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Genetic diversity of *Clavispora lusitaniae* isolated from *Agave fourcroydes* Lem, as revealed by DNA fingerprinting

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This study characterized *Clavispora lusitaniae* strains isolated from different stages of the processing and early fermentation of a henequen (*Agave fourcroydes*) spirit produced in Yucatan, Mexico using a molecular technique. Sixteen strains identified based on morphological features, obtained from different substrates, were typed molecularly. Nine different versions of the divergent D1/D2 domain of the large-subunit ribosomal DNA sequence were identified among the *C. lusitaniae* strains. The greatest degree of polymorphism was found in the 90-bp structural motif of the D2 domain. The MSP-PCR technique was able to differentiate 100% of the isolates. This study provides significant insight into the genetic diversity of the mycobiota present during the henequen fermentation process, especially that of *C. lusitaniae*, for which only a few studies in plants have been published. The applied MSP-PCR markers were very efficient in revealing polymorphisms between isolates of this species.

Keywords: *Agave fourcroydes*, *Clavispora lusitaniae*, D1/D2 large-subunit rDNA polymorphism, DNA fingerprinting, genetic diversity

Introduction

In Mexico, the cultivation and integral use of several agave species (*Agave* spp.) dates back to pre-Hispanic times. One of these agave species is henequen (*Agave fourcroydes* Lem.), an economically important crop on the Yucatan Peninsula that has been widely used in food, fiber, cloth, feed, paper, and medical products as well as to obtain cellulose, saponins, and other materials (Cazaurang *et al.*, 1990; Brumann, 2000).

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As part of the establishment of industrial production of a distilled alcoholic spirit known as mezcal (the generic name of all agave spirits) from henequen (Larque-Saavedra *et al.*, 2004), the mycobiota present during different stages of the processing and spontaneous fermentation of the must extracted from cooked henequen core or pines was isolated and characterized. This approach was applied to evaluate the use of native strains in the fermentation process, as has been accomplished in the wine industry. Sixteen of the 153 yeast strains isolated were identified as *Clavispora lusitaniae*. This species is one of the predominant species in the must recently extracted from cooked agave and in the early fermentation stages (Lappe *et al.*, 2004).

The genus *Clavispora* was proposed by Rodrigues de Miranda and comprises two species: *C. lusitaniae* and *C. opuntiae*. *Clavispora* spp. are characterized by the formation of clavate ascospores with indistinct warts. *Clavispora lusitaniae* is a cosmopolitan, saprobial, fermentative yeast. This species has been associated with clinical specimens from immunocompromised patients and is now recognized as an opportunistically infectious organism (De Hoog *et al.*, 2000). It has also been isolated from various other sources, including humans and other warm-blooded animals, insects, industrial wastes (e.g., the effluents of chocolate, mezcal, and tequila factories), and plant substrates (e.g., decomposed fruits, decaying fruits, and cladodes of prickly pear, and necrotic tissues of cactus species), and it has recently been detected in several agave species and during the fermentation process of agave must (mezcal and tequila) and sap (pulque) (Rodrigues de Miranda, 1979; Lachance, 1995; Lappe-Oliveras *et al.*, 2008; Kurtzman *et al.*, 2011). Bright red-to-purple areas have been observed in the harvested hearts of *Agave*. These areas release an odor that is typical of fermentation, and the dominant yeasts in these rots are *Clavispora lusitaniae* and *Metschnikowia agaveae* (Lachance, 1995). It has been reported that in the spontaneous fermentation of *Agave* spp., the first aroma production is carried out by so-called *non-Saccharomyces* strains, such as *Clavispora lusitaniae* and other yeast species; these strains can produce high concentrations of some compounds with a remarkable influence on the sensory qualities of the mature spirit (Escalante-Minakata *et al.*, 2008).

The present tendency is to use different molecular techniques for strain identification and the quantification of genetic diversity in yeast populations; these techniques have replaced traditional physiological tests (Cadez *et al.*, 2002; Naumova *et al.*, 2004; da Silva-Filho *et al.*, 2005; Blanco *et al.*, 2006). However, most of the published studies that address diversity in yeasts or the procurement of species-specific fingerprints have employed PCR-RFLP analysis of the ITS

region and/or of the D1/D2 domain, generally combined with only one mini-microsatellite primer (MSP) (Gadanho *et al*., 2006; Villa-Carvajal *et al*., 2006; Daniel *et al*., 2009).

