Folia Microbiologica (2018) 63:685–693
https://doi.org/10.1007/s12223-018-0607-2

ORIGINAL ARTICLE

MALDI-TOF MS Supplementary database for species identification employing the yeast diversity encountered on southern Brazil grapes

Bruna Carla Agustini[1] · Gildo Almeida da Silva[1] · Tania Maria Bordin Bonfim[2]

Received: 4 August 2017 / Accepted: 25 April 2018 / Published online: 11 May 2018

Abstract

The study of grape microflora is of interest when autochthonous yeasts, which are related to typical wine characteristics, are intended to be used in winemaking. The election of matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) as the first method for yeast identification was based on its accuracy and rapidity compared to alternative laboratory protocols for identification. The aims of this study are to consolidate the MALDI-TOF MS Supplementary database for environmental yeasts already constructed, to expand it through the addition of standard spectra of not included yeast species, and to discuss the grape microflora encountered in Southern Brazil. A total of 358 strains, isolated from grape berries, were submitted to protein profiling employing Biotyper and Supplementary database. Molecular biology techniques were used as alternatives to identify 6.4% of strains not promptly designated by protein profiling. These strains corresponded to the species *Candida californica*, *Zygoascus meyerae*, *Candida akabanensis*, *Candida azyma*, and *Hanseniaspora vineae*. The MALDI-TOF MS spectra of the identified species were added to Supplementary database. The presented results strengthen the need for further expansion of the mass spectra database to broaden its microbiological application.

Introduction

Grape berries surface harbors a diversity of microorganisms including yeasts, molds, and bacteria. The yeast species commonly encountered belong to the genera *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Candida*, *Kluyveromyces*, *Zygossaccharomyces*, and *Saccharomyces* (Fleet 2008; Settanni et al. 2012; Jara et al. 2016). *Saccharomyces cerevisiae* represents the most important species for alcoholic fermentation of grape must, dominating the process as ethanol concentration increases. On first stages of fermentation, non-*Saccharomyces* species are also present and have a great contribution to wine aroma and complexity (Jolly et al. 2014). Winemaking can be conducted in a spontaneous manner, in which only the grape-resident yeasts conduce the process; or vinification can be carried out by inoculated yeasts either commercial or autochthonous one. The use of autochthonous yeast can increase wine typicity and thus regional character (Varela et al. 2009; Orlic et al. 2010). Brazil are investing in delimitating its wine producing areas to reinforce the peculiarities of each region (Ramos and Fernandes 2012) and the research regarding microbial terroir is also an important attribute (Bokulich et al. 2014; Knight et al. 2015). The first geographical indication for Brazilian wines and sparkling wines was achieved in 2002 for Vale dos Vinhedos area, located in Rio Grande do Sul State (Valente et al. 2012).

In this context, the grape microflora of Brazilian wine-producing areas are being studied over the past few years but only *Saccharomyces cerevisiae* strains had been identified, regarding its main importance to winemaking. The remaining strains were cryopreserved without identification concerning their utility for future biotechnological interests. In 2010, these microorganisms started to be incorporated on a Culture Collection belonging to Brazilian Agricultural Research Corporation (EMBRAPA). In that period, all the species started to be identified before entering the Collection.

The election of MALDI-TOF MS as the main method for yeast identification was based on its accuracy and rapidity compared to alternative laboratory protocols for identification (Marklein et al. 2009; Pan et al. 2011). Moreover, a robust Supplementary database for protein profiling is being constructed focusing on environmental yeasts by our research

✉ Bruna Carla Agustini
brunagustini@gmail.com

[1] Laboratory of Applied Microbiology, Embrapa Grape and Wine, Bento Gonçalves, RS, Brazil

[2] Laboratory of Enzymology and Fermentation Technology, Pharmacy Department, Federal University of Paraná, Curitiba, PR, Brazil

team (Agustini et al. 2014) in order to complement the Biotyper database (Bruker Daltonics, Bremen, Germany).

Environmental yeast species and their identification may set a challenge when the type strains are not included in the MS database, leading to their misidentification as related species (Marklein et al. 2009; van Veen et al. 2010). It is worth noting that species identification relies on the comparison of the obtained spectrum from an unknown isolate with a previously available database. As commercially databases comprise mostly clinically relevant microorganisms, studies focusing on environmental isolates are required to extend such databases. When a robust database is employed, the percentage of identification has been enhanced (Christensen et al. 2012; Lau et al. 2013).

The aims of this study are to consolidate the MALDI-TOF MS Supplementary database already constructed, to expand it through the addition of new species spectra and to discuss the grape microflora encountered in Southern Brazil.

Material and methods

Yeast strains and culture conditions

Three hundred fifty-eight strains were isolated from eight samples collected in the vineyards located in Rio Grande do Sul State on five harvests from cultivars Cabernet Sauvignon, Cabernet Franc, Tannat, and Riesling Italico (Table 1).

Yeast isolation was performed by crushing ripened grapes in closed and previously sterilized plastic bags. On sterile conditions, grape juice was diluted to 10^{-4} and 10^{-5} with sterile distilled water. One hundred microliters of the dilutions were plated on must agar (250 mL/L Lorena grape must; 10 g/L yeast extract; 20 g/L agar). The plates were incubated at 28 °C for 72 h. For each colony type, one or more representative colonies were subcultured individually.

The pure cultures are deposited in Yeast Culture Collection (WDCM 1056) of the National Center for Research on Grapes and Wine (Bento Gonçalves, RS, Brazil) belonging to Brazilian Agricultural Research Corporation (EMBRAPA). An electronic system named Alelo was created to manage all the culture collections of this institution and to make this database available for the international community (http://alelomicro.cenargen.embrapa.br/InterMicro/Home/index.xjs). Each strain that enters the Alelo Catalogue receives a universal code comprising the acronym BRM followed by a sequential number.

