Identification of yeast and bacteria involved in the mezcal fermentation of *Agave salmiana*

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ABSTRACT

Aims: The objective of this work was to identify the yeast and bacteria present in the mezcal fermentation from *Agave salmiana*.

Methods and Results: The restriction and sequence analysis of the amplified region, between 18S and 28S rDNA and 16S rDNA genes, were used for the identification of yeast and bacteria, respectively. Eleven different microorganisms were identified in the Mezcal fermentation. Three of them were the following yeast: *Clavispora lusitaniae, Pichia fermentans* and *Kluyveromyces marxianus.* The bacteria found were *Zymomonas mobilis* subsp mobilis and *Zymomonas mobilis* subsp pomaceae, *Weissella cibaria, Weissella paramesenteroides, Lactobacillus pontis, Lactobacillus kefiri, Lactobacillus plantarum* and a new heterofermentative strain which was reported as *Lactobacillus farraginis*.

Conclusions: The phylogenetic analysis of 16S and ITS sequences showed that microbial diversity present in mezcal is dominated by bacteria, mainly LAB species and *Zymomonas mobilis*. It is important to note that the *L. pontis* and *Z. mobilis* species were the most abundant microorganisms present in the inoculum and that the presence of the *P. fermentans* and *K. marxianus* could be responsible for the production of some volatile compounds.

Significance and Impact of the Study: We identified the community of bacteria and yeast responsible of mezcal fermentation from for *Agave salmiana.*

Keywords: Mezcal, fermentation, phylogenetic analysis, ITS rDNA, 16S rDNA

INTRODUCTION

Mezcal is a traditional Mexican distilled beverage produced from the fermented juices of the cooked heart (or core), named or dubbed the “piñas”, of agave plants. The *Agave salmiana* is used in the Altiplano region for the mezcal production (De León-Rodríguez *et al*. 2006). The “piñas” are cooked in stone ovens to hydrolyze the inulin into fructose (Peña-Alvarez *et al*. 2004). During the process, the syrup obtained is naturally fermented with its own microorganisms. Along the fermentation the sugars are converted mainly into ethanol, high alcohols, and other compounds such as esters and organic acids (De León-Rodríguez *et al*. 2007). Therefore the taste and aroma of mezcal is determined by the composition of a mixture of compounds produced during the fermentation and others extracted from the *Agave* plant. It is worth to mention that a spontaneous fermentation constrains the mezcal process. Then, in order to provide a homogeneous quality between batches is convenient to initiate the fermentation process using well-identified inocula. For this, the analysis of the microbial diversity and its role in the sensorial features is crucial.

Many techniques have been previously developed for the identification of microorganisms. For instance, Lachance (1995) used microbiological methods and 13 different yeast species of the microorganisms involved in the fermentation of tequila (spirit obtained from *Agave tequilana weber*). The use of the molecular biology methods has allowed a rapid and accurate identification of yeast and bacteria. Methods based on the analysis of restriction fragment length polymorphism (RFLP) of the DNA that encodes the ribosomal RNA genes (5S, 5.8S, 18S and 26S) and the non-coding internal transcribed spacers (ITS) have been

applied successfully by several groups (Esteve-Zarzoso *et al*. 1999; Fernandez-Espinar *et al*. 2000; Las Heras-Vazquez *et al.* 2003). Flores Berrios (2006) used the Amplified Fragment Length Polymorphism (AFLP) technique to study the genetic yeast diversity, and showed the underlying relationship between the molecular profiles, strain origin and fermentation process for yeast isolated from Mexican *Agave* spirit (tequila, raicilla, sotol and mezcal), Italian and African wines.

Recently, the bacteria identification in natural environment has been extensively studied in several works (Dojka *et al.* 1998; Escalante *et al.* 2004; Coton *et al.* 2005). The phylogenetic analysis of the 16S rDNA region was proved to be a powerful tool to explore the biological diversity in such environments.

The aim of this work was the identification of the yeast and bacteria present in the mezcal fermentation from *Agave salmiana*. For this purpose molecular techniques such as PCR-RFLP were used to compare a specific ribosomal DNA region for both organisms classes. Those microorganisms with differences in the restriction pattern were identified by cloning, sequencing and lastly, compared against the GenBank database.

