YEAST SPECIES ASSOCIATED WITH HONEY: DIFFERENT IDENTIFICATION METHODS

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

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ADDITIONAL KEYWORDS

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PALABRAS CLAVE ADICIONALES

Identificación levaduras de la miel. Gen 26S rDNA. Región 5.8S-ITS. API 20C AUX. PCR-RFLP.

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*.

RESUMEN

En este estudio, se han utilizado tres métodos para identificar levaduras aisladas de la miel de Trás-os-Montes, Portugal. Se han identificado un total de 24 aislados usando una secuencia parcial del gen rRNA 26S (rDNA), los patrones de restricción generados de la región de los espaciadores transcritos internos (ITS1 e ITS2) del gen rRNA 5.8S (rDNA) y el kit API 20C AUX. Fueron identificadas nueve especies distintas de levaduras que representan seis géneros distintos. Entre las muestras aisladas de la miel, *Rhodotorula mucilaginosa*, *Candida magnoliae* y *Zygosaccharomyces mellis* eran las especies predominantes. La secuencia parcial del rDNA 26S rindió los mejores resultados para la correcta identificación, seguida por el análisis del 5.8S-ITS. El kit comercial de identificación API 20C AUX. sólo identificó correctamente el 58% de los aislados. Se describen dos perfiles nuevos de 5.8S-ITS, correspondiendo a *Trichosporon mucoides* y *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

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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 H_2O_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

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

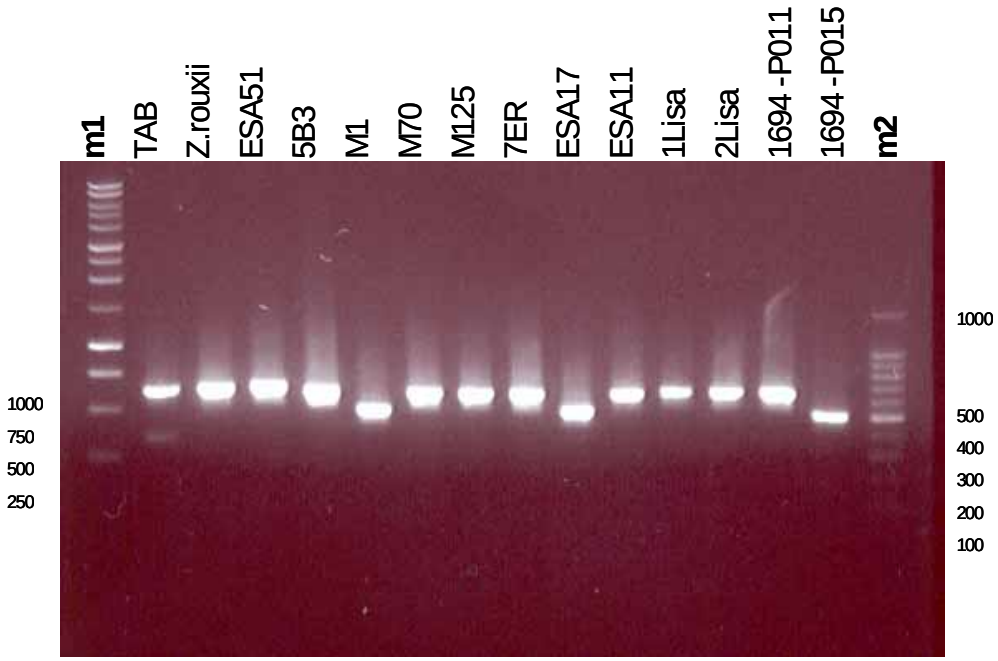

Figure 1. *Size of the PCR amplified 26S rDNA. Lanes m1 and m2 correspond to molecular size standards* (1 kb DNA Ladder *and* 100-bp DNA Ladder *from Promega*). (Tamaño de los productos amplificados por PCR del rDNA 26S. Los carriles m1 y m2 corresponden a los marcadores estándar de peso molecular: (*1 kb DNA Ladder* y *100-bp DNA Ladder* de Promega).

species identification and phylogeographical and other population genetic studies (Mukabayire *et al*., 1999; Wörheide *et al*., 2002).

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 microorganisms isolation, were effectuated without previously product heating.

YEAST STRAINS AND GROWTH CONDITIONS

Yeasts 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.

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'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and were synthesized according to the method of White *et al.* (1990).

The thermal cycling parameters were as follows: initial denaturing at 94ºC for 5 min; 35 cycles of denaturing at 94ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 2 min; and a final extension step at 72ºC for 10 min. PCR products (approximately 0.5 to 1.0 µg) were digested without further purification with *Hha*I, *Hae*III and *Hinf*I 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 Na_2-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'-GCATATCAATAAGCGG AGGAAAAG-3') and NL4 (5'-GGTCCGTG TTTCA AGACG C-3'). The PCR conditions were an initial denaturing at 94ºC for 1 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min, and extension at 72ºC for 2 min, with a final extension at 72ºC 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 no 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'Etoile, 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 M109 M125 5B1	99.47% AF335986	*Rhodotorula mucilaginosa*	AJ437347
ESA1 7ER	100% SLC9634	*Saccharomyces cerevisiae*	AJ437312
M41 ESAB21	100% U72163	*Zygosaccharomyces rouxii*	AJ783434
ESA51	100% AF335988	*Trichosporon mucoides*	AJ508385
5B3 M70 ESAB2	100% CPU45754	*Candida parapsilosis*	AJ749821
M1 M26 M27 ESA17 M16 ESAB4	99.52% CMU45722	*Candida magnoliae*	AJ550624
ESA11	98.94% U75725	*Pichia membranifaciens*	AJ511348
1Lisa 2Lisa 1694-P011	99.64% U72164	*Zygosaccharomyces mellis*	AJ511349
1694-P015 ESAB20	100% AY008842	*Candida sorbosivorans*	AJ783433

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

Reference strains	Species	AP[a] (bp)	Restriction fragments (bp) *H*haI	*Hae*III	*Hinf*I
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 M16 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 (Esteve-Zarzoso *et al.*, 2001), randomly amplified polymorphic DNA_s, 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

correct identification percentage.

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 *Hha*I, *Hae*III and *Hinf*I, 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 mellis*. One strain of *Zygosaccharomyces mellis* 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)		
		*Hha*I	*Hae*III	*Hinf*I
Trichosporon mucoides	500	300+100	500	300+150
Candida sorbosivorans	400-450	200+100+70+50	210+190+50	230+200

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

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*

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,

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

Identification method	Correct hits		Misidentified	Unidentified
	Species level	Genus level		
26S rDNA	9	6		
ITS profiles	7	6		2
API 20C	5	4	7	3

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 ccompare 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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