YEAST SPECIES ASSOCIATED WITH HONEY: DIFFERENT IDENTIFICATION METHODS

ESPECIES DE LEVADURAS ASOCIADAS A LA MIEL: MÉTODOS DIVERSOS DE IDENTIFICACIÓN

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SUMMARY
In the present study, three different methods were used to identify yeast isolated from Trás-os-Montes, Portuguese honey. A total of 24 isolates were identified using a partial sequence of the 26S rRNA gene (rDNA), restriction patterns generated from the region spanning the internal transcribed spacers (ITS1 and ITS2) of the 5.8S rRNA gene and the API 20C AUX kit. Nine different yeast species were identified representing six different genera. Among the isolated honey samples, Rhodotorula mucilaginosa, Candida magnoliae and Zygosaccharomyces mellis were the predominant species. Partial sequence of the 26S rDNA yielded the best results in terms of correct identification, followed by-5.8S-ITS analysis. The commercial identification kit API 20C AUX was able to correctly identify only 58% of the isolates. Two new 5.8S-ITS profiles were described, corresponding to Trichosporon mucoides and Candida sorbosivorans.

INTRODUCTION
Honey has been highly appreciated as an alimentary product, and has been largely used, since ancient times, either preventing or curing infirmity and illness, as well as in cosmetic manufacturing (Kang et al., 2007).

Honey is a sugary substance obtained from the nectar of the flowers or from the secretions which either come from or lie on the living parts of the plant and which honey bees crop, transform and combine with their own specific substances, and store in the honeycomb of the beehive. Honey is a product extremely rich in sugars of which glucose and fructose are outstanding; it also possesses vitamins, mineral salts and
enzymes (Oh and Cheon, 2007). The mechanism of antimicrobial action of honey is most likely a combination of a number of different factors. Its low water activity is likely to play a major role when honey is applied topically, but studies comparing its effect to sugar syrups of the same concentration have show that honey has superior activity. Hydrogen peroxide is produced within honey by the action of the bee-derived enzyme glucose oxidase (which transforms glucose into glucanic acid plus $\text{H}_2\text{O}_2$) and has known antimicrobial activity. The hydrogen peroxide produced enzymatically in honey is considered by several investigators as one of the main reasons for such biological activity of honey. This effect is more accentuated in honeys with high pH and moisture content, due to the inhibition of glucose oxidase enzyme at low pH and gluconic acid.

Fermentation is an irreversible phenomenon that can run in honey mainly during storage, causing significant economical losses. In such a case, honey presents a characteristic odor, increasing acid flavor and gas bubbles (Perez-Perez et al., 2007).

The honey of Trás-os-Montes, a region in the North of Portugal, is characterized by its dark color, accentuated floral aroma and intense, persistent odor. Designation of Origin (D.O.P - Denominação de Origem Protegida) was registered and protected by CE Regulation Nº 1107/96, 12-06. This honey has been chosen because it is produced in the influence area of the Escola Superior Agrária of Bragança-Portugal (ESAB). In Portugal, the apiculture involves more than 25 000 beekeepers, with a production over 4500 tons per year. The Trás-os-Montes region is mainly agriculturist, being the honey production the source of income of a large population. To our knowledge, this is the first research work about the molecular isolation and characterization of Trás-os-Montes honey yeasts.

Knowledge of the moisture and temperature conditions influencing growth of microorganisms in honey has long been used to control the spoilage of honey (Marvin, 1930). However, the need for additional microbiological data on honey desirable, as new uses of honey develop. Microorganisms in honey may influence quality and safety. Due to the natural properties of honey and control measures in the honey industry, honey is a product with minimal types and levels of microbes. The microbes of concern in honey are primarily yeasts and spore-forming bacteria. Total colony-forming units (CFU) from honey samples can vary from zero to tens of thousands per gram for no apparent reason. Most samples of honey contain detectable levels of yeasts. Although yeast counts in many honey samples are below 100 plate counts per gram, yeasts can grow in honey to very high numbers (Snowdon and Cliver, 1996).