The strains have been stored in cryogenic vials at − 80 °C using 32.5% of glycerol as cryoprotectant. For microorganism reactivation, the cryogenic vials were defrosted at room temperature. Aliquots of 3 μL were transferred to Petri dishes containing YEPD medium (10-g/L yeast extract; 20-g/L peptone; 20-g/L dextrose) and were cultured at 25 °C for 24–48 h.

Identification of yeast strains

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based identification

All strains were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The microorganisms were prepared by the cell extraction method as described previously (Agustini et al. 2014) and the matrix used was CHCA (α-cyano-4-hydroxycinnamic). MALDI-TOF MS analysis of all strains was performed on a MicroFlex LRF mass spectrometer (Bruker Daltonics, Bremen, Germany). The spectra were recorded in the linear positive mode at a laser frequency of 60 Hz within a mass range from *m/z* 2000 to 20,000. For each spectrum, 240 laser shots in 40-shot steps from different positions of the target spot were collected and analyzed. The spectra were externally calibrated by using *Escherichia coli* ribosomal proteins (Bruker Daltonics, Bremen, Germany).

To identify an unknown microorganism, the spectrum acquired was loaded with a database (including bacteria and fungi spectra) and analyzed by the use of a standard pattern-matching algorithm (Biotyper, Bruker Daltonics, Bremen, Germany), which compared the spectrum acquired with those present in the library. FlexAnalysis Software (Bruker Daltonics, Bremen, Germany) was also used for

Table 1 Information about the grape samples from which the yeast strains were isolated, number of isolates per sample and number of species per sample

Grape variety	Sample code (harvest year)	Number of isolates per sample	Number of species per sample
Cabernet Franc	VVT (1997)	33	4
	VVT (1999)	61	8
Riesling Italico	VVB (1997)	31	6
Cabernet Sauvignon	VVT (2002)	68	5
	CNPUV (2002)	55	11
	CSPB (2012)	22	4
Tannat	TPB (2012)	40	5
	TASL (2015)	48	13

visual inspection and mass spectra processing such as smoothing, normalization, baseline subtraction, and peak picking. When appropriate, the MALDI-TOF mass spectra was transformed in pseudo gel-view by mMass software (version 5.5.0) (Strohalm et al. 2008).

For Supplementary database implementation, the Main Spectra Projection (MSP) was created using four replicates of six separated colonies from the species to be included in the user-generated library. The main spectra were generated considering the 24 spectra obtained and are saved apart from the Biotyper database (Bruker Daltonics, Bremen, Germany). A good average is achieved by measurements of a minimum of 20 spectra, according to the manufacturer orientations (Maier et al. 2008). The addition of a given reference strain was made using the "MSP creation" function of the MALDI Biotyper software (version 3.0) comprising Bruker's default parameters (Max. Mass Error of each single spectrum: 2000; Desired Mass Error for the MSP: 200; Desired Peak Frequency Minimum: 25%; Ma. Desired Peak Number for the MSP: 70). This Supplementary database is physically located on Mass Spectrometry Laboratory of Genetic Resources and Biotechnology Unit of EMBRAPA (Brasília, DF, Brazil).

Molecular biology identification

Strains unsuccessfully identified by MALDI-TOF MS analysis were investigated through RFLP technique employing PCR products from the ribosomal region spanning the ITS1 (internal transcribed spacer), the 5.8S rRNA gene, and the ITS2. DNA extractions, PCR, and RFLP experimental conditions were described by Agustini et al. (2014).

To assess the taxonomic identity of the resulting groups of ITS-RFLP used to create the Supplementary database, the PCR product of the D1/D2 region of the large subunit (LSU) of the 26S ribosomal RNA gene of at least one isolate of each group was sequenced. The sequencing primers employed were NL-1 and NL-4 (Kurtzman and Robnett 2003). Sequences were analyzed using Blast search at NCBI (http://www.ncbi.nlm.nih.gov/blast) and were deposited on GenBank (accession numbers are given along the text).

Regarding the species *Candida diversa*, specific primers were designed and named as 28CANDIV3 (5′-CTTTAAGTCTTTACCAAAGAGTCGAGTT-3′) and 28CANDIV4 (5′-CACAATGTGGTTCAAAACTTCTTATC-3′). The PCR mix was the same as for PCR-ITS. Amplifications were carried out using the following PCR condition: 94°C for 5 min followed by 33 cycles of 94°C for 30 s, 66.5°C for 45 s, 72°C for 30 s, and a final step at 72°C for 5 min.

Distinction of species with identical restriction profiles or similar 26S rRNA gene sequences was performed using biochemical reactions (Kreger-van Rij 1984; Barnett et al. 2000) or PCR-RAPD according to previous literature (Smith et al. 2005).

Results

A total of 358 yeast strains were isolated from grape berries from eight samples comprising four different varieties in five vintages years (Table 1).

MALDI-TOF MS employing Biotyper database (version 3.1, Bruker Daltonics, Bremen, Germany), which included standard spectra from bacteria (5302 MSP) and fungi (690 MSP) species, and also the Supplementary database (35 MSP) constructed elsewhere (Agustini et al. 2014) were used as the primary identification methodology and only 6.4% of the strains tested remained without identification. On the other hand, employing exclusively Biotyper database in this first round of identification, 44.7% of the strains would be unidentified, reinforcing the importance of a comprehensive in-house library.

The strains which remained unidentified even with the Supplementary database already constructed were submitted to ITS-RFLP to create groups of similar restriction profiles. From the 23 unidentified strains, five groups were formed. At least one strain of each group was submitted to domains 1 and 2 sequencing of the 26S nuclear ribosomal gene. The BLAST search for these strains pointed to the species *Hanseniaspora vineae* (Genbank accession number: KX421391), *Candida akabanensis* (KP058521), *Candida californica* (KP058519), *Candida azyma* (KX421390), and *Zygoascus meyerae* (KX610375).

