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Comparison between morphophysiological and molecular methods for the identification of yeasts isolated from honey

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Abstract

Honey has a characteristic microbiological profile depending on their physical and chemical composition, results of the concentration sugar, with a high degree of resistance to the proliferation of microorganisms. Thus, the aim of the present study was to compare the morphophysiological and molecular methods for identification of yeast isolated from the honey obtained from the *Apis mellifera* L. bee. To this end, 97 samples of floral honey acquired at two of Piauí's, Brazil, honey cooperatives were analyzed. Yeast identification was conducted by the morphophysiological method and by the molecular analysis of the sequences of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region. Six yeast strains were isolated, two of them new isolated species; however comparisons between the morphophysiological and molecular methods showed divergence in the identification all the isolated species. Thus, it is concluded that morphophysiological identification tests alone are unreliable as a single yeast identification means and that molecular methods showed higher effectiveness in the identification of yeast isolates.

Introduction

Beekeeping constitutes an activity in which good economic results can be achieved, and has attracted the interest of many breeders in several institutions in Brazil, besides being very important to the agricultural sector at the national level (Evangelista-Rodrigues *et al.*, 2005).

The honey production in Brazil was 37.820 tons in 2015, compared to the previous year there was a reduction of 1.7% produced honey. The drop in honey production is due to lack of rainfall in the main producing regions. Despite the problems, Brazil is still among the world's ten largest honey producers in accord Brazilian Institute of Geography and Statistics (IBGE, 2015) and the Brazilian Association of Exporters of Honey (ABEMEL, 2016).

Honey is the result of the dehydration and transformation of nectar (Crane, 1983), and is constituted basically of a complex mixture of highly concentrated sugars while also containing, besides the sugars in solution, organic acids, enzymes, vitamins, flavonoids, minerals and a variety of organic compounds that contribute to its nutritional and sensory characteristics (Serrano, 1994; Bárbara *et al*., 2015; Atanassova *et al*., 2016).

In general, the microbiological quality of honey is directly related to its extraction and processing. Factors such as the use of appropriate hygiene procedures for equipment and facilities, observing the incidence of winds and the absence of insects and pets on the processing site, are some of the attributes that affect the microbiological quality of honey (Souza, 2007).

Honey microbiota is very variable and comes from microorganisms originating from primary sources, introduced by the bees themselves, and secondary sources, resulting from inadequate forms of hygiene during handling of the hives and manipulation of the honey. The microbiological profile in honey consists of common microorganisms, such as bacteria from the genus *Bacillus* and *Clostridium*, present in the sporulated state, filamentous fungi from the genera *Penicillium, Mucor* and *Saccharomyces* yeasts, which can negatively influence the final quality as they multiply in honey exposed to the action of external factors, such as handling conditions, contamination with bacterial spores in high temperature storage and

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high relative humidity (Snowdon and Cliver, 1996; Sodré, 2007; Grabowski and Klein, 2015).

The identification and characterization of yeast species have traditionally been based on morphological and, mainly, physiological characteristics regarding their capabilities (Barnett *et al*., 1990; Pitt and Hocking, 2009; Kurtzman *et al*., 2011). However, molecular techniques have become increasingly used as tools for the identification of yeast, especially when dealing with species with morphological features difficult to distinguish. The applications of these techniques have generated a greater number of studies on the classification, identification and ecology of yeast species (Guillamón *et al*., 1994). The present study aimed to compare the morphophysiological and molecular methods for identification of yeasts isolated from honey of the *Apis mellifera* L. bee.

Material and Methods

Sample collection

Ninety-seven samples of floral honey from Appis melifera were collected between March and May 2013. The samples were acquired at two of Piauí's honey cooperatives, with 50 samples acquired from a honey warehouse at the micro-region of Picos (Cooperative A) and 47 from the micro-region of Simplício Mendes (Cooperative B). The samples were collected aseptically into sterile vials with a capacity of 40 g. They were then was referred to the Microbiological Control Laboratory studies of the Center for Food Studies, Processing and Research (NUEPPA) of the Agricultural Sciences Center at the Federal University of Piauí.

Yeast isolation

From each sample, 10 g of honey were weighed aseptically and added to 90 mL of a 0.1% peptone water containing glucose 20%, obtaining an initial dilution of 10^{-1}, and, from this dilution, fold dilutions were prepared to 10^{-3}. From each dilution aliquots of 0.1 mL (duplicate) were inoculated by the scattering by surface seeding method in agar yeast extract of peptone dextrose (YPD). The plates were incubated at 25°C for seven days in microbiological incubators. To obtain pure isolates, each colony was isolated on YPD plates by streak plate method. The isolates obtained from a single CFU were subcultured in inclined tubes in Malt Extract Agar (MEA) for species identification.