Identification and characterization of yeast species have been traditionally based on morphological traits and especially on their physiological capabilities (Barnett et al., 1990; Kreger-Van Rij, 1984). These characteristics are strongly influenced by culture conditions and can give uncertain results (Yamamoto et al., 1991). The conventional methodology for yeast identification requires evaluation of some 60-90 tests, and the process is complex, laborious and time consuming (Deák, 1995; Deák and Beuchat, 1996). In recent years, rapid kits for yeast identification have been developed to overcome this complexity. However, they were initially designed for clinical diagnosis and their application is restricted to 40-60 yeast species of medical interest (Deák, 1993). In general, it is necessary to do around 100 tests to obtain a reliable identification of yeasts at the species level, and 1-3 weeks are often necessary to obtain a final result (Kreger-van Rij, 1984; Lin and Fung, 1987; Barnett et al., 1990). The API 20C AUX system is one of these methods, consisting of 19 assimilation tests, and has been widely used.

Recent progress in molecular biology
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has lead to the development of new techniques for yeast identification. Microbial identification has undergone a revolutionary change by the introduction of PCR-based methodologies. The polymerase chain reaction (PCR) amplification of specific sequences for the identification of organisms has become common because of the relative easiness of manipulation and the high reproducibility (Guillamón et al., 1998).

Conventional yeast identification based on phenotypic characteristics is often misleading and inconclusive, and usually needs to be corroborated by molecular methods. Currently, one of the most commonly adopted methods is the sequencing of the 26S rDNA D1/D2 region of the rRNA gene. Although it has already been demonstrated that some species have significant variability in the D1/D2 domain, sequencing of this region is generally accepted as a means for species delimitation, especially for yeasts with ascomycetic affinity (Ramos et al., 2005).

Ribosomal RNA genes (rDNA) have been the most widely employed nuclear sequence in evolutionary analyses, primarily because concerted evolution occurs among its repeated copies and conserved universal primers are available, which facilitate the amplification of the target sequences by the PCR (Hillis and Davis, 1988; White et al., 1990; Hillis and Dixon, 1991). Due to their rapid rates of evolution and easy amplification, the internal transcribed spacer (ITS) regions-ITS1 and ITS2 located between the 18S, 5.8S and 28S genes (or their homologues), have been popular in phylogenetic inference for closely related taxa (Miller et al., 1996; Maoxian et al., 2004), cryptic

Figure 1. Size of the PCR amplified 26S rDNA. Lanes m1 and m2 correspond to molecular size standards (1 kb DNA Ladder and 100-bp DNA Ladder from Promega). (Tamaño de los productos amplificados por PCR del rDNA 26S. Los carriles m1 y m2 corresponden a los marcadores estándar de peso molecular; 1 kb DNA Ladder y 100-bp DNA Ladder de Promega).
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One of the most successful methods for yeast identification is restriction fragment length polymorphism (RFLP) analysis of the 5.8S rDNA gene and the two flanking internal transcribed sequences (ITS1 and ITS2). This technique consists of PCR amplification using conserved oligonucleotide primers for the 26S and 18S rDNA genes (figure 1), followed by endonuclease restrictions analysis of the amplified product (Arias et al., 2002). Because ribosomal regions evolve in a concerted fashion they have low intraspecific polymorphism and high interspecific variability (James et al., 1996).

Previous works (Guillamón et al., 1998; Esteve-Zarzoso et al., 1999) established a database containing region endonuclease restriction patterns for the 5.8S-ITS region of 132 yeast species isolated from numerous sources. This 5.8S-ITS database combines reference yeast strains from different origins and can be more useful for environmental or wild yeast strain identification than the clinically oriented commercial databases (Arias et al., 2002).

In the present study, three different methods were used to identify yeast isolated from honey. The first objective was to isolate the yeast species. A second objective was to compare different methodologies for yeast identification and to establish which method could be more useful for routine analysis. In this sense, we used a commercial identification method based on phenotypic traits (API 20C AUX System) and two DNA sequence-based protocols (5.8S-ITS profiles and partial sequence of the 26S rDNA gene). We decided to use the partial sequence of 26S rDNA gene, since it has a universally recognized role in yeast taxonomy and the available database include all yeast species described to date.