MATERIALS AND METHODS

Mezcal origin and sampling

A sample of inoculum was kindly provided by Ing. Juan Zarur from the Mezcal factory “La Perla” located in San Luis Potosí State, Mexico.

Sampling, isolation and selection of bacteria and yeast

The inoculum for mezcal production were collected aseptically and stored at -80°C with glycerol. The sample was diluted in sterile saline solution (NaCl, 0.85% wt v^{-1}) and plated on seven different media prepared for bacteria and/or yeast growth respectively (Table 1). The plates were incubated at 30°C and 28°C for bacteria and yeast for a period of 48h. Three colonies with the same morphological features were selected for further characterization. The purification from each colony was performed on the same medium by successive subculturing at the same temperature (Beuchat 1993; Deák *et al*. 1998).

DNA extraction

Bacterial DNA was isolated using a physical breakdown method with glass beans and for yeast the DNA was isolated according to Querol et al. (1992) with some modifications described below.

Physical breakdown

Bacteria cells were grown overnight in 5ml of the same broth in which they were isolated. Cells were washed with distilled water, centrifuged and resuspended in 0.2ml of extraction mixture (2% triton, 1% SDS, 10mM NaCl, 10mM TRIS-HCl pH 8, USB, Cleveland, USA), EDTA 1mM, adding 0.2ml of 25:24:1 phenol:chloroform:isoamylic alcohol, 0.06g of 0.5-mm glass beads and 0.1ml sterile distilled water. Finally, the cells were homogenized by vortex during 1 min at high speed for 8 times. The tubes were chilled on ice for 1 min between runs. The upper layer was recovered and transferred to a clean tube and 1 volume of pure chloroform was added, the tube was mixed carefully and centrifuged at 13 000 *g* for 5 min. The aqueous phase was saved in a clean tube and 20µg ml^{-1} RNAase

was added and incubated for 1 hour at 37°C. After 1 volume of 24:1 chloroform:isoamylic alcohol was added and centrifuged again. The aqueous phase was transferred to a clean tube with 10 µl of isopropanol and 4µl of ammonium acetate (4M) and carefully mixed and incubated for 15 min at room temperature. Supernatant precipitated was centrifuged for 10 min and the DNA was washed with 70% v v^{-1} ethanol, vacuum-dried and dissolved in 30µl of TE (10mM Tris-HCl, 1mM EDTA pH 7.5).

Enzymatic breakdown

Yeast cells were grown overnight in 5ml of TGYC broth, then the cells were centrifuged, at 6000 *g* for 5 min, washed with distilled water and resuspended in 500µl of 0.9M sorbitol (Sigma), 0.1M EDTA pH 7.5, and 40µl of 2.5mg ml^{-1} Zymolyase 20T (Seikagaku, Tokyo, Japan). Tubes were incubated at 37°C for 20 min. After centrifugation for 5 min at 7000 *g*, spheroplasts were resuspended in 500µl of 50mM TRIS-HCl, 20mM EDTA pH 7.4, adding 2.5µl of 10% w v^{-1} SDS, and the mixture was incubated at 65°C for 30 min. Immediately, 200µl of 5M potassium acetate was added, and the tubes were placed on ice for 1-2 hours. Then, they were centrifuged at 13000 *g* at 4°C during 20 min. The supernatant was precipitated by adding 1 volume of isopropanol, and after incubation at room temperature for 15 min, was centrifuged for 10 min. The DNA was washed with 70% v v^{-1} ethanol, vacuum-dried and dissolved in 30µl of TE (10mM Tris-HCl, 1mM EDTA pH 7.5).