Morphophysiological identification

Each of the strains was identified to genus and species using the taxonomic key proposed by Pitt and Hocking (2009).From each pure isolated obtained in the MEA tubes, a suspension was made in tubes containing 5 mL of 0.1% peptone water, which was seeded in duplicate by means of splines and exhaustion in the following culture media: Agar malt extract (MEA) at 25ºC and 37ºC, Agar Czapek (Cz), Agar malt acetic acid (AMA), yeast extract and glucose 50% (MY-50G), agar yeast extract, NaCl 10% and glucose 12% (MY10-12).

The growth on the plates was examined at seven days of incubation. The presence of growths and the morphological characteristics of the colonies, such as color, size and appearance were observed in different media. For each colony a sheet from the MEA plate at 25°C was prepared in compliance with the microscopic characteristics such as size and cell shape, reproduction mode, cell arrangement (single, in pairs, chains), presence of pseudo-mycelium and ascospore production also were observed. The culture media were purchased from Himedia (Mumbai, India) and chemicals by Vetec (Rio de Janeiro, Brazil).

Molecular identification

Genomic DNA was extracted according to the protocols described by Loque *et al.* (2010). Yeast previously identified by the morphophysiological technique were confirmed by sequencing of the variable domains D1/D2 of the large rRNA gene subunit; the divergent domains D1/D2 were amplified by PCR as described by Lachance *et al.* (1999) using the primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'). The species were also confirmed by sequencing the ITS-5.8S rRNA gene domain using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as described by White *et al.* (1990). The ITS amplification and sequencing were performed as described by Rosa *et al.* (2009).

Sequencing of the obtained DNA fragments was performed using an automatic sequencer (ABI 3730). Comparisons among the obtained sequences and those contained in the GeneBank were performed using the BLASTn program (Basic Locus Serch Alignment Tool), available at the NCBI (National Center for Biotechnology Information) website *(http: // www.ncbi.nlm.nih.gov/blast/)*.

Results and Discussion

A total of five yeast species were isolated from floral honey from the *Apis mellifera* L. bee. Based

Table 1. Comparison between the identification of yeast species by molecular and morphophysiological techniques

Identification code	Morphophysiological Identification	Molecular Identification
L1A	*Kloeckera apiculata*	*Sporisorium elionuri*
L3A	*Pichia anomala*	*Zygosaccharomyces mellis*
L4A	*Brettanomyces bruxellensis*	*Pseudozyma sp.*
L5A	*Zygosaccharomyces bailii*	*Aureobasidium pullulans*
L6A	*Kloeckera apiculata*	*Sympodiomycopsis sp.*
L7A	*Pichia anomala*	*Zygosaccharomyces mellis*

on the morphology and macroscopic aspects of the yeast culture media for different purposes by the Pitt and Hocking taxonomic key (2009) the following species were identified: *Pichia anomala, Kloeckera apiculata, Zygosaccharomyces bailii* and *Brettanomyces bruxellensis* (Table 1).

This identification method is a quick and simple system that allows for the identification of the main yeast species related to food spoilage. However, this is a system that relates only a few species by limiting the search for others which arise as occasional contaminants, and, thus, some isolates may be misclassified. Despite being a simple and incomplete method, this technique was chosen for this study due to its low cost compared to conventional methods, speed, using a reduced number of culture media and being recommended by Pitt and Hocking (2009) for identifying food spoilage yeasts.

Comparisons between the morphophysiological and molecular methods showed divergence in identifying all isolated species, since the Pitt and Hocking (2009) key shows no species identified by the molecular technique, demonstrating a great failure in the morphophysiological method (Table 1). Because of these yeast identification difficulties, molecular techniques are an option for enabling the identification of a large number of species, since they are more reliable due to their high level of specificity (Guillamón *et al*., 1996; Hierro, 2004; Kurtzman *et al.*, 2011).

Some studies comparing other identification methods also found large differences when compared to molecular techniques. Carvalho *et al.* (2010) found that 58% of the yeast were correctly identified using the API 20CAUX commercial kit, and the correctly identified genera were *Rhodotorula, Candida* and *Saccharomyces*. However, the species of the *Zygosaccharomyces* genus could not be identified because they are not described in that kit. Many species of this genus are xerophilic and some, for example *Z. bailii,* cause big problems in the food industry, to control the processes food must be pasteurized or sterilized (Pitt and Hocking, 2009; Spencer *et al.*, 2011).

