



Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Development of species-specific primers for rapid identification of *Debaryomyces hansenii*

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ARTICLE INFO

Article history:

Received 25 June 2014

Received in revised form 26 September 2014

Accepted 7 October 2014

Available online xxxx

Keywords:

Debaryomyces hansenii

Yeasts

Species-specific primers

PCR

Rapid-identification

Foods

ABSTRACT

In this work, we developed a specific PCR assay for *Debaryomyces hansenii* strains that uses a putative homologous *PAD1* region (729 bp) present in this yeast species as a target. The amplification of this sequence with the *D. hansenii* specific primer pair (DhPADF/DhPADR) was found to be a rapid, specific and an affordable method enabling identification of *D. hansenii* from other yeast strains. Primers were tested in almost 100 strains, 49 strains from Type Culture Collection belonging to the genus *Debaryomyces* and to other yeast species commonly found in foods or related genera. These primers were able to discriminate between closely related species of *Debaryomyces*, such as *Debaryomyces fabryi* and *Debaryomyces subglobosus*, with a 100% detection rate for *D. hansenii*. Also, the method was tested in 45 strains from different foods. Results confirmed the specificity of the PCR method and detected two earlier misidentifications of *D. hansenii* strains obtained by RFLP analysis of the 5.8S ITS rDNA region. Subsequently we confirmed by sequencing the D1/D2 domain of 26S rDNA that these strains belonged to *D. fabryi*. We call attention in this work to the fact that the RFLPs of the 5.8S ITS rDNA profiles of *D. hansenii*, *D. fabryi* and *D. subglobosus* are the same and this technique will thus lead to incorrect identifications.

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1. Introduction

The yeast species *Debaryomyces hansenii* is widespread in nature (Kurtzman et al., 2011) and has been extensively studied because of its halotolerance and potential industrial applications (Wrent et al., 2014). In fact, it is one of the most frequently isolated yeasts among secondary microorganisms involved in the ripening process of traditional cheese (Dolci et al., 2009), contributing to distinctive organoleptic characteristics (Padilla et al., 2014; Petersen et al., 2002). Likewise, it is an important contribution to flavor development in traditional dry-cured meat products such as sausages or ham as has been reported (Cano-García et al., 2014; Martín et al., 2006). In addition, *D. hansenii* seems to be suitable as a biocontrol agent for reducing risk caused by mycotoxin producing molds in dry-cured ham, dairy products and fruits (Andrade et al., 2014; Hernandez-Montiel et al., 2010; Liu and Tsao, 2009).

For laboratories and industries an accurate, quick and affordable identification is very useful. Yeast identification based on morphological

and physiological criteria is nowadays little used because it is much slower and less accurate than molecular techniques. Based on genetic differences previously reported (Corredor et al., 2000; Groenewald et al., 2008; Prillinger et al., 1999; Quirós et al., 2006), strains that formerly were included in *D. hansenii* have been recently reinstated as new species of the genus, i.e. *Debaryomyces fabryi*, *Debaryomyces subglobosus* (Kurtzman et al., 2011). They show close physiological similarity to *D. hansenii*, resulting in numerous misidentifications.

Identification by sequence analysis is expensive and time consuming when it comes to large scale work, and can take days if the sequencing facilities are outside the workplace (Hulin and Wheals, 2014). Gente et al. (2007) developed primer-pairs that are able to discriminate between a large number of different yeast species directly from the surface of smear-ripened cheese, among them *D. hansenii*, but there was no reproducible DNA amplification with the strain *D. hansenii* CBS 766 (one of the three assayed). Other methods such as RAPD and mtDNA have been proposed as an alternative to traditional characterization at species- and at intra-species levels (Nikolaou et al., 2007; Querol et al., 1992). However, Andrade et al. (2010) concluded that RAPD was not able to discriminate between species such as *D. hansenii* and *Candida zeylanoides* and though mtDNA was more reliable, and some of the isolates could be characterized directly as belonging to *D. hansenii* species, it failed to identify the majority. Corredor et al. (2000) developed two probes for *D. hansenii* var *hansenii*, but this

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Table 1

Yeast strains and PCR products obtained with the DhPadF/DhPadR pair of primers.