PCR amplification and cloning of 16S rDNA and ITS rDNA

The 16S rDNA regions of the bacteria were amplified using the forward primer 533F 5'-GTG CCA GCA GCC GCG GTA A -3' and the reverse primer 1492R 5'-GGT TAC CTT GTT ACG ACT T -3'. (Dojka *et al.* 1998). To amplify the ITS rDNA region from yeast the primers used were ITS1 5'- TCC GTA GGT GAA CCT GCG-3' and ITS4 5'- TCC TCC GCT TAT TGA TAT GC -3' which were described elsewhere by White *et al.* 1990. PCR amplification of ITS region was performed in 50µl volume on a thermocycler (Peltier PTC-200, M.J. Research, Wattham. MA, USA). Each reactions contained 2µl template, 8µl of dNTP's mixture (2.5mM, Roche Diagnostics, Germany), 5 µl Extaq buffer 10X, 5µl $MgCl_2$ 25 mM, 0.25µl of Extaq polymerase 5U (Takara, Japan), and 1 µl of each primer (100pM, Invitrogen, Carlsbad, CA). Cycling conditions were 94°C for 4 min, followed by 30 cycles of (94°C for 1 min), annealing temperature of 55°C for 30 seconds), extension (72°C for 2min) and a final extension of 72°C for 10 min. PCR products were analyzed by electrophoresis with a 1.5% agarose gel in TAE buffer (40mM Tris-acetate, 1mM EDTA), 100 bp DNA ladder was used for the characterization of band size, following ethidium bromide staining and UV illumination.

The 16S rDNA from each bacterial colony was amplified by PCR using the pair of primers 533F and 1492R. The PCR was performed in 50µl volume containing 0.25µl of Taq Polymerase (Invitrogen, Carlsbad, CA), 5µl of Taq Polymerase buffer 10X, 8µl of dNTP mixture, 1.0µl of each primer (100pM, Invitrogen, Carlsbad, CA) and 2 µl of bacterial DNA extracted from each different colony as template in a final reaction volume on 50 µl. For the amplification of 16S rDNA, the samples were incubated for 14 min at 94°C

to denature the target DNA and then cycled 30 times at 94°C for 1 min, 50°C for 45s and 72°C for 2 min. The samples were then incubated for 12 min at 72°C for a final extension and were maintained at 4°C until tested.

Each PCR product amplified (16SrDNA and ITS region respectively) were clonated into the pCR 4-TOPO vector kit sequencing (Invitrogen, Carlsbad, CA). Two microliters of ligation reaction were used to chemically competent *E. coli*, One-shot TOP10 cells included in the TOPO TA cloning kit. Transformed cells were plated on LB-ampicillin plates. For bacteria, positive clones carrying approx. 1000 bp, 16S rDNA were identified by colony PCR. In contrast, the yeast presented different size of amplified product (between 300-800 bp) and they were identified by colony PCR using one ITS primer and one primer of the cloning kit.

A 15µl of each PCR amplified product (ITS and 16S rDNA) was digested with 1µl of restriction enzyme *HaeIII* (New England Biolabs Ltd., Hertfordshire, England) and 2µl of buffer and sterile water. The mixture was incubated for 2 hours at 37°C and inactivated for 20 min at 80°C. The restriction fragments were separated on a 3% (w v^{-1}) agarose gel. The resulting gels were stained with ethidium bromide and photographed. The lengths of both amplified products and restriction fragments were estimated by comparison against a 100 bp DNA ladder (Gibco-BRL).

Identification and phylogenetic analysis

In order to identify unique sequences of bacteria and yeast, we performed amplified ribosomal DNA restriction analysis (ARDRA) profiling for each positive clone. The unique ARDRA profiles belonging to partial sequences corresponding to *E. coli* 16S rDNA and ITS, were submitted to the non-redundant nucleotide database at GenBank using the BLAST program (www.ncbi.nlm.nih.gov) in order to determine the identity of the clone inserts.

A multiple alignment of 16S rDNA clones and reference 16S rRNA or rDNA sequences retrieved from GenBank database was performed using Clustal W program. A distance matrix calculation of nucleotide substitution rates and a phylogenetic tree was constructed with the Kimura 2-parameter and the neighbor-joining (NJ) method, respectively, using MEGA 3.1. In order to provide confidence estimated for the tree topology in the NJ method bootstrap methods were used (1000 replicates).