Considering the strains promptly identified by MALDI-TOF MS, at least one and at maximum of three strains of each species identified had their identification confirmed by other methods, such as PCR-RFLP, sequencing and/or PCR employing specific primers. This step confirmed the efficacy of MALDI-TOF MS in discriminating the species predicted, since all the species pointed out by the second methodology was compatible with the mass spectrometry previous results. Most of the species identified by protein profiling presented ITS-restriction patterns compatible with the ones encountered in the literature (Guillamón et al. 1998; Esteve-Zarzoso et al. 1999; Bautista-Gallego et al. 2011; Settanni et al. 2012; Agustini et al. 2014); hence, a third identification technique was unnecessary. Exception made to strains of the species *Candida diversa* and *Candida inconspicua*, as explained below.

The analyzed strains of *Candida diversa* have failed in the amplification using universal primers ITS1 and ITS4 probably due to polymorphisms affecting the primer annealing. This inconvenience was already detected in other research (Agustini et al. 2014). Concerning the high frequency of this occurrence between the isolates of *Candida diversa* in

Southern Brazil, a pair of specific primers were constructed using the D1/D2 domain sequence acquired for the strain 1VVT99/BRM 42321 (KJ173775), in order to simplify this identification when amplification of ITS1/ITS4 failed. The expected amplicon size was 292 bp. The specificity of the designed primers was tested in vitro by performing a search on BlastN and when optimizing the PCR conditions, a positive and negative control were always present. Figure 1 demonstrates the amplification in 292 bp of two positive controls that correspond to strains of *Candida diversa*, and expounds the inability of the designed pair of primers to amplify five other species used as negative controls (Fig. 1). Hence, all the strains identified as *Candida diversa* by MALDI-TOF MS had their identity confirmed by the amplification with these specific primers.

Still in the context of confirming MALDI-TOF MS identifications, the two strains identified as *Candida inconspicua* (standard spectrum in Biotyper database acquired from DSM 70631) revealed a restriction pattern not compatible to the one described by Frutos et al. (2004). The strains of the present study have shown a 480-bp ITS-amplicon and the following fragmentation with the endonucleases *Cfo*I (170, 100, 90 bp), *Hae*III (320, 80 bp), and *Hin*fI (280, 200 bp). Since the abovementioned restriction pattern was compatible with the species *Pichia membranifaciens* (Villa-Carvajal et al. 2006; Stringini et al. 2008), D1/D2 domain sequencing was done to resolve this impasse and endorsed the PCR-RFLP result. *P. membranifaciens* (standard spectrum in Biotyper database acquired from CBS 107) was the second best match pointed out by Biotyper database as a possible identification for the strains of the present study. This similarity on protein profiling between *C. inconspicua* and *P. membranifaciens* was carefully investigated and the information collected from the website of DSMZ (German Collection of Microrganisms and Cell Culture) indicated an update on the information received by the depositor concerning the strain DSM 70631, reclassifying it as *P. membranifaciens*. Hence, protein profiling identification was precise; the drawback was on the outdated denomination of the strain present in Biotyper database (version 3.1).

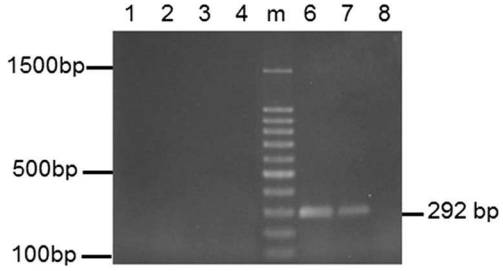


Fig. 1 Electrophoretic profile of amplicons resulting from designed primers 28candiv3 and 28candiv4. Line 1: *Issatchenkia terricola* 24VVT97/BRM 41725; line 2: *Hanseniaspora uvarum* 15VVB97/BRM 41705; line 3: *Issatchenkia hanoiensis* 59VVT99/BRM 42356; line 4: *Pichia galeiformis* 14TASL15/BRM 33362; line m: 100 bp ladder; line 6: *Candida diversa* 1VVT99/BRM 42321; line 7: *Candida diversa* 44TASL15/BRM 36498; line 8: *Saccharomyces cerevisiae* 1VVT97/BRM 4687

All yeast strains isolated from the eight grape samples analyzed belong to 22 different species that are described in Table 2 along with their frequency and their relative abundance. *Hanseniaspora uvarum* and *Issatchenkia terricola*, both with a frequency of 75%, were the most frequent species on the studied samples, followed by *Hanseniaspora opuntiae* and *Saccharomyces cerevisiae*. Regarding the relative abundance, the species *Pichia myanmarensis* followed by *Hanseniaspora uvarum* were the most abundant among the samples.

Discussion

The 22 different species recovered in this study are predominant yeasts on grape microflora (Fleet 2003; Barata et al. 2012; Jara et al. 2016) with the exception of *Pichia galeiformis*, *Pichia myanmarensis, Candida akabanensis*, and *Candida azyma*. *Pichia galeiformis* was found on table olive (Rodríguez-Gómez et al. 2010; Bautista-Gallego et al. 2011). *Pichia myanmarensis* was reported as inhabitant of sugar palm (Nagatsuka et al. 2005). *Candida akabanensis* was first isolated from insect frass in bark of a grapevine (Nakase et al. 1994) and was also encountered in sugarcane leaf (Limtong et al. 2014). *Candida azyma* was first reported to be associated with the sugarcane crop (de Azeredo et al. 1998) and more recently was retrieved from grape berries of Bangalore blue and Cabernet varieties in India (Chavan et al. 2009).