Species	Strains	Other collections	Origin	Amplification with DhPadF/DhPadR (400 bp)
				DhPadF/DhPadR (400 bp)
<i>Candida cretensis</i>	CECT 12038		Pork sausage	—
<i>Debaryomyces fabryi</i>	CBS 6066	ATCC 22262	Tanning fluid	+
	CECT 11365	CBS 4373	Dry white wine	—
	CECT 11370 ^T	ATCC 20278; CBS 789	Interdigital mycotic lesion	+
<i>Debaryomyces hansenii</i>	CBS 116		Cherries	+
	CBS 164		Cheese	+
	CBS 766	ATCC 10623	Cheese	+
	CBS 1102	CECT 11364	Beef-and-pork sausage	+
	CBS 1792	CECT 11363	Chilled beef	+
	PYCC 4745		Sea water	+
	CECT 10019		Frass on <i>Philadelphus coronarius</i>	+
	CECT 10026		Salt cod	+
	CECT 10038		Frass on <i>Alnus glutinosa</i>	+
	CECT 10517		Alpechin	+
	CECT 10284		Frass on <i>Cornus sanguinea</i>	+
	CECT 10352		Tomato	+
	CECT 10353		Tomato	+
	CECT 10360		Cheese	+
	CECT 10378		Date	+
	CECT 10386		Prune	+
	CECT 10414		Alpechin	+
	CECT 11369 ^T	NRRL Y-7426 ^T ; CBS 767 ^T	Carlsberg Laboratories	+
<i>Debaryomyces maramus</i>	CBS 4264	CECT 11371	Cider	—
	CECT 11362 ^T	ATCC 11627 ^T ; CBS 1958 ^T	Atmosphere	—
<i>Debaryomyces prosopidis</i>	CBS 8450 ^T	ATCC 201611 ^T	Exudate of <i>Prosopis juliflora</i> (mesquite tree)	—
	DBVPG 7012	ATCC MYA-255	Exudate of mesquite trees	—
<i>Debaryomyces subglobosus</i>	CBS 792 ^T	JCM 1989 ^T	Infected nail	—
	CBS 1796		Skin lesion	—
<i>Debaryomyces udonii</i>	CBS 7057	JCM 7856	Soil	—
<i>Hanseniaspora uvarum</i>	CECT 10142		Cucumber	—
	CECT 11105	CBS 2589	Grape must	—
<i>Kluyveromyces lactis</i>	CECT 1132	ATCC 48432	Brine	—
	CECT 10361		Cheese	—
<i>Meyerozyma guilliermondii</i>	CECT 1456 ^T	ATCC 46036 ^T ; CBS 2030 ^T	Insect frass on <i>Ulmus americana</i> (elm tree)	—
<i>Priceomyces carsonii</i> ^a	CECT 10227 ^T	ATCC 58371 ^T ; CBS 2285 ^T	Slime flux of <i>Quercus kelloggii</i> (black oak)	—
<i>Rhodotorula glutinis</i>	CECT 10145 ^T	ATCC 96365 ^T ; CBS 2367 ^T	Fruit of <i>Phyllodendron</i> sp.	—
<i>Saccharomyces cerevisiae</i>	ATCC 7754	CBS 1368	Fleischmann bakers yeast	—
<i>Schwanniomyces pseudopolymorphus</i> ^b	CECT 11360 ^T	ATCC 24211 ^T ; CBS 2008 ^T	Tanning fluid, prepared from bark of sweet-chestnut	—
<i>Schwanniomyces yamadai</i> ^c	CBS 7035 ^T	ATCC 56471 ^T ; CECT 11416 ^T	Soil of grassland	—
<i>Torulaspora delbrueckii</i>	ATCC 66821 ^T	CBS 1146 ^T ; CECT 11199 ^T	Unknown	—
<i>Wickerhamomyces anomalus</i> ^d	CECT 1114 ^T	ATCC 8168 ^T ; CBS 5759 ^T	Unknown	—
	CECT 12806		Grape juice	—
<i>Yarrowia lipolytica</i>	CECT 10363		Butter	—
<i>Zygosaccharomyces bailii</i>	CECT 1898 ^T	ATCC 58445 ^T ; CBS 680 ^T	Brewery	—
<i>Zygosaccharomyces cidri</i> ^e	CECT 10657 ^T	ATCC 36238 ^T ; CBS 4575 ^T	Cider	—
<i>Zygosaccharomyces fermentati</i> ^f	CECT 11056 ^T	ATCC 58446 ^T ; CBS 707 ^T	Sediment of peppermint beverage	—
<i>Zygosaccharomyces mellis</i>	CBS 1091	DBVPG 6460	Fermenting honey	—
	CECT 10127		Honey	—
<i>Zygosaccharomyces rouxii</i>	CECT 1232 ^T	ATCC 2623 ^T ; CBS 732 ^T	Concentrated must of black grape	—

