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ORIGINAL ARTICLE

Potential production of 2-phenylethanol and 2-phenylethylacetate by non-*Saccharomyces* yeasts from *Agave durangensis*

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Abstract

Introduction The participation of non-*Saccharomyces* yeasts in fermentation processes is of great importance due to their participation in the formation of esters and superior alcohols, which confer characteristic aromas to beverages such as wine and mescal.

The aim The aim of this study was identify and evaluate the potential aroma production of yeast native of *Agave* fermentation by the mescal production in Durango, Mexico. Isolated yeasts were molecularly identified by 5.8s ribosomal gene; the potential production of aromas was carried out in fermentations with the addition of L-phenylalanine and evaluated after 24 h of fermentation. Extraction and quantification of aromatic compounds by headspace solid-phase micro-extraction (HS-SPME) and gas chromatograph mass spectrometry (GC-MS).

Results The isolated non-*Saccharomyces* yeasts could be classified into six different genera *Saccharomyces cerevisiae*, *Clavispora lusitaniae*, *Torulaspora delbrueckii*, *Kluyveromyces dobzhanskii*, *Kluyveromyces marxianus*, and *Kluyveromyces* sp. All probed strains presented a potential aroma production (ethyl acetate, isoamyl acetate, isoamyl alcohol, benzaldehyde, 2-phenylethyl butyrate, and phenylethyl propionate), particularly 2-phenylethanol and 2-phenylethylacetate; the levels found in the *Kluyveromyces marxianus* ITD0211 yeast have the highest 2-phenylethylacetate production at 203 mg/L and *Kluyveromyces marxianus* ITD0090 with a production of 2-phenylethanol at 1024 mg/L.

Conclusion Non-*Saccharomyces* yeasts were isolated from the mescal fermentation in Durango; the *Kluyveromyces* genus is the most predominant. For the production of aromas, highlighting two strains of *Kluyveromyces marxianus* produces competitive quantities of compounds of great biotechnological interest such as 2-phenylethanol and 2-phenylethylacetate, without resorting to the genetic modification of yeasts or the optimization of the culture medium.

Keywords Mescal · Bioconversion · Aroma · L-Phenylalanine · *Kluyveromyces marxianus*

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Introduction

The non-*Saccharomyces* yeasts are well recognized for their contribution to the aroma of fermentative beverages (Cordente et al. 2012; Ciani et al. 2016; Masneuf-Pomarede et al. 2016), especially wine. Their presence has also been reported in mescal and tequila. In Mexico, these alcoholic beverages are distinguished from each other, based on the agave species used in their production. For example, *Agave tequilana* Weber var. Azul (blue variety) is used for tequila, whereas *Agave salmiana* and *Agave durangensis*, (*or Agave duranguensis*) among others, are used for mescal production in various regions of Mexico (Lappe-Oliveras et al. 2008; Páez-Lerma et al. 2010; De los Rios-Deras et al. 2015; Kirchmayr et al. 2017). Mescal has elevated its economic importance in the last years (Kirchmayr et al. 2017). During the mescal production process, agave juice is naturally fermented by native yeasts, such as *Saccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Debaryomyces*, *Hanseniaspora*, *Kloeckera*, *Schizosaccharomyces*, *Torulaspora*, and *Zygosaccharomyces* (Lachance 1995; Díaz-Montaño et al. 2008; Escalante-Minakata et al. 2008). Previously published research on fermentations of agaves suggests that non-*Saccharomyces* yeasts have an important role in the initial fermentation process and influence the production of the volatile compounds (Lappe-Oliveras et al. 2008; Narváez-Zapata et al. 2010; Martell Nevárez et al. 2011). The potential use of these yeasts as inoculants has been described (Rodríguez-Sifuentes et al. 2014; Nuñez-Guerrero et al. 2016), as well as their participation in generating the volatile compounds in mescal, mainly esters (Martell Nevárez et al. 2011; Rutiaga-Quiñones et al. 2012; Hernández-Carbajal et al. 2013). Despite the increasing use of non-*Saccharomyces* yeasts in biotechnology, there are still many opportunities to improve native yeast exploration. These prospects have led to a great interest in further enhancing the number of non-*Saccharomyces* yeasts available, by selecting or developing strains with novel and attractive properties.

Flavor has a major impact on the quality perception of food and beverages, and fragrances are highly valued in the cosmetic and perfume industry. For natural aroma compounds that exist at low concentrations in their original sources, biotechnological processes represent an attractive alternative to the traditional preparation by extraction (Schrader et al. 2004). Due mainly to its sweet and rose-like taste and odor 2-phenylethanol (2-PE) and its more fruit-like form, acetate ester 2-phenylethylacetate (2-PEA), find use in various flavor compositions (Fabre et al. 1998). For food applications, the rising demand for natural products means natural flavor compounds are increasingly becoming a necessity (Etschmann and Schrader 2006, Morrissey et al, 2015).

Both 2-PE and 2-PEA can be produced by *de novo* synthesis or from L-phenylalanine (L-Phe) by non-*Saccharomyces* yeast whole-cell biocatalysis via the Ehrlich pathway (Etschmann et al. 2003), (Etschmann and Schrader 2006). 2-PE can also be metabolized to 2-PEA by a *trans*-esterification reaction, which involves the transfer of a group of acetyl-coenzyme A acetate to the hydroxyl group of 2-PE (Hazelwood et al. 2008; Pires et al. 2014). When L-Phe is the sole nitrogen source in the medium, large amounts of 2-PE are accumulated. Several biotechnological processes are known for producing 2-PE, based on this pathway, and considerable progress has been made on the development of this process. In this context, yeast biodiversity may be greatly impacted by the production of different aroma products derived from primary and secondary metabolism. The diversity of non-*Saccharomyces* yeasts responsible for many of the volatile compounds found in mescal, in the state of Durango, Mexico, has not yet been evaluated. This research aimed to identify the non-*Saccharomyces* microbiota present in fermentations in three different mescal-producing regions and assess the production potential of aromatic compounds the addition of L-Phe as an inductor.