The aim of the present study was to determine the genetic diversity of the *C. lusitaniae* strains present during the different stages of henequen spirit processing and fermentation through PCR fingerprinting with a set of MSPs with different motifs.

Materials and Methods

Yeast strains

Samples of *A. fourcroydes* were collected in the communities of Tixpehual and Baca on the Yucatan Peninsula and processed in the laboratory of GeMBio. Isolation of the mycobiota present during different stages of the production process of henequen mezcal (from cooked agave cores, agave must and press rinse water) was carried out according to standard methodologies (Harrigan and McCance, 1976). Each colony with different a morphology was isolated, purified and preserved in sterile water at 4°C until its use in subsequent tests.

The obtained yeasts were preliminarily characterized through morpho-physiological tests (Yarrow, 1998) and identified as described in different reports (Barnett *et al*., 2000; Kurtzman *et al*., 2011).

The sixteen *C. lusitaniae* strains that were characterized in this study are presented in Table 1.

rDNA sequence analysis

Total genomic DNA was extracted according to a method developed in the GeMBio laboratory (Tapia-Tussell *et al*., 2006). The DNA concentration was spectrophotometrically quantified, and DNA quality was determined as previously described (Sambrook *et al*., 1989).

The identity of the isolated *C. lusitaniae* strains was corroborated through sequence analysis of the amplified D1/D2 domain of 26S rDNA using the universal primers NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′) (O'Donnell, 1993). The sequences were obtained by Macrogen Inc. and then aligned and edited using the BioEdit Program, v 7.0.5. (Hall, 2005), and manually corrected. The obtained sequences were compared against those available in the GenBank database. The sequences were aligned, and a tree was constructed with the Mega 5 program (Tamura *et al*., 2011).

PCR fingerprint analyses

PCR-based fingerprinting was carried out using four 15-bp mini-microsatellite primers (MSP), according to previous reports: for M13 (5′-GAGGGTGGCGGTTCT-3′), an annealing temperature of 50°C was used (Cadez *et al*., 2002; Libkind *et al*., 2003); for $(TCC)_5$ and $(GACAC)_3$, the annealing temperature was 42°C (Bridge *et al*., 1997); and for $(CAG)_5$, the annealing temperature was 60°C (Talhinhas *et al*., 2002). PCR amplification was performed in a 25 µl reaction mixture containing 1× PCR buffer (10 × 200 mM Tris-HCl, 500 mM KCl, pH 8.4; Invitrogen), 0.25 mM each dNTP (Invitrogen), 2 mM $MgCl_2$, 0.8 µM primers, 10 ng of template DNA and 1 U of *Taq* polymerase (Invitrogen). DNA amplification was performed using a GeneAmp 9700 DNA Thermal Cycler (PerkinElmer), with an initial denaturing step at 95°C for 5 min, followed by 40 cycles of 40 sec at 95°C, 60 sec at the appropriate annealing temperature, and 60 sec at 72°C, and a final extension step of 5 min at 72°C. The amplified DNA fragments were separated via polyacryla-

Table 1. *C. lusitaniae* strains and their sources of isolation and D1/D2 sequence polymorphisms

Strain number	GenBank	Position and type of D1/D2 sequence polymorphisms				Isolation data	References
		177	197	394	410-490		
ExoC11	GU454736	AT	-	-	P1	Agave must, Mexico	This study
ExoC21	GQ396267	AT	-	-	P1	Agave must, Mexico	This study
ExoC26	GQ396268	AT	-	-	P1	Agave must, Mexico	This study
ExoC34	AY894824	AT	-	-	P1	Agave must, Mexico	This study
ExoC36	GQ396269	AT	-	-	P2	Agave must, Mexico	This study
ExoC55	GQ396270	AT	-	-	P1	Agave must, Mexico	This study
ExoC57	AY894825	CC	-	-	P3	Agave must, Mexico	This study
PC4		AT	TA	-	P2	Cooked agave, Mexico	This study
PC5	EF063132	AT	-	-	P4	Cooked agave, Mexico	This study
PC8		AT	-	-	P5	Cooked agave, Mexico	This study
PC14		AT	-	-	P1	Cooked agave, Mexico	This study
PC24		AT	-	-	P4	Cooked agave, Mexico	This study
AL16	GQ396271	CC	-	-	P3	Press rinse water, Mexico	This study
AL20		AT	-	-	P1	Press rinse water, Mexico	This study
AL26		CC	-	-	P6	Press rinse water, Mexico	This study
AL30		AT	-	-	P1	Press rinse water, Mexico	This study
14MCHS[rs]	EU669465	CT	-	TT	P4	Pulque, Mexico	Herrera-Solórzano (2008)
7MCHS[rs]	EU669469	CC	-	TT	P4	Agave sap. Mexico	Herrera-Solórzano (2008)
UWO(PS)94-423.2[rs]	AY190540	CC	-	-	P3	Pulque, aguamiel. Mexico	Lachance *et al.* (2003)