METHODS

HONEY STORAGE CONDITIONS

Chemical and bacteriological compositions of all analyzed honeys were inside the stipulated limits designed by Portuguese and European norms. The honey was stored in 0.5 and 1 kg glass bottles at room temperature. All analyses, including mainly the microorganism isolation, were efectuated without previously product heating.

YEAST STRAINS AND GROWTH CONDITIONS

Yeast were isolated in agar broth by the extinguishing dilution technique. A total of 200 Trás-os-Montes honey samples were used in this work. The honey was plated into Wikerham Medium broth containing 2% (w/w) glucose (from VWR (West Chester, PA, USA), 1% w/w peptone from (Franklin Lakes, NJ, USA); 0.5% (w/w) yeast extract and 2% (w/w); agar in demineralised water). The plates were incubated at 30 ºC for 48 h. Three to five colonies were randomly selected from each batch.

The cells were routinely grown on liquid cultures using the same medium and some conditions at 200 rpm.

API 20C AUX

The API gallery method has been chosen because has been recommended by several yeasts identification investigators, mainly the ones with clinic interest and further optimised in our laboratory. Molten (50ºC) API basal medium ampoules were inoculated with yeast cells picked from individual colonies and the resulting suspension was standardized to turbidity equal to a nº 2 McFarland standard. Each cupule was inoculated and trays were incubated at 30ºC for 72 h. Cupules showing turbidity significantly greater than that of the negative control were considered positive. Identification was made by generating a microcode and using the API 20C Analytical Profile index from the same manufacturer.
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5.8S-ITS ANALYSIS AND SEQUENCING

DNA was isolated according to J.M. Beckerich (Institut National de la Recherche Agronomique, France, personal communication) and diluted to 1-50 ng/µl. The rDNA region was amplified in a UNO II Thermocycler from Biometra® (Goettingen, Germany). PCR conditions for ITS amplification were described in a previously work (Esteve-Zarzoso et al., 1999). Primers pairs used to amplify the ITS region, ITS1 (5'-TCGTTAGGTGAACCTGCGG-3') and ITS4 (5'-TCTCCGCTTATTGATATGC-3') and were synthesized according to the method of White et al. (1990).

The thermal cycling parameters were as follows: initial denaturing at 94°C for 5 min; 35 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min; and a final extension step at 72°C for 10 min. PCR products (approximately 0.5 to 1.0 µg) were digested without further purification with HhaI, HaeIII and HinfI restriction endonucleases (from Promega, Madison, WI, USA). Amplified products and their restriction fragments were electrophoresed on 1.5 and 3% agarose gels, respectively, in 1X TAE (Tris, acetic acid and Na2-EDTA) buffer. Gels were stained with ethidium bromide, visualized, and photographed under UV light. Fragment sizes were estimated by comparison with the a DNA standards 100-bp DNA Ladder and 1kb DNA Ladder (Promega).

26S RDNA SEQUENCING

Amplification of the D1/D2 region of 26S rDNA sequences was carried out using primers NL1 (5'-GCATATCAAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTAAGCAGC-3'). The PCR conditions were an initial denaturing at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, with a final extension at 72°C for 5 min. Amplified products were purified using Gene clean® II Kit from Qbiogene (Carlsbad, CA, USA) before being sequenced.

The sequencing of the fragments of the DNA obtained in this work was carried through in an automatic sequencer ABI Prism 377™ from Applied Biosciences (Foster City, CA, USA). Four different fluorescent colors identify the four deoxynucleotides incorporated in the extension reaction (A; C; G and T). The automatic sequencer ABI Prism 377™ carries the electrophoretic separation and the detection of the fragments of DNA marked with fluorescence.

Sequence comparisons were performed using the BLAST search tool algorithm within the GenBank database, http://www.ncbi.nlm.nih.gov/blast

ANALYSIS OF THE DATA

An isolated sample was considered correctly identified when at least two methods ascribed it to the same species. When this situation did not occur, putative identification was based on a partial sequence of the 26S rDNA gene. Each identification method was evaluated (i) for its ability to identify the isolates to the species level, (ii) for its ability to identify the isolates to the genus level, (iii) for its discrepant identification, and (iv) for its failure to provide identification.