Materials and methods

Yeast strains

Thirty-four native strains, identified as non-*Saccharomyces* from *Agave durangensis* fermentation and obtained from the Collection of the Instituto Tecnologico de Durango, were isolated from three mescal-producing regions of Durango State, Mexico: Mezquital (23° 28′ 22″ N, 104° 24′ 40″ W), Nombre de Dios (23° 51′ 00″ N, 104° 14′ 00″ W), and Durango (24° 01′ N, 104° 40′ W). All yeast strains were conserved, as culture stock at − 20 °C in 30% (*v*/*v*) glycerol.

Molecular identification

Growth conditions

Yeast cells preserved in glycerol were first activated on YDP solid medium (glucose 20 g/L, casein peptone 20 g/L, yeast extract 10 g/L, and agar 20 g/L). DNA was then extracted at 24-h growth, using the method detailed by Sambrook and Russell (2001).

Polymerase chain reaction and amplification

Polymerase chain reaction (PCR) was carried out in 50-μL volumes, using 2.0 μL of DNA with ITS1 (5′–TCC GTA GGT GAA CCT GCG G–3′) and ITS4 (5′–TCC TCC GCT TAT TGA TAT GC–3′) primers to amplify the rDNA repeat unit that includes the 5.8S rRNA gene and the two non-coding regions designated the internal transcribed spacers (ITS1 and ITS4) (White et al. 1990). Amplification began with an initialization step of one cycle at 95 °C for 5 min, then 35 cycles of

95 °C for 1 min, 52 °C for 2 min, and 72 °C for 2 min, followed by a final elongation at 72 °C for 10 min (White et al. 1990). The PCR product was electrophoresed on 1% agarose gel with TAE 0.5× buffer (Promega, Madison, WI, USA), at 95 V for 45 min, stained with ethidium bromide (Sigma–Aldrich. St. Louis, MO, USA) and visualized under UV light (Benchtop UV transilluminator, Upland, CA, USA); DNA fragment sizes were determined using a 100-bp DNA ladder (Promega, USA). The PCR product was purified using $C_2H_7NO_2$ and C_2H_6O (> 99%) (Sigma–Aldrich). The rDNA sequences were acquired using an ABI PRISM Model 3730XL sequencer (Applied Biosystems, Inc., Foster City, CA, USA) at the National Genomics for Biodiversity Laboratory (Langebio) of Cinvestav (Irapuato, Mexico).

Phylogenetic analysis

The obtained sequences were aligned using the MUSCLE program (https://www.ebi.ac.uk/Tools/msa/muscle), and regions of local similarity between sequences were identified from the National Center for Biotechnology Information (NCBI) database of GenBank using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast). Phylogenetic analyses

Table 1 Strains used in this study

Species	Strain	Locality	Accession no.
Clavispora lusitaniae	ITD 0132	Mezquital	MH282797
Kluyveromyces marxianus	ITD 0002	Mezquital	MH282778
Kluyveromyces marxianus	ITD 0003	Mezquital	MH282779
Kluyveromyces marxianus	ITD 0090	Mezquital	MF797638.1
Kluyveromyces marxianus	ITD 0091	Mezquital	MH282784
Kluyveromyces marxianus	ITD 0092	Mezquital	MH282785
Kluyveromyces marxianus	ITD 0093	Mezquital	MH282786
Kluyveromyces marxianus	ITD 0128	Mezquital	MH282787
Kluyveromyces marxianus	ITD 0141	Mezquital	MH282790
Kluyveromyces marxianus	ITD 0142	Mezquital	MH282791
Kluyveromyces marxianus	ITD 0145	Mezquital	MH282792
Kluyveromyces marxianus	ITD 0211	Mezquital	MH282793
Kluyveromyces sp.	ITD 0040	Mezquital	MH282781
Kluyveromyces sp.	ITD 0041	Mezquital	MH282782
Kluyveromyces sp.	ITD 0089	Mezquital	MH282783
Kluyveromyces sp.	ITD 0136	Mezquital	MH282788
Kluyveromyces sp.	ITD 0137	Mezquital	MH282789
Torulaspora delbrueckii	ITD 0110	Mezquital	MH282795
Torulaspora delbrueckii	ITD 0129	Mezquital	MH282796
Saccharomyces cerevisiae	ITD 0109	Mezquital	MH282794
Clavispora lusitaniae	ITD 0095	Nombre de Dios	MH282804
Clavispora lusitaniae	ITD 0099	Nombre de Dios	MH282805
Clavispora lusitaniae	ITD 0103	Nombre de Dios	MH282806
Clavispora lusitaniae	ITD 0104	Nombre de Dios	MH282807
Clavispora lusitaniae	ITD 0107	Nombre de Dios	MH282808
Kluyveromyces marxianus	ITD 0102	Nombre de Dios	MH282801
Kluyveromyces marxianus	ITD 0264	Nombre de Dios	MH282802
Kluyveromyces marxianus	ITD 0268	Nombre de Dios	MH282803
Kluyveromyces sp.	ITD 0046	Nombre de Dios	MH282798
Kluyveromyces sp.	ITD 0048	Nombre de Dios	MH282799
Kluyveromyces sp.	ITD 0049	Nombre de Dios	MH282800
Kluyveromyces marxianus	ITD 0069	Durango	MH282810
Kluyveromyces sp.	ITD 0062	Durango	MH282809
Kluyveromyces dobzhanskii	ITD 0157	Durango	MH282811
Kluyveromyces marxianus	CBS 600	Reference	KY103809.1

were conducted in MEGA7 Program. The sequences were deposited in GenBank.