RESULTS

Phylogenetic analysis of ITS and 16S rDNA

Figure 1 shows a gel of the different restriction patterns obtained of the ITS region amplified. The size of the PCR amplification products and their restriction patterns corresponding to three different yeast isolated in selective media and the positive control, are shown in Table 1. The PCR-RFLP analysis of the rDNA ITS region corresponded with the restriction patterns reported elsewhere by Esteve-Zarzoso *et al.* (1999). Therefore, the use of PCR-RFLP to identify yeast such as *Clavispora lusitaniae, Pichia fermentans* and *Kluyveromyces marxianus* in the mezcal fermentation was only a first approximation. In

order to perform a clear-cut positive identification the amplified ITS1, ITS2 regions and 5.8S rDNA were cloned and sequenced by duplicate. The results of the alignment of the sequenced region with the GenBank database confirmed the PCR-RFLP analysis. The organisms identified and their identities with the sequenced clones are shown in Table 2. The ITS sequences cloned as well as the sequences of the organisms identified in database were used to construct a phylogenetic tree using the neighbor joining method (Figure 2).

A total of 120 colonies of bacteria were isolated using the different culture media. The PCR product of each colony corresponding to 16SrDNA (1000 bp approximately), was digested with an endonuclease enzyme *HaeIII*. The results showed at least fifteen different restriction patterns. Some of them have shown slight differences, i.e., only one band had a different size. The sequences of these different clones were aligned with the database of GenBank. The bacteria identified were *Zymomonas mobilis subsp mobilis* and *pomaceae*, *Weissella cibaria, Weissella paramesenteroides, Lactobacillus pontis, Lactobacillus kefiri*, *Lactobacillus plantarum,* and *Lactobacillus farraginis* a new heterofermentative strain which is very close to *Lactobacillus hilgardii* (Endo and Okada 2007). Finally, the phylogenetic tree was constructed with the 16S rDNA sequences obtained and the sequences of the organisms identified as the closest neighbor in databases. The Figure 3 shows the phylogenetic tree and the bootstrap analysis revealed that most of the branches in the tree have bootstrap values up to 80%. Moreover, high bootstrap values validated the robustness of the branching pattern obtained.

DISCUSSION

Although, the mezcal is an ancient alcoholic beverage, its fermentation process is scarcely known. Recent reports confirmed that a complex mixture of volatile compounds such as acids, alcohols, esters, and terpenes are the responsible components for the spirit aroma (De León-Rodríguez *et al.* 2006; Lachenmeier *et al.* 2006). When referring to the aroma of the mezcal, it is important to distinguish between the compounds extracted from i) the raw material (*Agave* plant), ii) those compounds produced by fermentation, and iii) the ones corresponding to the bouquet produced by the transformation of some compounds during the ageing. Throughout the fermentation, the yeast present influences the ester production and the formation of aroma. Furthermore, the higher alcohols are important precursors of the esters during the ageing process (Clemente-Jimenez *et al*. 2005).

Our results point out the fact that the presence of *Kluyveromyces marxianus* and *Pichia fermentans*, may possibly be the key organisms for the mezcal fermentation process. Furthermore, the higher alcohols produced by yeast are important precursors of the esters during the ageing process (Clemente-Jimenez *et al*. 2005).

In some wines, the spontaneous fermentation drives the process. Then, the first period of aroma production is carried out by the so-called *non-Saccharomyces* strains, such as *Kloeckera, Hanseniaspora, Candida, Pichia, Zygosaccharomyces, Schizosaccharomyces, Torulaspora, Hansenula* and *Metschnikowia* (Rojas *et al*. 2001; Clemente-Jimenez *et al*. 2005). The aforementioned strains produce high concentrations of some compounds with a remarkable influence on the sensory quality of wine (Rojas *et al*. 2001; Clemente-Jimenez

et al. 2005). It is known that *Hanseniaspora* and *Pichia sp*. promote the esterification of various alcohols such as ethanol, geraniol, isoamyl alcohol and 2-phenylethanol, increasing the concentrations of esters with a fruity aroma (Rojas *et al*. 2001). Conversely, *P. fermentans* has proved to be a good starter strains for must fermentation in the winemaking industry. This yeast has a high capacity to produce volatile compounds, which increase the aromatic properties of wine, although it has shown a low capacity to produce ethanol (Clemente-Jimenez *et al*. 2005). In spirits like Mezcal, the presence and concentration of volatile compounds is crucial for the quality of the beverage (De León-Rodríguez *et al*. 2006). Another identified yeast was the *K. marxianus* that belongs to the GRAS (Generally Regarded As Safe) group of microorganisms (Medeiros *et al*. 2000). It has been reported previously that *K. marxianus* was used to produce aroma compounds such as monoterpene alcohols and isoamyl acetate (fruity aroma) in liquid fermentations. Thus, this could be also used for goals in industry. *Clavispora lusitaniae* has been recovered from some cacti in Southern Mexico and also occurs in fruits, *Agave*, effluents, and warm-blooded animals (Starmer *et al.* 2003). There is no information about the production of volatile compounds during the fermentation with *C. lusitaniae* alone*;* this could be because they do not appear naturally in grape must.