From the 358 yeast strains isolated and analyzed, 93.6% have been successfully identified by MALDI-TOF MS. All the strains identified by mass spectrometry have showed a log-score > 1.9 for identification while the value that is considered for the species identification boundary by manufacturer recommendations is > 2.0. However, the log score values at 1.9 has been indicated as reliable in previous work (Agustini et al. 2014), when employing only the manufacturer database. Moreover, Stevenson et al. (2010) affirmed that all organisms that had a spectral score of 1.8 or greater has been successfully identified at species level. Pinto et al. (2011) also pointed out that all the genus identification (> 1.7 and < 2.0) in their study also resolved isolates to the correct species designations.

It is worth noting that the Supplementary database have favored the achievement of log scores values above 2.0. Considering the first 30 strains of *Saccharomyces cerevisiae* identified in this work employing Biotyper database, 57% of them achieved log scores above 2.0, while the others 43% resulted in values between 1.9 and 2.0. When the same spectra were confronted to the Supplementary database, where one of our strains, named 1VVT97, was added as a standard

Table 2 Species encountered on the eight analyzed grape samples with their frequency and mean relative abundance. The presence of the species on Biotyper or Supplementary databases are also indicated

Species	Biotyper database	Supplementary database	Frequency* (%)	Mean relative abundance (%)**
[illegible]		x	38	2.0
2. Candida apicola		x	25	3.4
[illegible]		x	13	0.3
[illegible]		x	25	1.1
5. Candida diversa		x	50	7.0
6. Pichia membranifaciens	x		25	0.6
7. Pichia occidentalis	x		13	0.3
8. Starmerella bacillaris		x	13	0.8
9. Hanseniaspora opuntiae	x	x	63	3.9
10. Hanseniaspora uvarum	x		75	20.1
11. Issatchenkia hanoiensis		x	13	0.8
12. Issatchenkia terricola	x	x	75	9.2
13. Metschnikowia pulcherrima	x		13	0.8
14. Issatchenkia orientalis	x		13	2.8
15. Pichia galeiformis/manshurica	x		25	2.5
16. Meyerozyma guillermondii	x		40	2.8
17. Pichia myanmarensis		x	50	26.3
18. Saccharomyces cerevisiae	x	x	63	10.1
[illegible]		x	25	2.0
20. Zygossaccharomyces bisporus	x		13	0.8
21. Zygossaccharomyces bailii	x		13	1.4
[illegible]		x	38	1.1

*Frequency = (number of samples containing a certain species/number of grape samples)×100

**Mean relative abundance = number of isolates from a certain species/total number of isolates

Species in bold were included in Supplementary database in the present study

spectrum for *Saccharomyces cerevisiae*, 90% of the 30 strains achieved log scores above 2.0. Christensen et al. (2012) also have demonstrated the increase in the log score values after incrementing the manufacturer database. In the same context, Lau et al. (2013) have developed a clinically comprehensive database for filamentous fungi identification and noted that, when employing an in-house database, the log score boundary established by the manufacturer could be maintained above 2.0 for species designation.

Considering the strains identified promptly by MALDI-TOF MS employing Biotyper and Supplementary database already constructed (Agustini et al. 2014), a confirmation stage was performed with at least one and a maximum of three strains from the 17 species designated. PCR-RFLP confirmed all the identifications except for the species *Candida diversa* that failed on the ITS1/ITS4 amplification, as abovementioned.

Regarding the 23 strains (6.4%) that remained without identification by protein profiling, the technique of ITS-RFLP was applied to gather them according to their restriction patterns and posterior D1/D2 26S rDNA sequencing of at least one strain of each cluster was performed. In Table 3, the closest match resultant of BlastN search are presented. All the species identified demonstrated similarity > 98%, boundary considered appropriate for identification purposes (Kurtzman and Robnett 2003; Gayevskiy and Goddard 2012).

The ITS-RFLP method is a broadcast technique for the identification of yeast species (Guillamón et al. 1998; Esteve-Zarzoso et al. 1999; Pham et al. 2011). This technique has a low cost when compared to sequencing, but has a disadvantage due to the frequent intraspecies variability and to the similar patterns relating to species with close genetic proximity. Regarding this last issue, the species *Zygoascus meyerae* had its RFLP profile not encountered in the literature for comparison; however, its fragmentation was almost identical to the fragmentation of *Zygoascus hellenicus* (Barata et al. 2008) when endonucleases *Cfo*I, *Hae*III, and *Hin*fI were employed. D1/D2 sequencing of the strain 13CNPUV02/BRM 42309 resulted in similar matches for both *Z. meyerae* and *Z. hellenicus* (Table 3). According to Smith et al. (2005), these species comprise the *Zygoascus* clade and could not be distinguished physiologically either. These authors suggest

Table 3 Size in bp of the ITS-PCR products, the restriction fragments of the species present in the yeast collection analyzed and closest matches from FASTA searches