P, Type of D2 sequence polymorphism.
[rs] Reference strains.

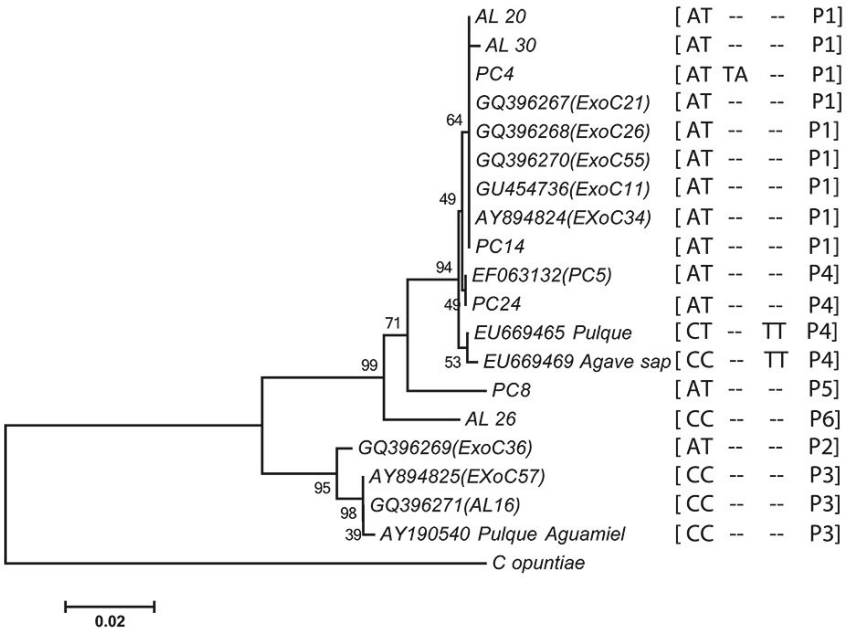

Fig. 1. Neighbor-joining phylogram showing the various polymorphic sequences of the D1/D2 domains of the large-subunit rDNA of *C. lusitaniae*. The bootstrap values were determined from 1,000 iterations. The sequence polymorphisms are described symbolically in brackets (see Table 1).

mide gel electrophoresis (6%) in 1× TG (Tris-borate-glycine) buffer at 100 V for 2 h. A molecular size marker (1 kb Plus DNA Ladder, Invitrogen) was added to each gel as a reference. The resultant DNA banding patterns were visualized through silver staining.

Data analyses

The PCR fingerprinting data were scored as discrete variables, using "1" to indicate the presence of a fragment and "0" to indicate the absence of a fragment. The binary data obtained by scoring the MSP profiles obtained with different primers, both individually and cumulatively, were employed to construct a similarity matrix using Jaccard's coefficients (Jaccard, 1908) and the unweighted pair group method. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to generate a dendrogram based on Jaccard's similarity coefficient with the sequential agglomerative hierarchical and nested (SAHN) clustering module of NTSYSpc software, version 2.02e (Rohlf, 2000). Principal coordinate analysis (PCoA) was also performed to separate the yeast strains.

The data were further employed to calculate polymorphism information content (PIC) values according to the following equation (Anderson *et al.*, 1992):

$$PIC=1-\sum_{i=1}^{k} P_i^2$$

where P_i is the frequency of the ith allele, and k is the total number of different alleles at the locus.