Production of volatile organic compounds

Chemicals and reagents

L-Phe (< 98%), 2-PE (> 99%), and 2-PEA (> 99%) were purchased from Sigma–Aldrich. $Na_2HPO_4.2H_2O$, $MgSO_4.7H_2O$ (Caisson Laboratory In., Smithfield, UT, USA), and citric acid were obtained from Fermont (Mexico City, Mexico). Glucose, yeast extract and casein peptone came from BD Bioxon (Mexico City, Mexico).

Bioconversion

The strains were pre-grown in 125-mL baffled Erlenmeyer flasks (Corning, Inc., USA) with vented top, containing a 50-mL operative volume of standard yeast medium YPD broth (20 g/L glucose, 20 g/L casein peptone, and 10 g/L yeast extract), at 30 °C for 12 h and 120 rpm. For fermentation, the strains were inoculated at a concentration of 10^7 cells/mL and incubated at 30 °C for 24 h and 120 rpm. Duplicate experiments were done for induction with L-Phe (9 g/L), in which the culture medium contained 30 g/L glucose, 35 g/L $Na_2HPO_4.2H_2O$, 10.5 g/L citric acid, 0.5 g/L $MgSO_4.7H_2O$, and 0.17 g/L yeast extract, in a 50-mL medium, in a 125-mL Erlenmeyer flask (Etschmann et al. 2004). The yeast *Kluyveromyces marxianus* CBS 600 (KY103809.1) was included as a reference.

Gas chromatography–mass spectrometry analysis

The volatile organic compounds were extracted by headspace solid-phase micro-extraction (HS-SPME) with a divinylbenzene/carboxen/polydimethylsiloxane fiber (Supelco, Bellefonte, PA, USA). One milliliter of the sample was taken from each fermentation at 24 h, placed inside a 4-mL vial, sealed tightly with a screw-top septum-containing cap, and allowed to stand at 35 °C for 1 h. The SPME needle was then inserted through the septum, the holder was secured, and the fiber was exposed to the headspace. After 1 h of sampling at 35 °C, the fiber was retracted and immediately inserted into the inlet of a HP 5890 Series II GC instrument directly coupled to an HP 5972 mass-selective detector (Hewlett–Packard, Palo Alto, CA, USA) and equipped with an HP-FFAP capillary column (25 m × 0.320 mm i.d., film thickness 0.50 m; Hewlett–Packard), for thermal desorption. The injection was accomplished by desorption of the fiber at 230 °C for 6 min with the injector operated in the splitless mode for 1 min. An additional 5-min exposure in the injection port allowed the fiber to be cleaned of any compound that may not have been desorbed during the initial minute (Calvo-Gómez et al. 2004). Helium was used as the carrier gas, at a linear flow of 2 mL/min. The injector and detector temperatures were 230 and 260 °C, respectively. The oven temperature was increased from 40 to 240 °C, using the following program: the initial temperature was maintained for 3 min, ramped at 4 °C/min to 100 °C, held for 1 min, and then ramped at 4 °C/min to 240 °C and held for 10 min. The ionization voltage was 70 eV. All the assays were performed twice. The analyzed compounds were identified by comparing their mass spectra with those in the NIST database (Calvo-Gómez et al. 2004). In addition, the volatile compounds of interest (2-PE and 2-PEA) were quantified by standard curves.

Statistical analysis

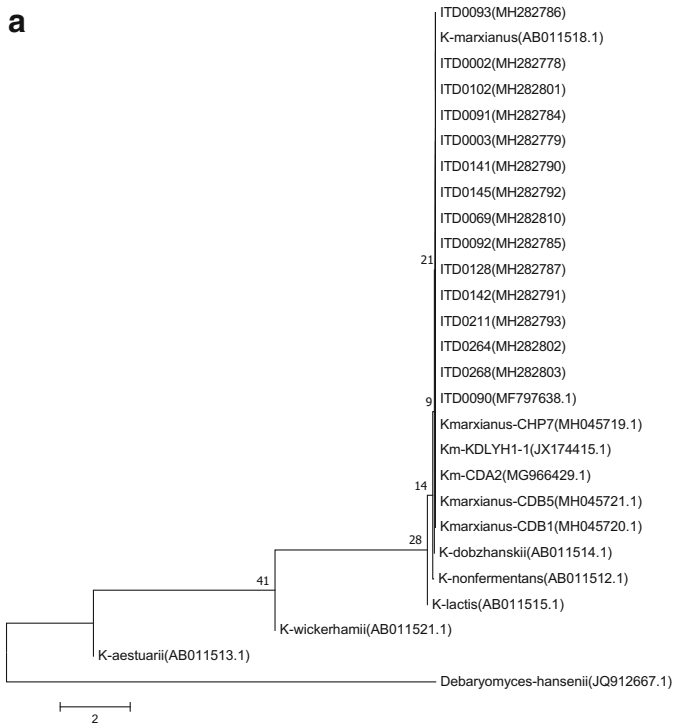
Data of the volatile compounds, 2-PE and 2-PEA, were evaluated by the HSD–Tukey–Kramer comparison test, at $\alpha = 0.01$ All statistical analyses were done using JMP software version 13.2 (SAS Institute, Inc., NC, USA).