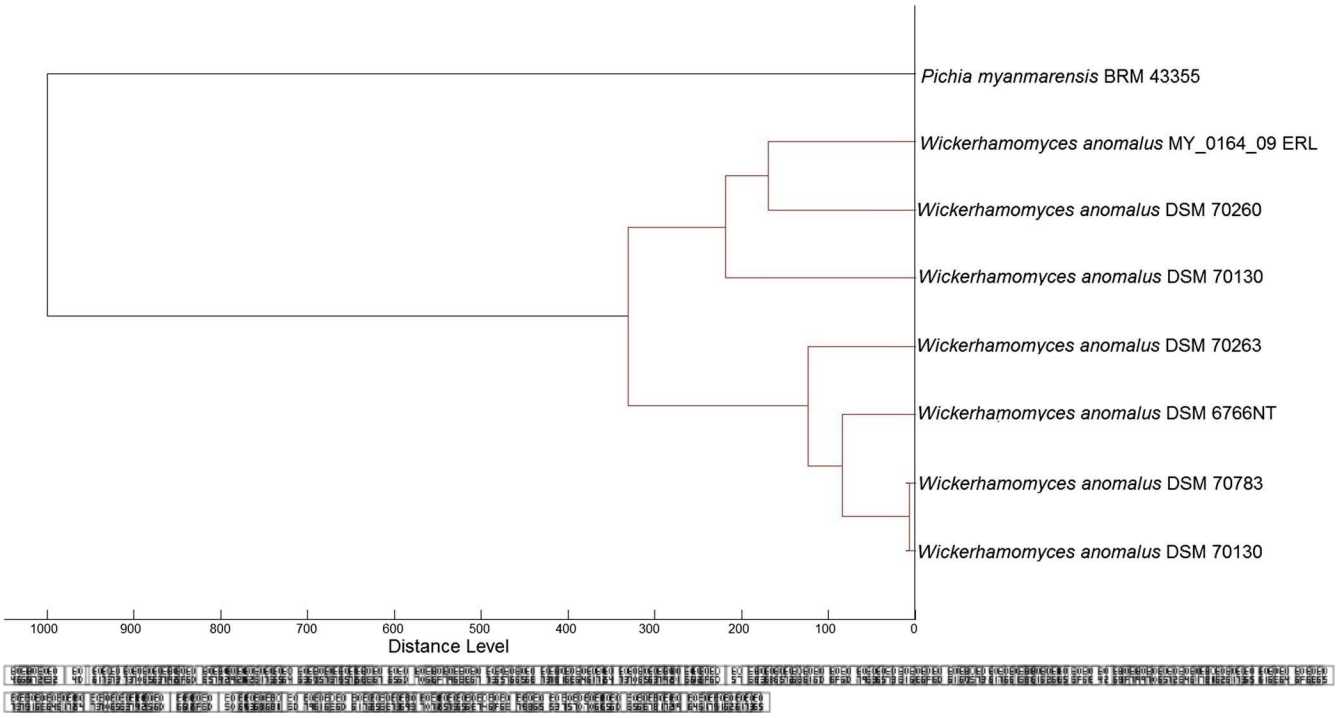
Isolates (accession numbers)	ITS-PCR sizes (bp)	Size of restriction fragments (bp) *Cfo*I *Hae*III *Hin*fI			Identity/query cover (%)	Closest match (accession number)
Candida akabanensis 33VVT99/BRM 42307 (KP058521)	390	200, 180	390	200, 190	99/100	*Candida akabanensis* (EU100744.1)
					98/96	*Candida dosseyi* (FJ614694.1)
					93/98	*Candida blattae* (FJ614695.1)
Candida azyma 11TASL15/BRM 41458 (KX421390)	450	195, 185	450	230, 200	99/99	*Candida azyma* (JN004198.1)
Candida californica 75VVT02/BRM 42308 (KP058519)	430	220, 90, 50, 40	330, 80	230, 200	99/100	*Candida californica* (JN615563.1)
Zygoascus meyerae 13CPUV02/BRM 42309 (KX610375)	650	320, 320	630	340, 170, 130	100/99	*Zygoascus meyerae* (KT175535.1)
					100/98	*Zygoascus hellenicus* (AB719056.1)
Hanseniaspora vineae 20TASL15/BRM 40909 (KX421391)	750	280, 160, 140	690	390, 380	99/100	*Hanseniaspora vineae* (KU316707.1)

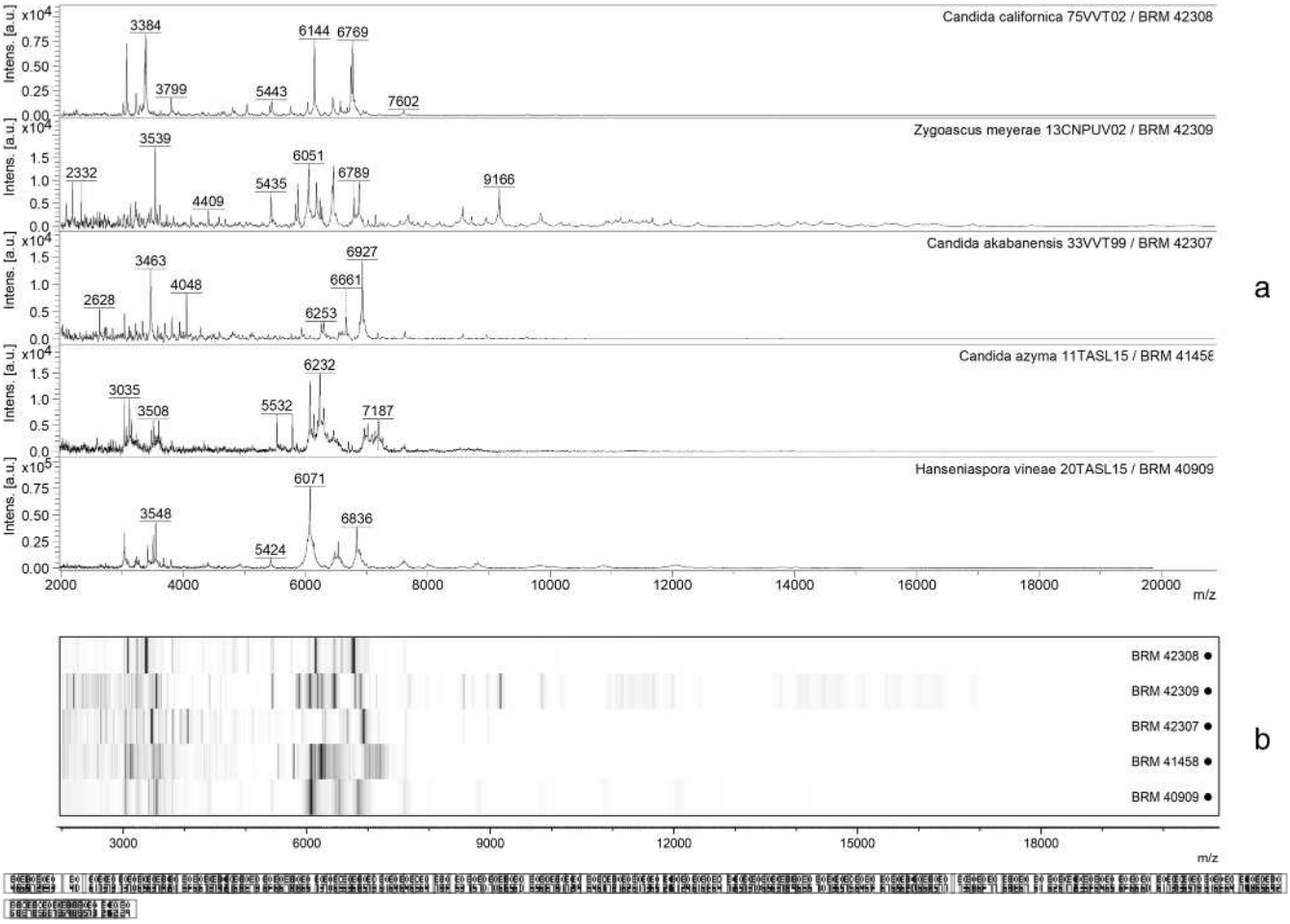
PCR-RAPD as a possible alternative for the differentiation of these species. Employing primer M13, the strains analyzed in the present study resulted in similar pattern (data not shown) as the strains of *Z. meyerae* CBS 6173, CBS 8425, and CBS 7115 presented by Smith et al. (2005), corroborating the best match on sequencing results.