Results and Discussion

The yeast species *Clavispora lusitaniae* was found to be one the most abundant species in the mycobiota isolated from different substrates related to henequen spirit production. This result is consistent with the findings of another study on the yeast community associated with *Agave tequilana* fermentation (Lachance, 1995). The frequency at which this species has been isolated from agave plants and agave products and its immediate adaptation to must (obtained from crushed cooked agave cores) during spontaneous fermentation support the hypothesis that this yeast occurs in a wide variety of products of plant and animal origin as well as in industrial wastes and clinical specimens (Lachance, 1995; De Hoog *et al.*, 2000; Lachance *et al.*, 2003).

rDNA sequence analysis

Sequence analysis of the D1/D2 region of 26S rDNA from the sixteen strains revealed 99–100% identity with *C. lusitaniae* species, thus confirming the identity of the strains. The GenBank accession numbers of each sequence are listed in Table 1.

The D1/D2 domain of 26S rDNA has proven to be useful for yeast identification. Extensive investigations conducted over the last 25 years have shown that there are sufficient differences between yeast D1/D2 domains to allow the identification of yeast species as well as the prediction of intra- and inter species relationships and the establishment of fingerprints (Kurtzman and Robnett, 1998; Lopandic *et al.*, 2006).

In this study, we identified a substitution (AT/CC) at position 177 and a double deletion of (TA) at position 197 in the D1 domain. This double deletion was observed in all strains except the PC4 strain. A double deletion (TT) at position 394 in the D2 domain was also observed in all strains. The *C. lusitaniae* strains exhibited nine different D1/D2 region sequences (Fig. 1). The greatest degree of polymorphism was found in the 90-bp structural motif of the D2 domain (Fig. 2). This finding is consistent with a report by Lachance *et al.*

