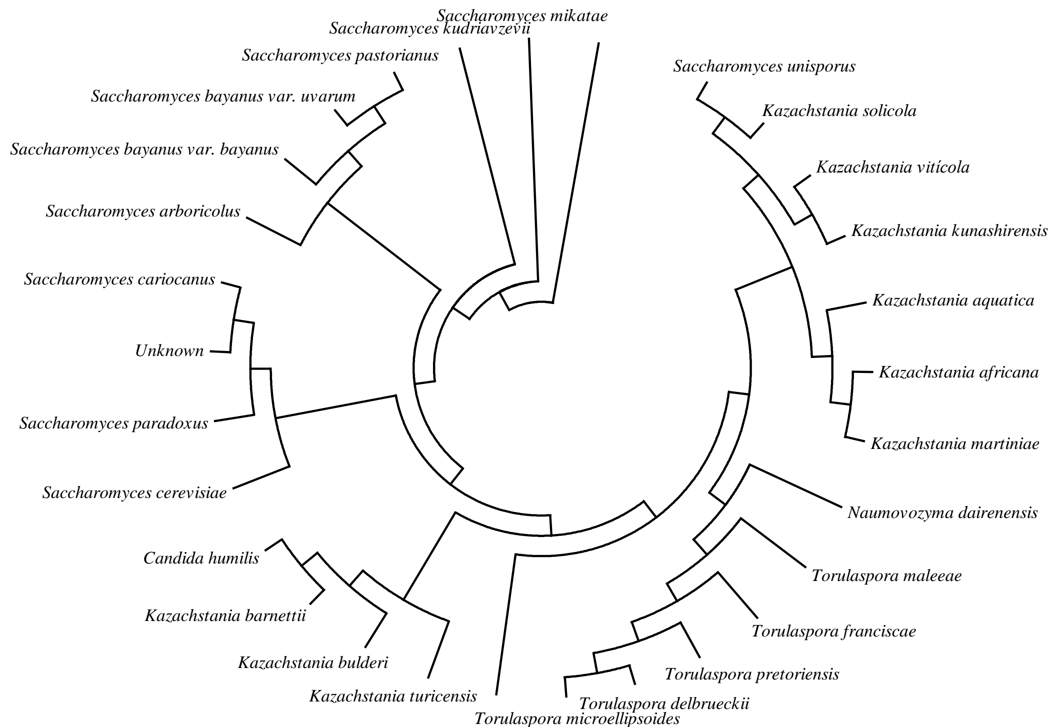

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Figure. 4. Phylogenic analysis of the yeast sequences obtained from the pulque samples and its relationship with different yeasts involved in fermentative processes. The program MEGA 3.0 was utilized and the tree was constructed with the Parsimony analysis.

4. CONCLUSIONS & RECOMMENDATIONS

Finally, with the PCR-DGGE were obtained different pattern of banding of the yeasts and were identified some yeasts involved in the fermentation process pulque through by complementing both molecular techniques. However, a low identification efficiency was found since it could not differentiate between species of the selected bands, possibly because the size of the fragments of the D1/D2 region were short and/or the highly conserved region. For these reasons, would be useful to analyze other ribosomal regions or fragments larger and more nucleotide variable. Herein, the band profiles obtained using DGGE are the first on its kind and provide more information of the identification compared to the traditional isolation and identification. Thus the DGGE, due its simplicity can be an effective tool for the microbial diversity analysis in the pulque fermentation and perhaps another beverage as wine or beer, especially on those beverages with a large diversity of *Saccharomyces sp.,* whenever required to be rapidly identified. Furthermore, this methodology is uncomplicated and fast, becomes especially attractive when a large number of samples from different sources, allowing individual analysis of each of the sample and even serve to isolate strains of biotechnological interest for the production of fermented beverages or producers of metabolites with nutraceutical properties.

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