Result and discussion

Molecular identification and phylogenetic analyses

Table 1 indicates the molecular identification of the studied strains, for each geographic region. These strains corresponded to six different genera: *Clavispora lusitaniae*, *Kluyveromyces* sp., *Kluyveromyces dobzhanskii*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii*. Previous investigations of the yeasts associated with mescal production in Mexico, described the presence of non-*Saccharomyces* strains, such as *K. marxianus*, *C. lusitaniae*, and *Pichia fermentans* from *Agave salmiana* fermentation, in San Luis Potosí State (Escalante-Minakata et al. 2008). In another Vinata, from the same region, the non-*Saccharomyces* yeasts were: *K. marxianus*, *Pichia kluyveri*, *Zygosaccharomyces bailii*, *C. lusitaniae*, *T. delbrueckii*, and *Candida ethanolica* (Verdugo-Valdez et al. 2011). In mescal produced using the species *Agave durangensis* in Durango, the predominant non-*Saccharomyces* yeasts belonged to *Candida* genus, including *Candida lusitaniae*, *Candida kefir*, *Candida glabrata*, *Candida laurentii*, and

Fig. 1 Neighbor-joining trees were constructed from the evolutionary distance data for ITSI-5.85 rDNA-ITS2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). **a** *Kluyveromyces marxianus* tree of group one. **b** *Kluyveromyces dobhzankii* tree. **c** *Kluyveromyces sp of group two* tree. The accession numbers of reference sequences used in this tree are as follows: *K. nonfermentans* (AB011512.1), *K. lactis* (AB011515.1), *K. wickerhamii* (AB011521.1), *K. aestuarii* (AB011513.1), *K. marxianus* (AB011518.1), *K. marxianus* (MH045720.1), *K. marxianus* (MH045721.1), *K. marxianus* (MH045719.1), *K. marxianus* (MG966429.1), *K. marxianus* (JX174415.1), *K. dobzhanskii* (ABO11514.1), and *D. hansenii* (JQ912667.1). Evolutionary analyses were conducted in MEGA7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ITD0268(MH282803)
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Km-CDA2(MG966429.1)
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Kmarxianus-CDB5(MH045721.1)
Kmarxianus-CDB1(MH045720.1)
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K-lactis(AB011515.1)
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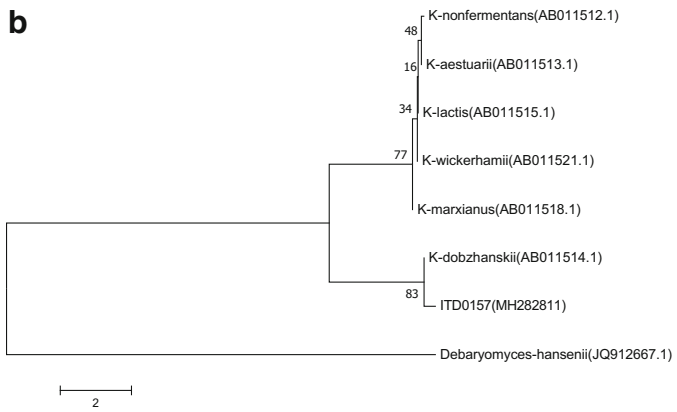
K-aestuarii(AB011513.1)
Debaryomyces-hansenii(JQ912667.1)
2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K-wickerhamii(AB011521.1)
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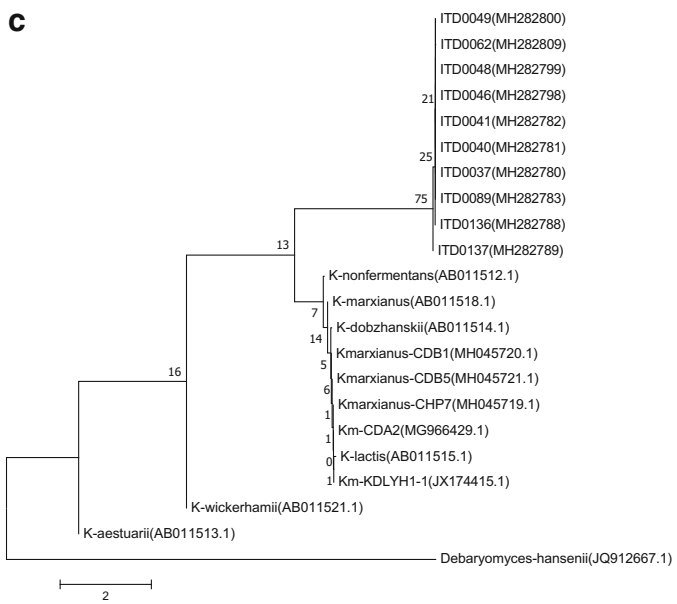
2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ITD0062(MH282809)
ITD0048(MH282799)
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ITD0046(MH282798)
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ITD0040(MH282781)
25
ITD0037(MH282780)
75
ITD0089(MH282783)
ITD0136(MH282788)
13
ITD0137(MH282789)
K-nonfermentans(AB011512.1)
7
K-marxianus(AB011518.1)
14
K-dobzhanskii(AB011514.1)
5
Kmarxianus-CDB1(MH045720.1)
16
6
Kmarxianus-CDB5(MH045721.1)
1
Kmarxianus-CHP7(MH045719.1)
1
Km-CDA2(MG966429.1)
0
K-lactis(AB011515.1)
1
Km-KDLYH1-1(JX174415.1)
K-wickerhamii(AB011521.1)
K-aestuarii(AB011513.1)
Debaryomyces-hansenii(JQ912667.1)
2

Candida tropicalis (Páez-Lerma et al. 2010). Equally, in Durango State, Páez-Lerma et al. (2013) observed diverse microorganisms at the beginning of fermentation: *S. cerevisiae*, *T. delbrueckii*, *K. marxianus*, *Candida diversa*, *P. fermentans*, and *Hanseniaspora uvarum*, but only *T. delbrueckii* and *S. cerevisiae* were found at the end of the fermentations. Recently, Kirchmayr et al. (2017) mentioned *K. marxianus*, *Zygosaccharomyces rouxii*, *Z. bisporus*, *T. delbrueckii*, and *Pichia membranifaciens* as the main microbiota present, after *S. cerevisiae*, during mescal production in Oaxaca State. In our study, Mezquital region presented the greatest number and diversity of isolated non-*Saccharomyces*, which included *K. marxianus*, *T. delbrueckii*, and *C. lusitaniae*. Both *K. marxianus* and *C. lusitaniae* were also detected in mescal from Nombre de Dios. In fermentation of agave in Durango, the species identified were *K. marxianus* and *K. dobzhanskii*. This article is the first report where the strain *K. dobzhanskii* has been found in natural fermentation processes. This genus has been cataloged as the closest *Kluyveromyces lactis* relative of wild or native strains, so it has been used for modeling population genetics (Belloch et al. 1997, 2002; Sukhotina et al. 2006; Lane and Morrissey 2010).