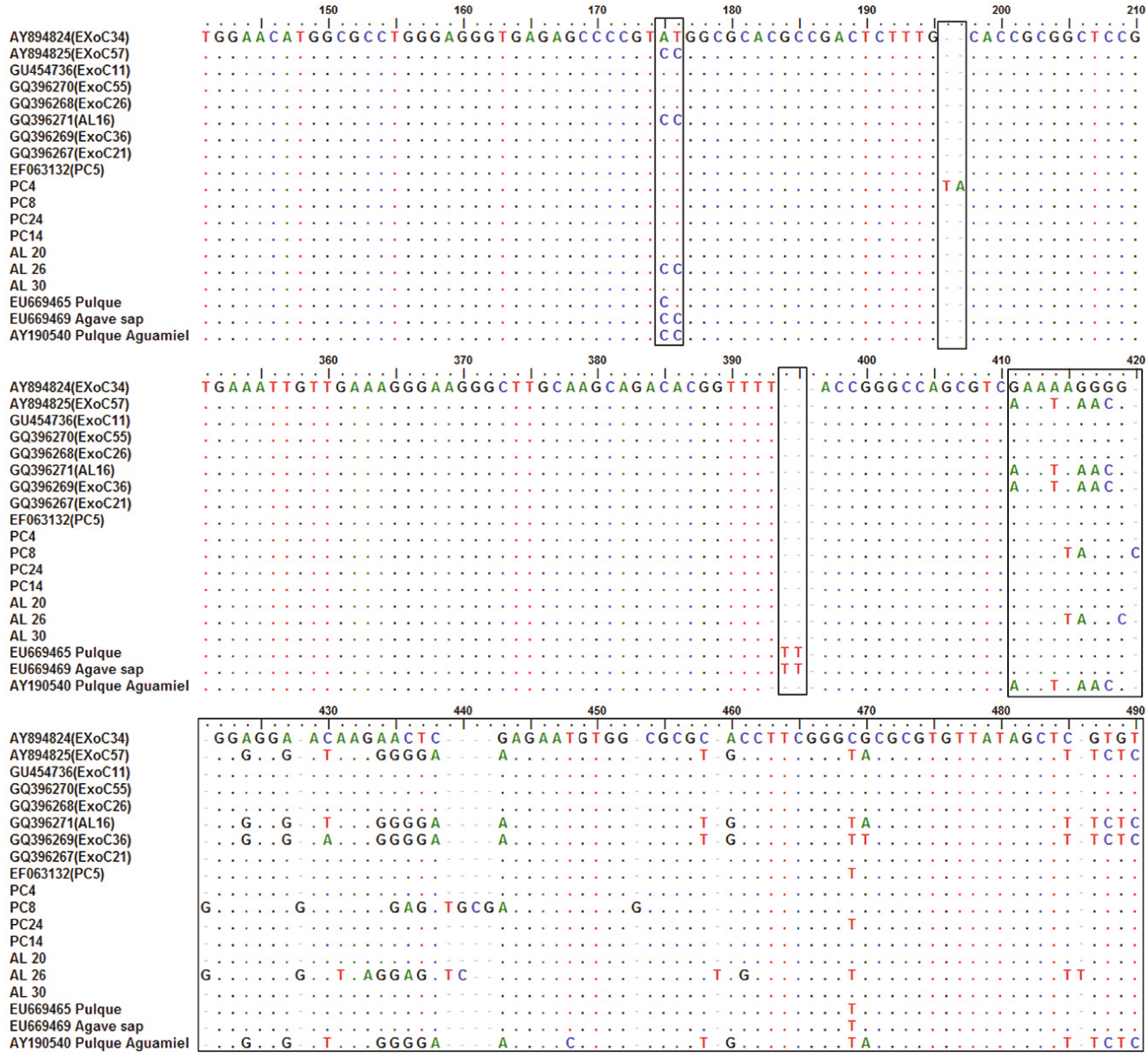

Fig. 2. **Position and type of the D1/D2 sequence polymorphisms found in *C. lusitaniae* strains.**

(2003), who assigned the sequences of the D1/D2 domains of *C. lusitaniae* isolates from different substrates to 10 categories and identified one major polymorphism located in a 90-bp structural motif of the D2 domain. The large number of categories identified by Lachance *et al.* (2003) within the sequence of the D1/D2 domain may be associated with the isolation of strains from different substrates.

PCR fingerprint analyses

PCR fingerprinting using four microsatellite primers (MSP-PCR) resulted in different types of patterns. This molecular

Table 2. **Polymorphism obtained by MSP analysis in 16 *C. lusitaniae* strains**

MSP	Loci amplified	Polymorphic loci	PIC	Approx. band range size (kb)
$(TCC)_5$	18	18	0.86	760 – 1650
$(CAG)_5$	55	55	0.94	150 – 3000
$(GACAG)_3$	38	38	0.79	450 – 2500
M13	15	15	0.84	350 – 2000
Total	126	126	0.86	150 – 3000

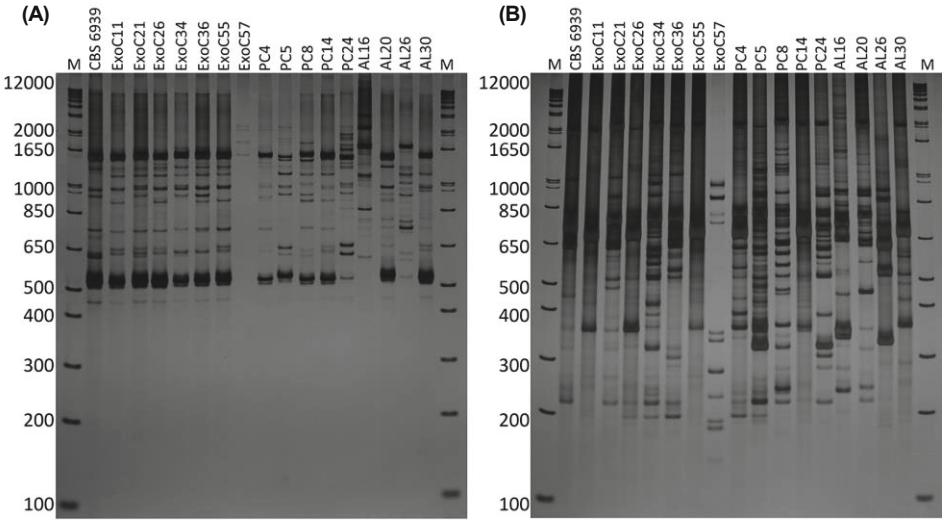

Fig. 3. Banding patterns obtained with the $(GACAC)_3$ (A) and $(CAG)_5$ (B) primers for the strains examined in this study. Lanes: M, DNA marker (1 kb DNA ladder).

technique allowed 100% of the isolates to be discriminated. The mean PIC value recorder over all four MSP primers was 0.86 (Table 2).