RESULTS AND DISCUSSION

YEAST SPECIES PRESENT IN HONEY

A total of 24 yeast strains isolated from Trás-os-Montes honey were identified in this study (table I). Nine species were identified, representing six genera. Candida magnoliae (25%), Rhodotorula mucilaginosa (17%), and Zygosaccharomyces mellis (12.5%) were more common among isolates. Candida spp. represented more than 45% of the honey isolates.

COMPARISON OF THE IDENTIFICATION METHODS

In recent years, several identification
methods have been proposed as alternatives to cumbersome classical yeast identification techniques. Among these methods, commercial miniaturized systems such as Vitek, API 32C, API 20C AUX from BioMerieux (Marcy L’Étoile, France), Yeast Star from Clarc Laboratories (Heerlen, The Netherlands), Auxacolor from Sanofi (Paris, France), and RapID Yeast Plus system from Thermo Fisher Scientific (Lenexa, KS, USA) were designed to shorten the identification time of clinical yeast isolates and are extensively used in clinical diagnosis (Arias et al., 2002). However, yeast importance is not confined to human pathogenesis. This large and divergent group of microorganisms has an important role in food science for its beneficial activities (i.e., alcoholic beverages

Table I. Results obtained with the 26S rDNA sequence. (Resultados obtenidos con la secuencia 26S rDNA).

| Reference strains | Identity | Classification | Genbank number |
|-------------------|----------|----------------|----------------|
| TAB               | 99.47%   | Rhodotorula mucilaginosa | AJ437347 |
| M109              |          |                |                |
| M125              |          |                |                |
| M125              |          |                |                |
| 5B1               |          |                |                |
| ESA1              | 100%     | Saccharomyces cerevisiae | AJ437312 |
| 7ER               | SLC9634  |                |                |
| M41               | 100%     | Zygosaccharomyces rouxii | AJ783434 |
| ESA21             | U72163   |                |                |
| ESA51             | 100%     | Trichosporon mucoides | AJ508385 |
| 5B3               | 100%     | Candida parapsilosis | AJ749821 |
| M70               | CPU45754 |                |                |
| ESA21             |          |                |                |
| M1                | 99.52%   | Candida magnoliae | AJ550624 |
| M26               | CMU45722 |                |                |
| M27               |          |                |                |
| ESA17             |          |                |                |
| M16               |          |                |                |
| ESA4              |          |                |                |
| ESA11             | 98.94%   | Pichia membranifaciens | AJ511348 |
| 1Lisa             | U75725   |                |                |
| 2Lisa             |          |                |                |
| 1694-P011         |          |                |                |
| 1694-P015         | 100%     | Candida sorbosivorans | AJ783433 |
| ESAB20            | AY008842 |                |                |

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Table II. Size in base pairs (bp) of the PCR products and the restriction fragments of the 5.8S-ITS analysis. (Tamano en pb de los productos de PCR y de los fragmentos de restriccción del análisis 5.8S-ITS).

| Reference strains | Species                        | AP* (bp) | HhaI (bp) | HaeIII (bp) | HinfI (bp) |
|-------------------|--------------------------------|----------|-----------|-------------|------------|
| TAB M109, M125, 5B1 | *Rhodotorula mucilaginosa*     | 640      | 320+240+80 | 425+215     | 340+225+75 |
| ESA1 7ER          | *Saccharomyces cerevisiae*     | 880      | 385+365+130 | 320+230+180+150 | 365+180+155 |
| M41 ESAB21        | *Zygosaccharomyces rouxii*     | 750      | 290+200+170+90 | 400+210+90  | 350+260+140 |
| 5B3 M70 ESAB2     | *Candida parapsilosis*         | 550      | 300+240     | 400+115     | 290+260    |
| M1 M26 M27 ESA17 ESAB4 | *Candida magnoliae*   | 425      | 200+190     | 285+140     | 225+200    |
| ESA11             | *Pichia membranifaciens*      | 500      | 175+110+90+75 | 330+90+50  | 275+200    |
| 2Lisa             | *Zygosaccharomyces mellis* (Pattern 1) | 850 | 350+250+210 | 560+200+90 | 400+270+180 |
| 1Lisa 1694-P011   | *Zygosaccharomyces mellis* (Pattern 2) | 850 | 350+250+200+150 | 800+400 | 400+250+100 |