Another study by Spencer *et al.* (2011) that evaluated an assimilation test, known as BiologYT MicroPlate, showed that 67% of erroneously identified yeasts were new species and recently reported in the literature. Thus, because of the wide variety of yeast species present in nature, the molecular methods, although more expensive and cumbersome, are more effective in their identification.

Among other identification methods, commercial miniaturized systems, such as Vitek, API 32C, API 20C AUX from BioMerieux (Marcy L'Etoile, France), Yeast Star from Clarc Laboratories (Heerlen, Netherlands), Auxacolor Sanofi (Paris, France) and RapIDYeast System Plus from Thermo Fisher Scientific (Lenexa, KS, USA) were designed to shorten the identification of clinical yeast isolates and are widely used in medical diagnoses. However, the importance of the yeast is not limited to human pathogenesis. This large, divergent group of microorganisms has an important role in the science of food due to their beneficial activities and the transformation of food characteristics, such as fermented honey (Arias *et al.*, 2002).

Five different species were identified by molecular identification by DNA sequencing, representing five genera (Table 2). The species *Zygosaccharomyces mellis* represents two identified isolates. Other works have also reported the presence of this yeast in honey. Carvalho (2005 and 2010) identified a total of nine yeast species isolated from honeys obtained from Trás-os-Montes, Portugal with *Rhodotorula mucilaginosa, Candida magnolia* and *Zygosaccharomyces mellis* among the most frequently isolated species. This species belongs to the *Zygosaccharomyces* genus, which is considered an important osmotolerant food yeast and commonly found in honey (Esteve-Zarzoso *et al*., 1999) and require a low water activity for growth (Kurtzman *et al*., 2011).

Čadež *et al.* (2015) isolated 34 yeast samples from bees and honey. They found *Zygosaccharomyces* as prevalent genus. As a result, six strains were identified as *Z. rouxii,* five strains as *Z. mellis* and three strains as *Z. siamensis.* Four strains showed different growth behavior in agar yeast extract-glucose-peptone (GPY) and other common culture media for yeast and, after several trials, a new species was characterized as *Zygosaccharomyces favi.*

In this study a species of *Aureobasidium pullulans*

Table 2. Identification of yeast strains by DNA sequencing

Molecular identification	Query cover	Identity	Top BLAST search results [GenBank accession number]	Number of isolates
Sporisorium elionuri	100%	100%	Stoll *et al.* [AY740157]	1
Pseudozyma sp.	100%	100%	Sugita,T [DMST 17137]	1
Aureobasidium pullulans	100%	99%	Korhola *et al.* [HG532101.1]	1
Sympodiomycopsis sp.	100%	96%	Freitas,L.F.D. and Rosa,C.A. [KJ410160.1]	1
Zygosaccharomyces mellis	99%	99%	Sinacori,M [KC692235.1]	2

was isolated as yeast, however, it is reported as a filamentous fungus in the literature. The fungal genus *Aureobasidium* is successively isolated as yeast due to its initial growth behavior on common culture yeast media, with white or pink colonies, which become filamentous (black color) after weeks of isolation (Spencer et al., 2011).

Two new species were isolated, *Pseudozyma* sp. and *Sympodiomycopsis* sp. with the anamorphic yeast belonging to the genus *Pseudozyma, Ustilaginales* order (Ustilaginomycetes, Ustilaginomycotina), which has a close relationship to teleomorphic species of the *Ustilago* and *Sporisorium* genera, which, in turn, are responsible for serious diseases in plants. The *Pseudozyma* sp. isolated in the present study has not yet been described in the literature and no description of its morphological, physiological and biochemical characteristics is available, however it is known that many species of this genus have been isolated from plants (Sugita *et al.* 2003;. Wang *et al.,* 2006; Oliveira *et al.*, 2013; Mekha *et al*., 2014), from blood samples and from coral reef waters (Statzell-Tallman *et al.*, 2010).

The anamorphic genus *Sympodiomycopsis,* which is a member of the Microstromatales family, was proposed by Sugiyama *et al.* (1991) to accommodate a basidiomycete with a formation of true hyphae, which in culture of malt extract agar (MEA) has a cream-color and smooth and soft texture, but not mucoid (Kurtzman *et al*., 2011). The biodiversity of yeast species present in honey is still largely unknown and the isolation of two new species in the present study shows the importance of research into the morphological, physiological and molecular characteristics of these yeasts, as they may have some important role in the quality of honey.

Conclusion

Morphophysiological identification tests alone are not reliable as a single yeast identification means. Molecular methods, however, showed greater effectiveness in identifying yeast isolates. In spite of this, the association of morphophysiological and molecular methods has an important role in morphological, physiological and molecular characterization in the identification of common yeast in honey, as well as species that are not described in the scientific literature.

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