The restriction patterns of the 5.8S-ITS region in Figure 2 shows it is an easy and rapid method of identification of yeast. The confirmation of the identity of the yeast was performed by means of the alignment with redundant sequences in the database. On the other hand, the phylogenetic tree showed the formation of three different branches and the bootstrap values of 96-100% validating the robustness of the branching pattern obtained.

Analyzing the PCR-amplified 16S rDNA sequences, it was found that several Lactic acid bacteria (LAB) species are the most abundant in the inoculum used for mezcal production.

Lactic acid bacteria such as *L. plantarum* (facultative heterofermentative), *L. hilgardii*, and *L. kefir* (obligately heterofermentative LAB) have been reported as members of the normal microbiota present in fermentations carried out by a mixed yeast/bacteria microbial population (Van Beek *et al*. 2002; Simpson *et al*. 2001). The heterofermentative LAB such as *L. hilgarddi* and *L. kefir*, produce lactic acid, ethanol, acetic acid and carbon dioxide as main products, but also diacetyl, acetoin, 2-3 butanediol and formate in minor proportions depending on the oxidizing potential of the environment (Liu *et al*. 2003). Therefore, it is possible that these organisms have a negative impact on mezcal flavor. The presence of *Lactobacillus pontis* was identified; it is a typical *Lactobacillus* isolated from endemic cereal fermentations (Müller *et al*. 2000). It is interesting to note that one of the identified microorganisms the *Weissella cibaria* belongs to the very recent detected species; it is a species with both genomic and phenotypic similarities to *Weissella confuse* (Björkroth *et al*. 2002). A common characteristic of both species that allows them to be distinguished from each other from *Weissella* species is the ability to grow at 45°C. *W. cibaria* had been isolated for the first time from Greek traditional wheat sourdoughs (Vuyst *et al*. 2002). *W. cibaria* and *W. paramesenteroides* have not been previously isolated from *Agave* fermented beverage.

Zymomonas mobilis is one of the few bacteria able to produce ethanol as final product by an almost quantitative conversion of glucose into ethanol and CO_2 via the Entner-Doudoroff pathway (Coton *et al*. 2005). Due to its ability to decouple growth and ethanol production and yields of up to 12% (w v^{-1}) ethanol, it has been proposed as a potential candidate for fuel ethanol production. (Buchholz *et al*. 1987; O'Mullan *et al*. 1995). *Z. mobilis* is responsible for the natural fermentations of sugar cane, *Agave* sap and palm sap (Swings and DeLey 1977). Another biotechnological interest of this bacterium includes its

production of acetaldehyde, a molecule often used as a flavor compound (Coton *et al.* 2005). The *Zymomonas* could be the responsible for the highest production of ethanol. This is supported by the fact that the identified yeast does not produce elevated concentrations of ethanol. Moreover, it has been previously proposed that *Z. mobilis* is an essential microorganism in the fermentation of *pulque* (Mexican beverage), along with yeast for ethanol production (Sanchez-Marroquín 1967). In addition, several strains of *Z. mobilis* have been previously isolated from pulque samples and have been reported as high ethanol producers (Escalante *et al.* 2004).

The phylogenetic analysis of 16S rDNA and ITS rDNA sequenced clones showed that microbial diversity present in mezcal is dominated by bacteria, mainly LAB species and *Zymomonas mobilis*. This result could be interpreted as an indicator of selective influences in the relative bacterial diversity induced by the must of *Agave* and the environment. It is important to note that the *L. pontis* and *Z. mobilis* species were the most abundant microorganisms present in the inoculum and that the presence of the yeast, *P. fermentans* and *K. marxianus* could be responsible for the contribution of the volatile compounds. Thus, a good selection of the starter cultures will improve the quality of this beverage. To the best of our knowledge, this work provides the first evidence of mixed cultures in the alcoholic fermentation of mezcal.