and bread making). Such microorganisms are also relevant for the economic losses that they cause when growing as saprotrophs in food and manufactured goods. For this reason, rapid and accurate identification methods are needed for wild environmental strains, to monitor biotechnological processes or to identify sources of food spoilage. Unfortunately, commercial kits for yeast identification were designed based on clinical strains and their use for identification of wild yeasts is often inconclusive (Arias et al., 2002).

Recently, new identification methods, mainly based on nucleic acid sequences, have been modified to detect wild environmental yeast isolates. Some of these are highly discriminative, such as those using mitochondrial DNA restriction analysis (Estève-Zarzo et al., 2001), randomly amplified polymorphic DNA, karyotyping, and intron splice site-specific PCR amplification (Barros-Lopes et al., 1996), and have been used mainly for intraspecific characterization and strain identification.

In this study, nucleotide sequence of the 26S rDNA (Table II) provided the highest
The majority of partial 26S rDNA gene sequences showed identities of 98% or higher to sequences in the GenBank database <http://www.ncbi.nlm.nih.gov/blast> (table I). Most sequences perfectly matched sequences present in the database. Analysis of the 5.8S-ITS endonuclease restriction pattern enabled us to correctly identify 78% of the strains at the species level (table II). Two isolates could not be identified, represented new 5.8S-ITS patterns that have not been previously described (table III).

Using the restriction endonucleases HhaI, HaeIII and HinfI, we found two different profiles corresponding to Trichosporon mucoides and Candida sorbosivorans. The species of the genus Zygosaccharomyces are important osmotolerant food spoilage ascomycetous yeasts (Esteve-Zarzoso et al., 1999), and one of the most common in honey. With this method we identified two species corresponding to Zygosaccharomyces rouxii and Zygosaccharomyces mellsii. One strain of Zygosaccharomyces mellsii showed different pattern indicating that this taxon includes two different types of strains.

Nuclear ribosomal sequences are often assumed to be homogeneous within individuals and populations of a species through concerted evolutionary processes (Hillis and Davis, 1988). However, multiple copies of ITS regions exist in the rDNA tandemly repeated units. If new mutations occurred faster than the homogenization processes, then one would expect to see multiple variants of ITS sequences at both the intraindividual and intraspecific levels (Maoxian et al., 2004).

Only 58% of isolates were correctly identified to the species level by API 20C AUX (table IV). Five reference strains, Trichosporon mucoides (ESA51), Candida magnoliae (M1, M26), Pichia membranifaciens (ESA11) and Candida sorbosivorans (1694-P015) were misidentified (table V). The isolates correctly identified belonged to the genera Rhodotorula, Candida, and Saccharomyces. The genera Zygosaccharomyces can not be identified because they are not described in the API 20C AUX database.

**CONCLUSIONS**

In this study, we compared the performance of narrower identification methods, typified by one commercial method (API 20C AUX), with analysis of the 5.8S-ITS region and contrasted them with broader identification tools, typified by a partial sequence of the 26S rDNA. Overall, the results demonstrate good reliability of the 5.8S-ITS analysis as a routine technique for identification of honey yeast isolates. This method allows identification in less than 8 h from colony isolation. There were two strains that had restriction digest profiles that were not present in the published database and could not be identified using this technique. In these cases, species assignment was based on partial sequence of the 26S rDNA gene. Based on identification using other methods, the two new 5.8S-ITS profiles corresponded to the species Trichosporon mucoides and Candida sorbosivorans.