In phylogenetic studies of *Kluyveromyces* strains (Fig. 1), three groups were recognized. The first two groups comprised strains directly related to the genus *K. marxianus* and *K. dobzhanskii*, respectively (Fig. 1a, b). The third group had direct relationship to the genera of the *Kluyveromyces* family (Fig. 1c). These strains were present in all the regions, accounting for 35% (Mezquital), 50% (Nombre de Dios), and 30% (Durango) of the total of the isolated *Kluyveromyces* strains and can represent a particular genetic diversity for *K. marxianus* strains isolated from the fermentation process during the production of mescal. In a recent study of the genetic diversity of the genus *K. marxianus*, all the isolates from a lactic environment were either diploid or triploid, whereas non-lactic isolates were haploid (Ortiz-Merino et al. 2018). Additionally, the authors distinguished three clades, of which the strain UFS-Y2791, isolated from American agave juice and representing the third clade, proved to be more diverse than the others (Ortiz-Merino et al. 2018). So far, only the presence of *K. marxianus* strains from different mescal production has been reported in the literature, indicating that the current work is the first to show that *K. marxianus* strains isolated from agave fermentation (mescal or tequila) have distinct genetic differences between them. Páez-Lerma et al. (2013) noted these differences with *S. cerevisiae* strains in wine.

Additionally, phylogenetic analysis among the *Clavispora lusitaniae* yeasts from this study (Fig. 2a) evidenced the genetic variability between strains of *Clavispora lusitaniae*. These yeasts were found predominantly in fermentations from Nombre de Dios, with 45% of the strains identified as *C. lusitaniae.* Pérez-Brito et al. (2015) characterized the great genetic diversity of *C. lusitaniae* strains isolated from the fermentation of *Agave fourcroydes* Lem. There are numerous accounts of this species during the different stages of processing and fermentation of *Agave* to obtain traditional Mexican beverages, such as "pulque," mescal, and tequila (Rodrigues de Miranda 1979; Lachance 1995; Lappe et al. 2004; De León Rodríguez et al. 2008; Lappe-Oliveras et al. 2008; Páez-Lerma et al. 2010; Kurtzman et al. 2011; Verdugo-Valdez et al. 2011), where its presence has been associated with the sensory qualities of these beverages (Escalante-Minakata et al. 2008).

The species present in relatively low quantity was *T. delbrueckii*, found only in the region of the Mezquital. Figure 2 b shows the phylogenetic tree for strains ITD0110 and ITD0129. These strains have been linked to a high production of volatile compounds that impart unique characteristics to beverages, such as mescal, and also other flavor compounds, including terpenoids, esters, higher alcohols, glycerol acetaldehyde, acetic acid, and succinic acid (Moreira et al. 2005; Jolly et al. 2014). Rutiaga-Quiñones et al. (2012) profiled the volatile compounds in *Agave duranguensis* juice supplemented with NH_4Cl and fermented with the yeast *T. delbrueckii* ITD0110. However, the genetic diversity present in this genus was not established. Nuñez-Guerrero et al. (2016) isolated *S. cerevisiae*, *T. delbrueckii*, and *K. marxianus* from *A. duranguensis* fermentation and proposed the use of a mixture of 75% *S. cerevisiae* and 25% *T. delbrueckii* as an inoculant to make mescal.

Production of volatile organic compounds

Table 2 presents the volatile compounds produced by the non-*Saccharomyces* yeasts studied in this work. In general, all strains were producers of esters, fatty acids esters, and higher alcohols. Esters are key flavor compounds in fermented beverages, like mescal. Among the acetate esters, the synthesis of ethyl acetate, which is responsible for the bouquet and desirable fruity flavors, depends on the ethanol concentration while the synthesis of isoamyl acetate, isobutyl acetate and 2-PEA, relies on the concentration of their corresponding higher alcohol, by the action of an alcohol acetyltransferase (Gethins et al. 2015; Loser et al. 2014).

The ethyl esters of short-chain fatty acids present are 2-phenylethyl butyrate and Phenylethyl propionate, synthesized from 2-PE and short-chain fatty acids. Phenylethyl propionate is an ester desirable in wine, due to its floral aroma (Beckner Whitener et al. 2015; Padilla et al. 2016). The formation of benzaldehyde from Phe has been studied in several microorganisms, such as *Pseudomonas putida* and the white rot fungi, *Trametes suaveolens*, *Polyporus tuberaster*, *Bierkandera adusteand*, and *Phanerochaete chrysosporium* (Rojas et al. 2001; Liu et al. 2004). Hence, these yeasts seem to have benzaldehyde production potential. The principal volatile compounds found were 2-PE, which is considered to be one of the most important aromatic alcohols, and 2-PEA. The higher alcohols are predominantly