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FIGURE CAPTIONS

Figure 1. ARDRA patterns obtained from digestion with *HaeIII*. Lanes: L, size ladder (100 bp DNA ladder; Gibco-BRL); S, positive control (*Sacharomyces cerevisiae*); MY05, *Clavispora lusitaniae*; MY01, *Kluyveromyces marxianus*; MY03, *Pichia fermentans*.

Figure 2. Phylogenetic relationship of 5.8S-ITS region from mezcal clones and sequences of closest neighbor ITS rDNA from identified yeast in NCBI database. The sequences were aligned by using Clustal W. The distance matrix and phylogenetic tree were calculated by the Kimura 2-paramenter and Neighbor joining algorithms, respectively. Percent of identity with closest references ITS rDNA clones in the database is indicated in parenthesis. The sequence of *Zymomonas mobilis* served as outgroup sequence. Bootstrap 1000.

Figure 3. Phylogenetic relationship of 16S rDNA from mezcal clones and sequences of closest neighbor 16S rDNA from identified bacteria in NCBI database. The sequences were aligned by using Clustal W. The distance matrix and phylogenetic tree were calculated by the Kimura 2-paramenter and Neighbor joining algorithms, respectively. Percent of identity with closest references 16S rDNA rDNA clones in the database is indicated in parenthesis. The sequence of *Sulfolobus acidocaldarius* served as outgroup sequence. Bootstrap 1000.

Table 1. Materials to elaborate the different media (1L)

Media	Components
TYGC	Tripton 30g, yeast extract 10g, glucose 20g, agar 15g, chloramphenicol 100mg l^{-1}
MRS	Protease peptone No. 3 10g, beef extract 10g, yeast extract 5g, dextrose 20g, polysorbate 80 1g, ammonium citrate 2g, sodium acetate 5g, magnesium sulfate 0.1g, manganese sulfate 0.05g, dipotassium phosphate 2g, agar 15g
MYGP	Malt extract 3g, yeast extract 3g, glucose 10g, peptone 5g, agar 15g, actidione 20mg l^{-1}, ethanol 3%
PD	Potato starch 4g, dextrose 20g, agar 15g
AA	Pancreatic digest of casein 20g, sodium chloride 5g, dextrose 10g, agar 20g, sodium thioglycollate 2g, formaldehyde sulfoxylate 1g, methylene blue 0.002g
PCA	Tryptone 5g, yeast extract 2.5g dextrose 1g, agar 15g
MA	Potato starch 4g, dextrose 20g, agar 15g and filtered agave juice for obtained a final concentration of 5° Brix.

Table 2. Yeast identified, lengths (in bp) of the 5.8S-ITS region amplified by PCR and the fragments obtained after digestion with restriction endonuclease *HaeIII.*

Yeast identified	**Amplified product (bp)**	**Restriction fragments *HaeIII***
Saccharomyces cerevisiae	880	320+230+180+150
Clavispora lusitaniae (MY05)	390	360
Kluyveromyces marxianus (MY01)	740	655+80
Pichia fermentans (MY03)	450	340+80+30

Table 3. Organisms showing the highest percent of identity in the output result from analysis in the non-redundant nucleotide database from NCBI with BLAST program.

Organism identified	**Gene Bank Accession number**	**Clones identified and identity against Gene bank database**
Clavispora lusitaniae	AY939811	MYO5(99%), MYO6(100%)
Kluyveromyces marxianus	AY939806	MYO1(99%), MYO2(99%)
Pichia fermentans	AF411062	MYO3(98%), MYO4(98%),
Lactobacillus farraginis	AB262732	MB02(93%)
Lactobacillus kefir	AY363303	MB01(97%)
Lactobacillus plantarum	DQ239698	MB03(98%)
Lactobacillus pontis	AJ422032	MB04(95%), MB05(98%), MB06(97%), MB07(98%), MB08(98%)
Weissella cibaria	WC1422031	MB10(98%)
Weissella paramesenteroides	AB023238	MB09(98%)
Zymomonas mobilis	AF281033	MB14(97%), MB15(98%)
Zymomonas mobilis subsp mobilis	AY670648	MB11(96%), MB12(98%), MB13(97%)
Zymomonas mobilis subsp pomaceae	AY670649	MB15(98%), MB16(99%)