Pichia myanmarensis is a new species described by Nagatsuka et al. (2005) that has demonstrated high similarity with *Wickerhamomyces anomalus* concerning to 26S rDNA D1/D2 domain sequencing. This statement was evidenced when evaluating the D1/D2 sequence of our strain 7CNPUV02/BRM 43355 (KJ173777) which showed 100% of identity with entry *P. myanmarensis* (ABI126678.1) and 99% with the entry *W. anomalus* (EF694618.1). Besides the similarity encountered on D1/D2 domain, the ITS-RFLP profiles of these species were divergent especially concerning to the cleavage from endonuclease *Hin*fI. *P. myanmarensis* presented fragments on 320, 180, and 120 bp (Agustini et al. 2014) and *W. anomalus* has showed a double fragment on 315 bp (Stringini et al. 2008; Lv et al. 2013). Regarding phenotypic approaches, the MALDI-TOF MS protein profiling technique has showed a greater capability of distinguishing both species, as demonstrated on the dendrogram based on protein mass patterns (Fig. 2). In this figure, the relatedness is clear between the mass signals of the spectra for the seven strains of *W. anomalus* present on Biotyper database; but all of them showed a completely different mass signal pattern when compared to the standard mass spectrum obtained for *P. myanmarensis* that was included in the Supplementary database previously (Agustini et al. 2014). This outcome reinforces the advantages of using MALDI-TOF MS for species differentiation. Beyond all the evidence pointing out for the *P. myanmarensis* strain, biochemical tests had also been performed to guarantee the identity of the species. The strains of *P. myanmarensis* showed ability to assimilate D-arabinose and ability to grow at 40 °C, characteristics that distinguished it from *W. anomalus* (Nagatsuka et al. 2005).

Zygoascus meyerae had its ITS-RFLP profile described for the first time in this study, the same holds for the species *Candida akabanensis*, *Candida californica*, and *Candida azyma* (Table 3).

When the BlastN analysis was performed with the strain 33VVT99, it has showed 99% of identity with the species *Candida akabanensis* with 100% of query cover; it has also revealed 98% of similarity for the species *Candida dosseyi* ATCC MYA-4359 with 98% of query cover; and 93% of identity with the species *Candida blattae* ATCC MYA-4360 with 98% of query cover. These results were not unexpected, Ribeiro et al. (2011) and Sipiczki (2011) have already showed the genetic similarity between these three species. These similarities were also reflected on MALDI-TOF MS analysis. The strain *C. blattae* CBS 9871T, present in Biotyper database, has also been indicated as a second best match for the seven strains belonging to *C. akabanensis* species. Strains of the species *C. dosseyi* were absent in Biotyper database (version 3.1) to allow phenotypic comparison. This outcome reinforces that the similarity between *C. akabanensis* and *C. blattae* are verified both genetically and phenotypically, as the identification by mass spectrometry technique are mainly due to the analysis of ribosomal proteins (Mehta and Silva 2015). The insertion of more entries belonging to these three similar species would be interesting to search for biomarkers for each species, allowing a more discriminatory protein-profiling identification.


Pichia myanmarensis BRM 43355
Wickerhamomyces anomalus MY_0164_09 ERL
Wickerhamomyces anomalus DSM 70260
Wickerhamomyces anomalus DSM 70130
Wickerhamomyces anomalus DSM 70263
Wickerhamomyces anomalus DSM 6766NT
Wickerhamomyces anomalus DSM 70783
Wickerhamomyces anomalus DSM 70130
1000 900 800 700 600 500 400 300 200 100 0
Distance Level


Candida californica 75VVT02 / BRM 42308
3384 6144 6769 3799 5443 7602
Zygoascus meyerae 13CNPUV02 / BRM 42309
3539 2332 6051 6789 5435 9166 4409
Candida akabanensis 33VVT99 / BRM 42307
3463 6927 6661 4048 2628 6253
Candida azyma 11TASL15 / BRM 41458
6232 3035 3508 5532 7187
Hanseniaspora vineae 20TASL15 / BRM 40909
6071 3548 6836 5424
Intens. [a.u.]
2000 4000 6000 8000 10000 12000 14000 16000 18000 20000
m/z
a
BRM 42308
BRM 42309
BRM 42307
BRM 41458
BRM 40909
3000 6000 9000 12000 15000 18000
m/z
b

The strains that MALDI-TOF MS were not capable of identifying in the first attempt belonged to absent species either on the manufacturer or supplementary database. Once the strains were identified by molecular biology techniques, a standard spectrum of each species was added to the Supplementary database to expand it. After that, all the strains analyzed were tested for a second time against the incremented database and were correctly designated. Each species has showed a particular protein profile in the mass spectra acquired that permits its differentiation from the other added species (Fig. 3A). When the mass spectra are transformed in pseudo gel-view (barcode), it becomes easier to verify the differences in *m*/*z* ratios of the different species added to Supplementary database (Fig. 3B).