Figure 3 shows the results obtained with the two most polymorphic primers. In all cases, greater genetic diversity was observed in the isolates from cooked agave cores (PC) and press rinse water (AL); at time zero of fermentation, the obtained profiles differed from each other but showed a lesser degree of polymorphism, except for strain ExoC57, which showed a unique profile with respect to all the other isolates of *C. lusitaniae*.

As shown in Fig. 3A, when the $(GACAC)_3$ primer was used, polymorphisms were found in the isolates from agave must located at approximately 650 bp and in the interval from 850 to 1,000 bp. This result differed from the other isolates, in which these polymorphisms were distributed across the total range of the amplification profiles (450 to 1,650 bp). A total of 30 polymorphic fragments were obtained, with a PIC of 0.79.

The $(CAG)_5$ primer (Fig. 3B) was the most informative primer, revealing the highest degree of polymorphism among the isolates, regardless of their source. The range of observed polymorphic band sizes was approximately 170–1,000 bp. In this case, a total of 55 polymorphic fragments were obtained, with a PIC of 0.94.

The genetic distance data were used to construct a UPMGA dendogram (Fig. 4). This dendogram showed two major clusters. Cluster I and cluster II separated the agave must and cooked agave strains from the press rinse water strains, with a genetic distance of 0.50. The ExoC57 strain was clearly

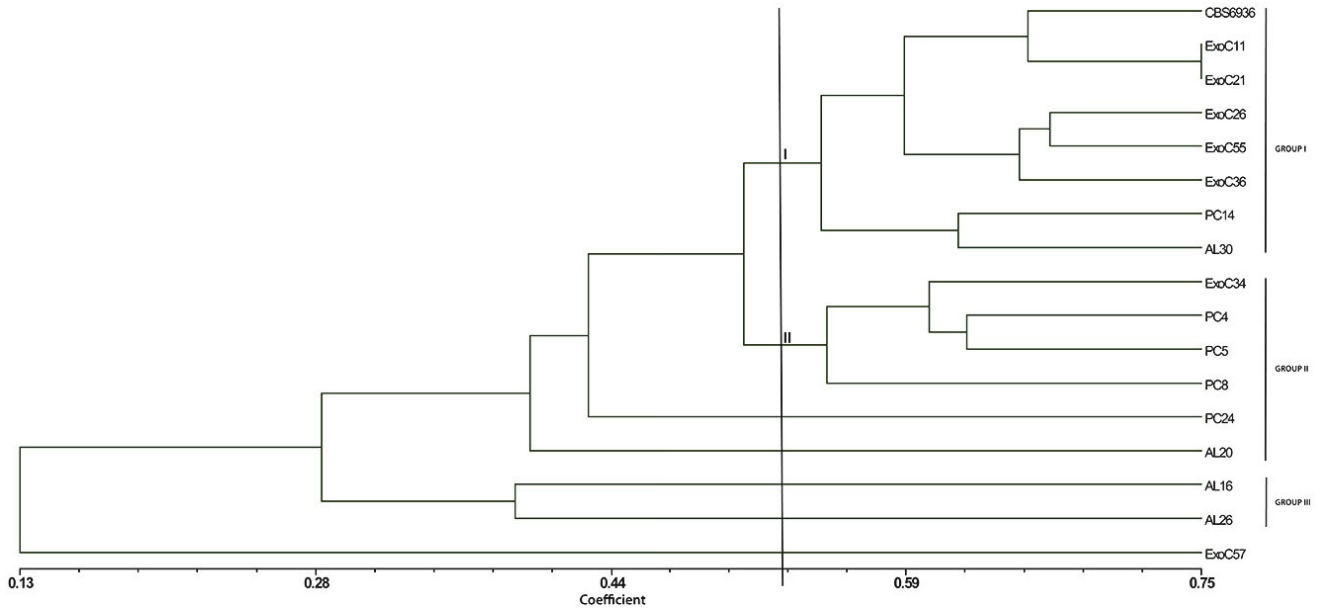

Fig. 4. UPGMA dendogram based on Jaccard's coefficient showing the relationships between *C. lusitaniae* strains determined using cumulative data. The yeast strains are named as in Table 1.