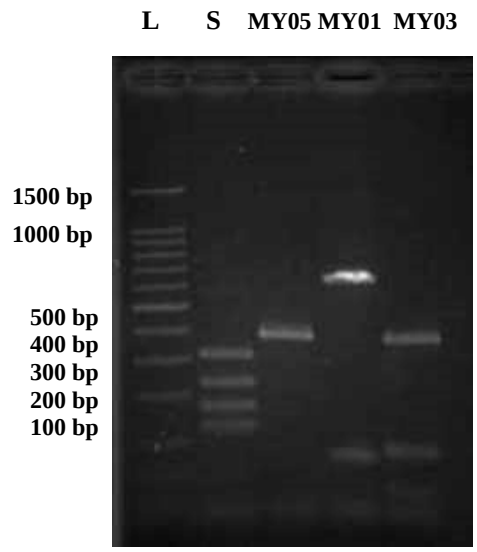
L
S
MY05
MY01
MY03
1500 bp
1000 bp
500 bp
400 bp
300 bp
200 bp
100 bp

Figure 1

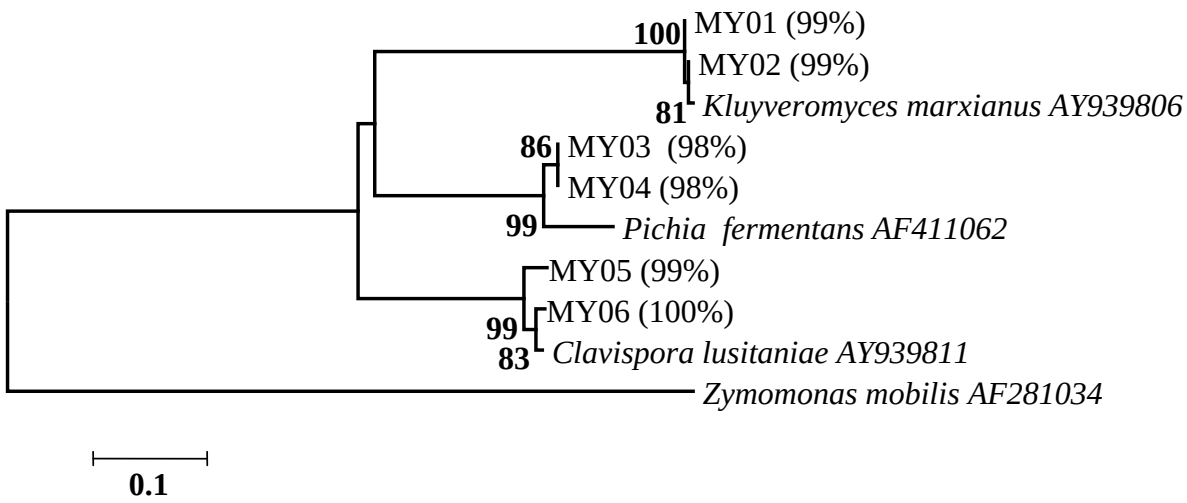
100
MY01 (99%)
MY02 (99%)
81
Kluyveromyces marxianus AY939806
86
MY03 (98%)
MY04 (98%)
99
Pichia fermentans AF411062
MY05 (99%)
99
MY06 (100%)
83
Clavispora lusitaniae AY939811
Zymomonas mobilis AF281034
0.1