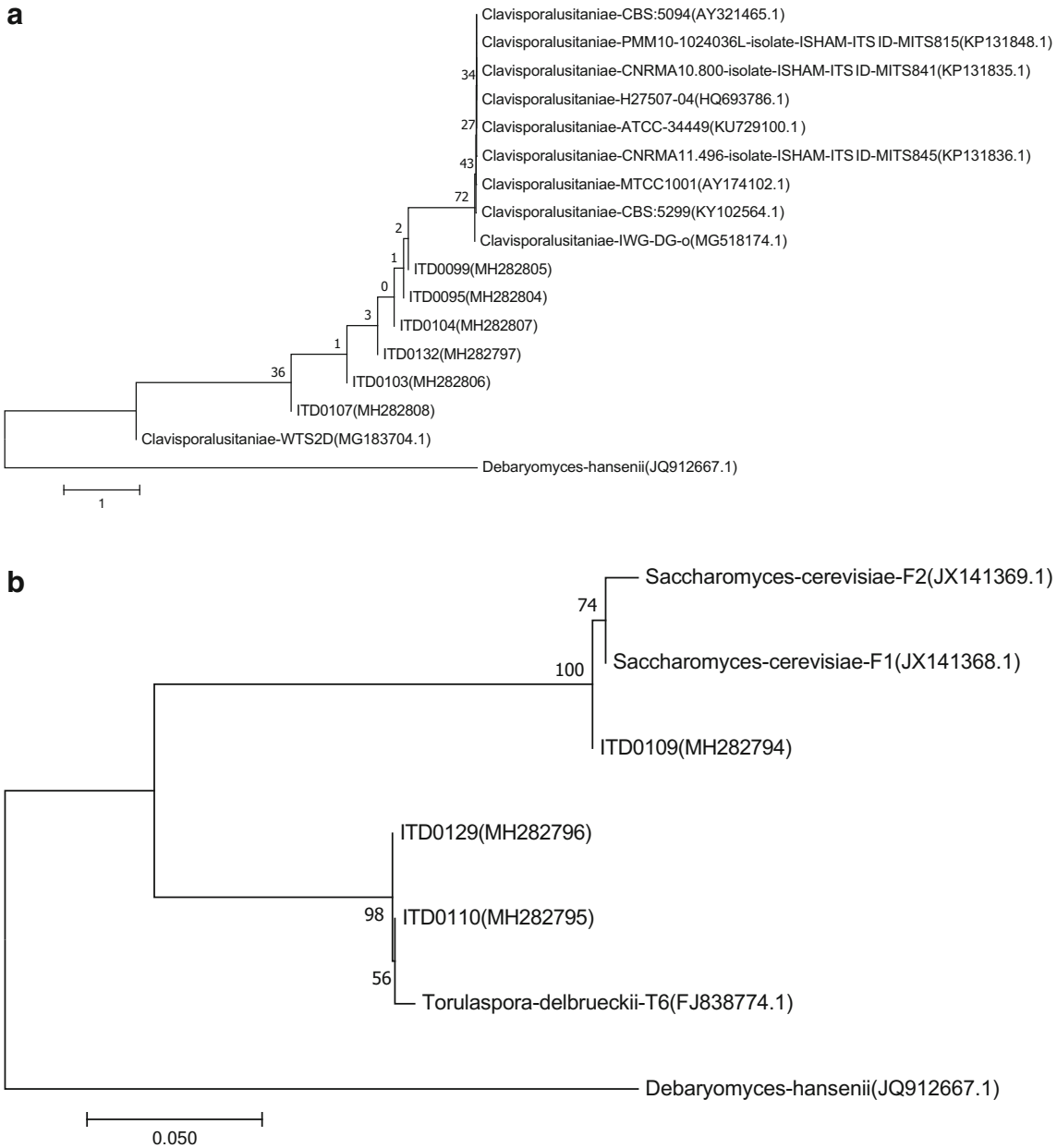

Fig. 2 Neighbor-joining trees were constructed from the evolutionary distance data for ITSI-5.85 rDNA-ITS2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). **a** *Clavispora lusitaniae* tree. **b** *S. cerevisiae–T. delbrueckii* tree. The accession numbers of reference sequences used in these trees are as follows: *C. lusitaniae* (AY321465.1), *C. lusitaniae* (KP131848.1), *C. lusitaniae* (KP131835.1), *C. lusitaniae* (HQ693786.1), *C. lusitaniae* (KU729100.1), *C. lusitaniae* (KP131836.1), *C. lusitaniae* (AY174102.1), *C. lusitaniae* (KY102564.1), *C. lusitaniae* MG518174.1), *C. lusitaniae* (MG183704.1), *S. cerevisiae* (JX141369.1), *S. cerevisiae* (JX141368.1), *T. delbrueckii* (FJ838774.1), and *D. hansenii* (JQ912667.1). Evolutionary analyses were conducted in MEGA7

formed by yeast during fermentation by the Ehrlich pathway, involving amino acid degradation, particularly Phe (Hazelwood et al. 2008; Styger et al. 2011) but can also be synthesized from glucose via pyruvate (Cordente et al. 2012). The Ehrlich pathway involves three steps: phenylpyruvate is decarboxylated to phenylacetaldehyde, then reduced to 2-PE (Etschmann and Schrader 2006), and finally esterified to 2-PEA.

The production of volatile organic compounds (2-PE and 2-PEA) presented significant statistical differences between by non-*Saccharomyces* yeasts is shown in Table 3.The most productive yeasts, in terms of 2-PE, were as follows: *Kluyveromyces marxianus* (ITD0090, ITD0091), as well as the yeasts *Kluyveromyces* sp. (ITD0046, ITD0089 and ITD0040), *Kluyveromyces dobzhanskii* ITD0157, and

Table 2 Volatile metabolites produced by non-*Saccharomyces* strains

Metabolite	RT	*m/z*	Sensorial description
Ethyl acetate	0.95	61-70-73-88	Pineapple, sweet, and fruit
Isoamyl acetate	4.04	55-70-87	Banana, sweet and fruit
Isoamyl alcohol	6.24	55-70-87	Alcohol, banana, and malt
Benzaldehyde	16.36	51-77-106	Almond, burnt sugar, cherry, and sweet
2-Phenylethylacetate	22.72	121-104-91-77	Floral, fruit, honey, and roses
2-Phenylethyl butyrate	23.68	117-104-91-77-71-65	Yeast, strawberry, floral, and sweet
Phenylethyl propionate	23.79	104-91-77-57	Floral, red fruit, and honey
2-Phenylethanol	24.22	103-91-77-65-51	Roses, fresh, and leafy