ATCC: American Type Culture Collection; CBS: Centraalbureau voor Schimmelcultures; CECT: Colección Española de Cultivos Tipo; DBVPG: Collezione dei Lieviti Industriali — Industrial Yeasts Collection; JCM: Japan Collection of Microorganisms.

PYCC: Portuguese Yeast Culture Collection.

^T Type strain.

^a Formerly *Debaryomyces carsonii* (Kurtzman and Fell, 1998).

^b Formerly *Debaryomyces pseudopolymorphus* (Kurtzman and Fell, 1998).

^c Formerly *Debaryomyces yamadai* (Kurtzman and Fell, 1998).

^d Formerly *Pichia anomala* (Kurtzman and Fell, 1998).

^e Now *Lachancea cidri* (Kurtzman et al., 2011).

^f Now *Lachancea fermentati* (Kurtzman et al., 2011).

method requires a Dot blot analysis of DNA to recognize the yeast species that hybridize with the probes, which makes it more expensive and slower than our proposal.

The *PAD1* gene in *Saccharomyces cerevisiae* encodes for phenylacrylic acid decarboxylase which confers resistance to cinnamic acid (Clausen et al., 1994). Other putative *PAD1* homologues have been found in yeast species such as *Candida albicans*, *Candida dubliniensis*, *D. hansenii* and *Wickerhamomyces anomalus*. All of them presented decarboxylation of sorbic acid (Stratford et al., 2007). During a study carried out in our

laboratories on decarboxylation of sorbic acid in *D. hansenii* we observed that the type strain presented a putative homologous region of the *PAD1* gene of *S. cerevisiae*. As significant differences in the homologous regions of the two were found, we thought that this sequence could be a good target for the *D. hansenii* specific detection and extended the comparison to other *D. hansenii* strains and yeast species.

The aim of the present study was to develop a specific primer for a rapid and affordable identification of the yeast species *D. hansenii* based on this region.

Table 2

Yeast strains and PCR products obtained with the DhPadF/DhPadR pair of primers.

Strains	Isolation source	Amplification with DhPadF/DhPadR
<i>Candida zeylanoides</i>	Ca2 ^a	Spanish sausage "Chorizo"
<i>Debaryomyces fabryi</i>	PR 18 ^a	Meat
	PR 66 ^a	Spanish sausage "Chorizo"
	Qba	Cheese
<i>Debaryomyces hansenii</i>	CH2 ^a	Spanish sausage "Chorizo"
	CH3 ^a	Spanish sausage "Chorizo"
	CH4 ^a	Spanish sausage "Chorizo"
	CYC 1265	Canned aubergine
	EPEC 1.3 ^b	Cheese
	EPEC 2.1 ^b	Cheese
	EPEC 2.2 ^b	Cheese
	EPEC 3.1 ^{b,d}	Cheese
	EPEC 4 ^b	Cheese
	EPDI 6 ^{b,c}	Cheese
	Es 4 ^a	Marzipan
	J-1 ^a	Iberican ham
	J-9 ^a	Iberican ham
	J-11 ^a	Iberican ham
	J-12 ^a	Iberican ham
	J-14 ^a	Iberican ham
	J-15 ^a	Iberican ham
	J-16 ^a	Iberican ham
	J-17 ^a	Iberican ham
	J-18 ^a	Iberican ham
	J-19 ^a	Iberican ham
	J-20 ^a	Iberican ham
	3C1.1 ^b	Cheese
	3L1.1 ^b	Cheese
	3T1.1 ^b	Cheese
	3hgE ^b	Cheese
	29C1.1 ^b	Cheese
	29C1.2 ^b	Cheese
	29C2p ^b	Cheese
	29I1.2 ^{b,d}	Cheese
	29 Inf 1 ^b	Cheese
	PR5 ^a	Spanish sausage "Chorizo"
	PR11 ^a	Spanish sausage "Chorizo"
	PR 13 ^a	Meat
	Yaa ^{b,c}	Spanish pastry "Pionono"
<i>Issatchenkia orientalis</i> ^e	PR 3 ^a	Cheese
<i>Meyerozyma guilliermondii</i>	Mi1 ^{a,b}	Jam
<i>Torulaspora delbrueckii</i>	CYC 1176 ^{a,b}	Plain yogurt
<i>Wickerhamomyces anomalus</i> ^f	MA 11.2 ^{a,b}	Jam
	TYN 1.3 ^{a,b}	Nougat
<i>Zygosaccharomyces rouxii</i>	T2R ^{a,b}	Nougat