Figure 2

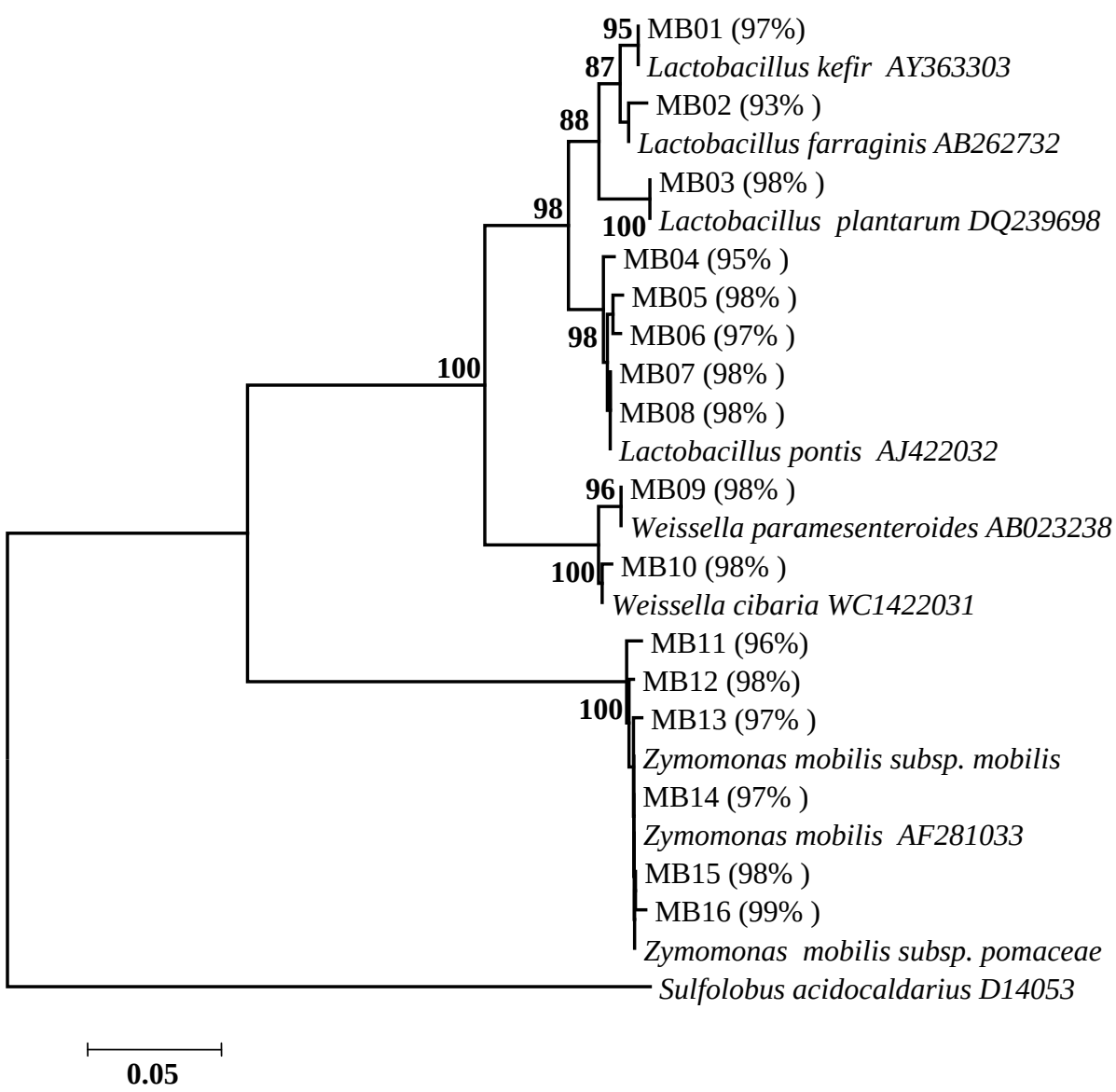
95
MB01 (97%)
87
Lactobacillus kefir AY363303
MB02 (93%)
88
Lactobacillus farraginis AB262732
MB03 (98%)
98
100
Lactobacillus plantarum DQ239698
MB04 (95%)
MB05 (98%)
98
MB06 (97%)
100
MB07 (98%)
MB08 (98%)
Lactobacillus pontis AJ422032
96
MB09 (98%)
Weissella paramesenteroides AB023238
100
MB10 (98%)
Weissella cibaria WC1422031
MB11 (96%)
MB12 (98%)
100
MB13 (97%)
Zymomonas mobilis subsp. mobilis
MB14 (97%)
Zymomonas mobilis AF281033
MB15 (98%)
MB16 (99%)
Zymomonas mobilis subsp. pomaceae
Sulfolobus acidocaldarius D14053
0.05

Figure 3