Clavispora lusitaniae ITD0107. *K. marxianus* yeast ITD0090 can be classified as the largest producer of 2-PE, even when compared with the most studied yeast *K. marxianus* CBS600 (Table 3). The concentrations obtained were similar to that reported by Etschmann et al. (2003) (890 mg/L in a molasses-based medium). Eshkol et al. (2009) evaluated the potential of stress-tolerant *Saccharomyces* strains to produce 2-PE under inductive conditions (Phe addition) and detected the concentrations between 340 and 460 mg/L at 48-h incubation, but these concentrations increased to 540 and 850 mg/L with selected yeast, when conditions were optimized with 10 g/L Phe addition, which were very similar quantities to those reported here, without the optimization process. In a related study, with the *K. marxianus* strain CBS6556, the optimization of the grape must culture medium with 3 g/L of L-Phe improved the 2-PE titer of 0.39 g/L after 84 h of culture to 0.47 g/L (Garavaglia et al. 2007). Mei et al. (2009) also used a yeast *Saccharomyces cerevisiae* BD and reported *in situ* product adsorption techniques, to obtain a better performance regarding the biotransformation of L-phenylalanine to 2-phenylethanol, reaching a concentration of 4.65 g/L of 2-PE with a content of 10 g/L of L-Phe in the medium. Chreptowicz et al. (2016) with yeast not genetically modified strain *Saccharomyces cerevisiae* JM2014 was isolated from a fermented milk drink (Turkey), producing a total concentration of 3.60 g/L of 2-PE after 72-h incubation at 30 °C batch culture with a medium containing 5 g/L of L-Phe in a 4-L bioreactor at laboratory scale. Recently, De Lima et al. (2018) evaluated the potential of yeast strain *K. marxianus* CCT 7735 in the 2-PE production and reported a production of 2.47 g/L of 2-PE, with the optimization in the medium through the optimal conditions achieving thus a production of 3.44 g/L of 2-PE. Lu et al. (2016) showed the 2-PE titer in a batch fermentation with the stress-tolerant yeast *Candida glycerinogenes* WL2002-5, reaching 5 g/L from L-Phe, under optimized culture conditions. Genetic modification strategies have also been considered, to further increase 2-PE production, such as *ARO8* and *ARO10* overexpression in *S. cerevisiae* SPO810 yeast the 2-PE reached 2.61 g/L after 60 h of cultivation (Yin et al. 2015).

Chreptowicz et al. (2018) reported yeast strains capable of producing over 2 g/L 2-PE through the L-Phe biotransformation in standard medium for 72-h batch cultures. *Clavispora lusitaniae* WUT17 strain reached the levels of 2.04 g/L of 2-PE in a standard medium and 0.95 g/L of 2-PE in an organic waste-based medium, which is superior to the one reported by Etschmann et al. (2003) of 0.33 g/L. It is well known that 2-PE synthesis is carried out by the Ehrlich pathway in yeast, such as *K. marxianus* and *Yarrowia lipolytica* (Fabre et al. 1998; Celińska et al. 2013). In a recent study, González et al. (2018) screened the 2-PE production potential of some non-*Saccharomyces* yeasts and discovered a 2-PE productive yeast (*T. delbrueckii*). However, in all cases, non-*Saccharomyces* species produce lower quantities than *S. cerevisiae*, indicating that the Ehrlich pathway may not be as active in non-*Saccharomyces* species as in *Saccharomyces*, at least under nitrogen-limiting conditions. Rutiaga-Quiñones et al. (2012) revealed the non-*Saccharomyces* yeasts potential for volatile compounds, particularly in A. *duranguensis* juice for mescal production; in this study, the strains *T. delbrueckii* ITD0110 and *K. marxianus* ITD0211 showed to be more productive of 2-PE under nitrogen-limiting conditions that the strain *S. cerevisiae* ITD0109. A possible theory for our observations, when the fermentations of different strains induced with L-Phe as the only source of nitrogen were evaluated, is that the Ehrlich route is working on these strains, but the metabolic plasticity differs for each of the strains studied. These results allow to raise genetic and biochemical differences between the strains of wine production and mescal, but additional studies are required to elucidate and describe them.

The Table 3 illustrates that 2-PEA production presents significant difference for each strain where that highlighting the *K. marxianus* strains (ITD0040, ITD0090, ITD0102, and ITD0211). A previous research by Rojas et al. (2001) described a very productive *H. guilliermondii* yeast, with a 2-PEA production of 83.83–163.8 mg/L, when using 2-PE as induction conditions and in the presence of extraction solvent. The present results describe a difference in the production potential from L-Phe induction, among all the strains studied, highlighting two strains, *K. marxianus* ITD0090 and *K. marxianus* ITD0211, due to the potential to overproduce 2-PE and 2-PEA, respectively. Etschmann et al. (2005)

Table 3 Production 2-phenylethanol and 2-phenylethylacetate obtained by different yeast strains non-*Saccharomyces* by HS-SPME