**Table III.** Nucleotide fragment length of new 5.8S-ITS profiles described in this study. (Tamaño de los fragmentos nucleotídicos de dos perfiles 5.8S-ITS nuevos descritos en el estudio).  

| Species                  | AP (bp) | Restriction fragments (bp) |
|--------------------------|---------|----------------------------|
|                          |         | HhaI | HaeIII | HinfI |
| Trichosporon mucoides    | 500     | 300+100 | 500    | 300+150 |
| Candida sorbosivorans    | 400-450 | 200+100+70+50 | 210+190+50 | 230+200 |

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As expected, the commercial method yielded the lowest number of correct results, since the major part of species found in honey are not present in their database. Nevertheless, new profiles can always be added to an existing database, as has been shown in the case of the 5.8S-ITS (Esteve-Zarzoso et al., 1999; Fernandez-Espinar et al., 2000). The simplicity and rapidity of commercial methods may be attractive enough to use in the food industry if the developed databases were robust (Arias et al., 2002).

As has been shown by several authors, a polyphasic approach may be the best way to achieve proper microbial identification (Arias et al., 2002). Integration of different classes of data and information leads to a consensus type of taxonomy and overcomes the limitations of each single identification method, thereby improving the reliability of the whole determination. This appears to be especially true for yeast identification since yeast taxonomy is incomplete and present-day classification is based on strains (Barnett et al., 2000). Although taxonomic descriptions should be as complete as possible, clinical diagnosis and industrial quality control laboratories demand rapid yeast identification methods. In general, commercial identification kits are usually faster, simpler to perform, and do not require special equipment. On the other hand, they rely on only a few tests, limiting their application in identifying environmental strains, although their usefulness for clinical isolates has been reported (Espinel-Ingroff et al., 1998; Graf et al., 2000).

Yeast identification based on 5.8S-ITS restriction analysis has proven to be a rapid, reliable, and accurate tool for yeast identification. In our hands, this technique provided good results in terms of time and accuracy, but the existent database should be further updated and improved. After we updated the previous database with the new profiles founded in this study, all of isolates were correctly identified. However,

| References | Incorrect identification | Correct Identification |
|------------|--------------------------|------------------------|
| M41        | Candida magnoliae        | Zygosaccharomyces rouxii|
| ESA51      | Cryptococcus laurentii   | Trichosporon mucoides   |
| M1         | Cryptococcus terreus     | Candida magnoliae       |
| M26        | Candida utilis           | Candida magnoliae       |
| ESA17      | Hansula polymorpha (Pichia angusta) | Candida magnoliae |
| ESA11      | Candida krusei           | Pichia membranifaciens  |
| 1694-P015  | Candida magnoliae        | Candida sorbosivorans   |

Table IV. Misidentification results obtained with the API 20C AUX. (Resultados incorrectos de identificación obtenidos con API 20C AUX).

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| Identification method | Correct hits | Misidentified | Unidentified |
|----------------------|--------------|---------------|--------------|
| 26S rDNA             | 9            | 6             |              |
| ITS profiles         | 7            | 6             | 2            |
| API20C               | 5            | 4             | 7            | 3            |

Table V. Performance of three different identification methods. (Comparación de tres métodos distintos de identificación).

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as more profiles are added to the database, identification may become increasingly difficult due to no or slight differences between the 5.8S-ITS profiles (Arias et al., 2002). Unfortunately, similar or identical 5.8S-ITS patterns do not necessarily belong to related species (Esteve-Zarzoso et al., 1999). Furthermore, it has to be considered that one single mutation in the 5.8S-ITS region could lead to the loss or gain of a restriction site, resulting in a completely different pattern (Arias et al., 2002). One alternative to overcome such an occurrence would be to sequence either the 26S rDNA or the 5.8S-ITS region and compare them with the presently available databases (Arias et al., 2002). Both regions, but especially the 26S rDNA, have been shown to provide enough variability to distinguish between most yeast species due to their high taxonomic value (Kurtzman and Fell, 1998). The sequencing time requirement and cost are still too high to facilitate use in common quality control labs but may be affordable in the future. Until that time, we propose the use of the 5.8S-ITS analysis as the best method for rapid and accurate identification of yeasts isolated from honey and the 26S rDNA sequencing for further corroboration.

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