Protein profiling by MALDI-TOF MS has been proved to be an accurate and fast technique for microorganism identification once the microorganisms tested belong to a species already included in the database. The continual expansion of the mass spectra database is desirable to consolidate the strengths and weaknesses of this technique. It has been demonstrated here that mass spectrometry was crucial for the identification of the species *P. myanmarensis*. On the other hand, *C. akabanensis* presented mass fingerprint too similar to *C.blattae* requiring more refined analysis to its differentiation.

Acknowledgments The authors gratefully acknowledge Dr. Carlos Bloch and Dr. Luciano Paulino Silva for their assistance in mass spectrometry.

Compliance with ethical standards

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

References

Agustini BC, Silva LP, Bloch C Jr, Bonfim TM, da Silva GA (2014) Evaluation of MALDI-TOF mass spectrometry for identification of environmental yeasts and development of supplementary database. Appl Microbiol Biotechnol 98:5645–5654

Barata A, Malfeito-Ferreira M, Loureiro V (2012) The microbial ecology of wine grape berries. Int J Food Microbiol 153:243–259. https://doi.org/10.1016/j.ijfoodmicro.2011.11.025

Barata A, Seborro F, Belloch C, Malfeito-Ferreira M, Loureiro V (2008) Ascomycetous yeast species recovered from grapes damaged by honeydew and sour rot. J Applied Microbiol 104:1182–1191. https://doi.org/10.1111/j.1365-2672.2007.03631.x

Barnett JA, Payne RW, Yarrow D (2000) Yeasts: characteristics and identification, 3rd edn. Cambridge University Press, Cambridge

Bautista-Gallego J, Rodríguez-Gómez F, Barrio E, Querol A, Garrido-Fernández A, Arroyo-López FN (2011) Exploring the yeast biodiversity of green table olive industrial fermentations for technological applications. Int J Food Microbiol 147:89–96. https://doi.org/10.1016/j.ijfoodmicro.2011.03.013

Bokulich NA, Thorngate JH, Richardson PM, Mills DA (2014) Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. P Natl Acad Sci USA 111:E139–E148. https://doi.org/10.1073/pnas.1317377110

Chavan P, Mane S, Kulkarni G, Shaikh S, Ghormade V, Nerkar DP, Shouche Y, Deshpande MV (2009) Natural yeast flora of different varieties of grapes used for wine making in India. Food Microbiol 26:801–808. https://doi.org/10.1016/j.fm.2009.05.005

Christensen JJ, Dargis R, Hammer M, Justesen US, Nielsen XC, Kemp M (2012) Matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis of Gram-positive, catalase-negative cocci not belonging to the *Streptococcus* or *Enterococcus* genus and benefits of database extension. J Clin Microbiol 50:1787–1791. https://doi.org/10.1128/JCM.06339-11

de Azeredo LA, Gomes EA, Mendonça-Hagler LC, Hagler AN (1998) Yeast communities associated with sugarcane in Campos, Rio de Janeiro, Brazil. Int Microbiol 1:205–208

Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A (1999) Identification of yeasts by RFLP analysis of the 5.85 rRNA gene and the two ribosomal interna transcribed spacers. Int J Syst Bacteriol 49:329–337

Fleet GH (2003) Yeast interactions and wine flavour. Int J Food Microbiol 86:11–22. https://doi.org/10.1016/s0168-1605(03)00245-9

Fleet GH (2008) Wine yeasts for the future. FEMS Yeast Res 8:979–995

Frutos RD, Fernández-Espinar MT, Querol A (2004) Identification of species of the genus *Candida* by analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Anton van Leeuw 85:175–185

Gayevskiy V, Goddard MR (2012) Geographic delineations of yeast communities and populations associated with vines and wines in New Zealand. ISME J 6:1281–1290

Guillamón JM, Sabaté J, Barrio E, Cano J, Querol A (1998) Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Arch Microbiol 169:387–392

Jara C, Laurie F, Mas A, Romero J (2016) Microbial terroir in Chilean valleys: diversity of non-conventional yeast. Front Microbiol 7:1–10. https://doi.org/10.3389/fmicb.2016.00663

Jolly NP, Varela C, Pretorius IS (2014) Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res 14:215–237. https://doi.org/10.1111/1567-1364.12111

Knight S, Klaere S, Fedrizzi B, Goddard MR (2015) Regional microbial signatures positively correlate with differential wine phenotypes: evidence for a microbial aspect to terroir. Sci Rep 5:14233. https://doi.org/10.1038/srep14233

Kreger-van Rij NJW (1984) The yeasts: a taxonomic study, 3.Ed edn. Elsevier, Amsterdam

Kurtzman CP, Robnett CJ (2003) Phylogenetic relationships among yeasts of the '*Saccharomyces* complex' determined from multigene sequence analyses. FEMS Yeast Res 3:417–432

Lau AF, Drake SK, Calhoun LB, Henderson CM, Zelazny AM (2013) Development of a clinically comprehensive database and a simple procedure for identification of molds from solid media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 51:828–834. https://doi.org/10.1128/JCM.02852-12

Limtong S, Kaewwichian R, Yongmanitchai W, Kawasaki H (2014) Diversity of culturable yeasts in phylloplane of sugarcane in Thailand and their capability to produce indole-3-acetic acid. World J Microbiol Biotechnol 30:1785–1796