Species	ID strains	Production of 2-PE (mg/L)		ID Strains	Production of 2-PEA (mg/L)	
		Mean	S.d		Mean	S.d
Clavispora lusitaniae	ITD0107	764.80	$62.64^{a–f}$	ITD0095	22.43	$2.55^{h–j}$
	ITD0095	636.60	$47.41^{b–g}$	ITD0107	17.17	$4.52^{h–j}$
	ITD0132	511.89	$2.23^{d–j}$	ITD0104	13.16	$1.14^{i,j}$
	ITD0104	394.74	$42.54^{g–j}$	ITD0099	12.14	$0.29^{i,j}$
	ITD0099	370.89	$10.13^{g–j}$	ITD0132	11.89	$0.19^{i,j}$
	ITD0103	357.54	$29.41^{g–j}$	ITD0103	9.20	1.06^{j}
Kluyveromyces marxianus	ITD0090	1024.46	306.38^{a}	ITD0211	203.53	3.52^{a}
	ITD0091	848.37	$112.28^{a–c}$	ITD0102	202.07	26.28^{a}
	ITD0211	630.81	$7.13^{b–h}$	CBS600	166.24	$0.40^{a–c}$
	ITD0102	618.88	$46.90^{b–h}$	ITD0091	136.49	$28.48^{a–d}$
	ITD0093	596.41	$26.16^{b–i}$	ITD0128	134.27	$13.52^{a–e}$
	ITD0092	564.33	$1.90^{c–i}$	ITD0264	108.66	$5.95^{b–f}$
	ITD0069	507.95	$33.82^{e–j}$	ITD0002	108.54	$24.08^{b–f}$
	ITD0268	446.14	$29.03^{f–j}$	ITD0069	89.36	$2.79^{d–h}$
	ITD0145	436.08	$28.34^{g–j}$	ITD0142	86.96	$11.79^{d–i}$
	ITD0142	391.87	$3.21^{g–j}$	ITD0092	79.61	$0.47^{d–j}$
	ITD0041	383.35	$2.05^{g–j}$	ITD0268	71.79	$12.81^{d–j}$
	ITD0128	380.32	$3.41^{g–j}$	ITD0145	71.30	$4.80^{d–j}$
	ITD0003	371.87	$19.70^{g–j}$	ITD0141	60.54	$2.56^{e–j}$
	ITD0141	310.26	$5.96^{h–j}$	ITD0093	56.74	$4.87^{f–j}$
	ITD0002	273.18	$63.30^{i,j}$	ITD0003	12.10	$1.85^{i,j}$
	ITD0264	217.89	3.97^{j}	ITD0041	107.02	$4.44^{b–f}$
	CBS600*	806.30	$55.86^{a–e}$	ITD0090	177.36	$64.88^{a,b}$
Kluyveromyces sp.	ITD0046	901.78	$126.87^{a,b}$	ITD0040	165.34	$28.25^{a–c}$
	ITD0089	832.62	$60.34^{a–d}$	ITD0089	133.57	$22.87^{a–e}$
	ITD0040	804.55	$9.92^{a–e}$	ITD0062	131.25	$2.70^{a–f}$
	ITD0137	643.05	$22.86^{b–g}$	ITD0046	116.48	$6.13^{b–f}$
	ITD0049	559.25	$47.84^{c–i}$	ITD0136	98.46	$9.66^{c–g}$
	ITD0037	516.94	$51.03^{d–j}$	ITD0037	91.82	$15.70^{c–h}$
	ITD0048	492.63	$4.10^{e–j}$	ITD0049	67.32	$9.22^{d–j}$
	ITD0136	448.58	$19.08^{f–j}$	ITD0048	62.72	$7.84^{d–j}$
	ITD0062	442.38	$24.29^{f–j}$	ITD0137	59.89	$6.05^{e–j}$
Kluyveromyces dobzhanskii	ITD0157	789.36	$55.07^{a–e}$	ITD0157	122.41	$9.00^{b–f}$
Saccharomyces cerevisae	ITD0109	491.38	$70.73^{e–j}$	ITD0109	8.97	0.37^{j}
Torulaspora delbruekii	ITD0110	676.55	$27.27^{b–g}$	ITD0110	25.50	$7.37^{g–j}$
	ITD0129	506.33	$23.75^{e–j}$	ITD0129	20.18	$2.48^{h–j}$

Media with the same letter are not significantly different according to the HSD–Tukey–Kramer comparison test ($\alpha = 0.01$)

described that the yeast *K. marxianus* CBS600 produced 1.3 g/L of 2-PEA, and a maximum of 4 g/L, using an organophilic pervaporation technique for continuous in situ product removal (ISPR) in a previously optimized medium. The production of 2-PEA by starter of *Hanseniaspora vinae–Saccharomyces cerevisiae* has been reported, where the concentration was 0.81 to 1.70 mg/L in wine; 2-phenylethyl acetate levels in wine vary from traces to 0.96 mg/L whereas its aroma threshold is around 0.25 mg/L (Viana et al. 2011). Also, a patent has been granted for the production of 2-PEA in anaerobic conditions with the *Kluyveromyces marxianus* KY3 strain with a production of 435 mg/L (Chang et al. 2014). Recently, it has been reported solid-state fermentation processes (SSF) for 2-PEA and 2-PE production using agroindustrial residue sugarcane bagasse as sole carbon source for the biotransformation of L-phenylalanine using

Kluyveromyces marxianus strain as inoculum, showing effective results as in other systems submerged fermentation (Martínez et al. 2018).

Guo et al. (2017) designed and expressed a 2-PEA biosynthetic pathway in *E. coli* and in shake flask cultures with L-Phe (1 g/L) and recorded the generation of 268 mg/L of 2-PEA. This amount is very similar to the one reported in yeast ITD 00211, highlighting that the production by the strains of this study has not gone through the process of optimization.

Conclusions

This study presents preliminary evidence of differences between non-*Saccharomyces* yeasts found during fermentation of *A. durangensis* for the production of mescal. Particularly, *Kluyveromyces* yeasts have a high variability among them with respect to the production of volatile organic compounds, where it was evidenced that these have the extraordinary potential to produce aromas, particularly, 2 PE and 2 PEA.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animal This article does not contain any studies with human or animal.

Informed consent Not applicable.

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