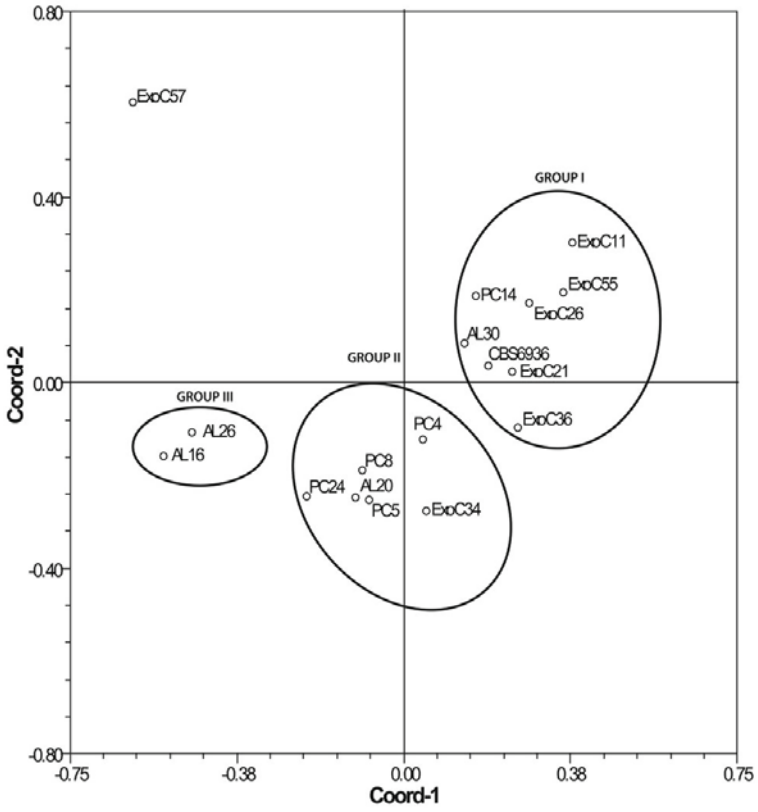

Fig. 5. Two-dimensional plot from the principal coordinate analysis showing the patterns of *C. lusitaniae* strains isolated from *Agave fourcroydes*; three genetic groups were identified.

separated from remainder of the yeasts, with a similarity coefficient of 0.13.

PCoA was carried out to estimate the genetic similarity between the *C. lusitaniae* strains. The two-dimensional PCoA plot (Fig. 5) indicated that the first and second principal coordinates accounted for 17.84% and 11.23% of the total variation, respectively. The yeast strains were positioned in discrete groups: group 1 consisted of most (5 of 7) of the agave must strains; group 2 consisted of the largest number of cooked agave strains (4 of 5); and the third group consisted exclusively of press rinse water strains. Only one strain (ExoC57) did not fall into any group. The placement of the strains was related to the source of isolation. The PCoA plot showed that the isolates clustered according their molecular patterns and sources of isolation; this behavior is similar to that observed in other yeast studies (e.g., Lopes *et al*., 2006).

In the PCR fingerprint analysis, all four markers resulted in a discrete banding pattern and highly polymorphic profiles. We believe that the MSP-PCR technique may be more sensitive than analysis methods employing other molecular markers that are commonly used to distinguish closely related isolates from the same species (such as PCR-RFLP) because the MSP-PCR method relies on fingerprinting of the whole genome (Lachance *et al*., 2003; Villa-Carvajal *et al*., 2006).

This study provides significant insight into the genetic diversity of the mycobiota present during the fermentation of henequen (*A. fourcroydes*), especially that of *C. lusitaniae*, for which few studies are available in plants. Although there have been other studies on *C. lusitaniae,* these works were carried out in *A. tequilana* and suggested that this yeast species is dominant in the fresh agave habitat (Lachance, 1995).

In the present study, we observed that *C. lusitaniae* isolated from henequen showed a high level of intraspecific genetic variability.

MSP-PCR markers were found to be very efficient for revealing the intraspecific variability between isolates of this species obtained from different stages of the fermentation process. The MSP-PCR technique has also proven useful for the intraspecific characterization of different yeast species (Perez-Brito *et al*., 2007; Daniel *et al*., 2009).

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