Lv X-C, Huang X-L, Zhang W, Rao P-F, Ni L (2013) Yeast diversity of traditional alcohol fermentation starters for Hong Qu glutinous rice wine brewing, revealed by culture-dependent and culture-independent methods. Food Control 34:183–190. https://doi.org/10.1016/j.foodcont.2013.04.020

Maier T, Schwarz G, Kostrzewa M (2008) Microorganism identification and classification based on MALDI-TOF MS fingerprinting with MALDI Biotyper. Bruker Daltonics, Germany

Marklein G, Josten M, Klanke U, Muller E, Horre R, Maier T, Wenzel T, Kostrzewa M, Bierbaum G, Hoerauf A, Sahl HG (2009) Matrix-assisted laser desorption ionization–time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates. J Clin Microbiol 47:2912–2917

Mehta A, Silva LP (2015) MALDI-TOF MS profiling approach: how much can we get from it? Front Plant Sci 6:184

Nagatsuka Y, Kawasaki H, Seki T (2005) *Pichia myanmarensis* sp. nov., a novel cation-tolerant yeast isolated from palm sugar in Myanmar. Int J Syst Evol Microbiol 55:1379–1382. https://doi.org/10.1099/ijs.0.63558-0

Nakase T, Suzuki M, Takashima M, Rosadi D, Hermosillo AM, Komagata K (1994) *Candida akabanensis*, a new species of yeast isolated from insect frass in a bark of a grape vine. Microbiol Cult Coll 10:35–43

Orlic S et al (2010) Diversity and oenological characterization of indigenous Saccharomyces cerevisiae associated with [illegible]lavka grapes. World J Microbiol Biotechnol 26:1483–1489. https://doi.org/10.1007/s11274-010-0323-9

Pan Y-L, Chow N-H, Chang TC, Chang H-C (2011) Identification of lethal *Aspergillus* at early growth stages based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Diagn Microbiol Infect Dis 70:344–354. https://doi.org/10.1016/j.diagmicrobio.2011.03.007

Pham T, Wimalasena T, Box WG, Koivuranta K, Storgårds E, Smart KA, Gibson BR (2011) Evaluation of ITS PCR and RFLP for differentiation and identification of brewing yeast and brewery 'wild' yeast contaminants. J I Brewing 117:556–568

Pinto A, Halliday C, Zahra M, van Hal S, Olma T, Maszewska K, Iredell JR, Meyer W, Chen SCA (2011) Matrix-assisted laser desorption ionization-time of flight mass spectrometry identification of yeasts is contingent on robust reference spectra. PLoS One 6:e25712

Ramos BD, Fernandes LRRMV (2012) An overview of geographical indications in Brazil. J Intellect Prop Rig 17:133–140

Ribeiro JRA, Carvalho PMB, Cabral AS, Macrae A, Mendonça-Hagler LCS, Berbara RLL (2011) *Candida middelhoveniana* sp. nov., a new yeast species found on the rhizoplane of organically cultivated sugarcane. A van Leeuw J Microb 100:341–347

Rodríguez-Gómez F, Arroyo-López FN, López-López A, Bautista-Gallego J, Garrido-Fernández A (2010) Lipolytic activity of the yeast species associated with the fermentation/storage phase of ripe olive processing. Food Microbiol 27:604–612. https://doi.org/10.1016/j.fm.2010.02.003

Settanni L, Sannino C, Francesca N, Guarcello R, Moschetti G (2012) Yeast ecology of vineyards within Marsala wine area (western Sicily) in two consecutive vintages and selection of autochthonous *Saccharomyces cerevisiae* strains. J Biosci Bioeng 114:606–614. https://doi.org/10.1016/j.jbiosc.2012.07.010

Sipiczki M (2011) Dimorphic cycle in *Candida citri* sp. nov., a novel yeast species isolated from rotting fruit in Borneo. FEMS Yeast Res 11:202–208

Smith MT, Robert V, Poot GA, Epping W, de Cock AW (2005) Taxonomy and phylogeny of the ascomycetous yeast genus *Zygoascus*, with proposal of *Zygoascus meyerae* sp. nov. and related anamorphic varieties. Int J Syst Evol Microbiol 55:1353–1363. https://doi.org/10.1099/ijs.0.63277-0

Stevenson LG, Drake SK, Shea YR, Zelazny AM, Murray PR (2010) Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of clinically important yeast species. J Clin Microbiol 48:3482–3486

Stringini M, Comitini F, Taccari M, Ciani M (2008) Yeast diversity in crop-growing environments in Cameroon. Int J Food Microbiol 127:184–189

Strohalm M, Hassman M, Ko[illegible]a B, Kodí[illegible]k M (2008) mMass data miner: an open source alternative for mass spectrometric data analysis. Rapid Commun Mass Spectrom 22:905–908

Valente MER, Perez R, Ramos AM, Chaves JBP (2012) Indicação geográfica de alimentos e bebidas no Brasil e na União Europeia. Cienc Rural 42:551–558

van Veen SQ, Claas EC, Kuijper EJ (2010) High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. J Clin Microbiol 48:900–907

Varela C, Siebert T, Cozzolino D, Rose L, McLean H, Henschke PA (2009) Discovering a chemical basis for differentiating wines made by fermentation with 'wild' indigenous and inoculated yeasts: role of yeast volatile compounds. Aust J Grape Wine R 15:238–248. https://doi.org/10.1111/j.1755-0238.2009.00054.x

Villa-Carvajal M, Querol A, Belloch C (2006) Identification of species in the genus *Pichia* by restriction of the internal transcribed spacers (ITS1 and ITS2) and the 5.8S ribosomal DNA gene. Antonie Van Leeuwenhoek 90:171–181. https://doi.org/10.1007/s10482-006-9071-0