^a Identified by PCR-RFLP analysis of the IGS region of the rDNA.^b Identified by RFLPs of the 5.8S-ITS rDNA region.^c The D1/D2 26S rDNA gene sequences of these strains showed 100% sequence similarity with *Debaryomyces fabryi* CBS 789^T.^d The D1/D2 26S rDNA gene sequences of these strains showed 100% sequence similarity with *Debaryomyces hansenii* NRRLY-7426.^e Now *Pichia kudriavzevii* (Kurtzman et al., 2011).^f Formerly *Pichia anomala* (Kurtzman and Fell, 1998).

2. Materials and methods

2.1. Strain and culture conditions

A total of 94 strains were used in this work. Tables 1 and 2 show the yeast strains obtained from different Type Culture Collections, strains previously isolated and identified in our laboratory (Quirós et al., 2005; Romero et al., 2005) and strains recently identified for this work by PCR-RFLPs of the 5.8S-ITS rDNA region (Esteve-Zarzoso et al., 1999)

using primers ITS1 and ITS4 (White et al., 1990) and restriction enzymes *Hinf*I, *Hae*III and *Cfo*I. The patterns obtained were compared with the Yeast-id database (<http://www.yeast-id.com>). All strains were cultured at 28 °C in Yeast Morphology Broth and routinely maintained in the same culture medium plus Agar (YMA): 0.5% (w/v) yeast extract (Difco Laboratories, Detroit, Michigan, USA), 0.3% (w/v) Proteose-Peptone No. 3 (Difco), 0.3% (w/v) malt extract (Difco), 1% (w/v) glucose (Panreac Quimica S.A., Barcelona, Spain), and 2% (w/v) agar.

2.2. Primer design

The putative *PAD1* homologous region (729 bp) present in *D. hansenii* was used for primer design. The sequences were obtained from NCBI. The forward primer DhPadF, 5'-GCGACTATGAACAGGTTCC AACGA 3', was selected from nucleotides 101 to 125 and the reverse primer DhPadR, 5'-CCTTCAATGTAACATCAGCGGCC 3', from nucleotides 479 to 502 bp. Hairpin formation, 3'-complementarity and potential self-annealing sites were tested by Oligo Calc (<http://www.basic.northwestern.edu/biotools/OligoCalc.html>). The primers used were prepared by Conda Labs–Spain.

2.2.1. DNA template preparation and PCR conditions

The DNA template was obtained following the protocol described by Lööke et al. (2011) or from fresh colonies. PCR amplifications were performed in three different thermocycles and each strain was tried at least twice. The DNA amplifications were carried out in 25 µL reaction volume containing 100 ng of template DNA, 1.25 µL of each primer (20 µM), 2.5 µL of 10 × PCR buffer, 1 µL of MgCl₂ (50 mM), 0.2 µL of dNTPs (100 mM) and 0.2 µL of Taq DNA polymerase (5 U/µL) supplied by the manufacturer (Biotools, Madrid, Spain). Different annealing temperatures were tested, the lowest being 52 °C and the highest 68 °C. PCR conditions were as follows: initial denaturalization at 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 45 s at the T_m selected, 72 °C for 45 s; and then 1 cycle of 72 °C for 8 min. PCR-amplified DNA fragments were separated in 1% (w/v) agarose gels (Bio-Rad) stained with 0.05% (v/v) ethidium bromide (Bio-Rad) and visualized under UV light. The GeneRuler 100 bp Plus DNA Ladder (MBI Fermentas) was used as a molecular size marker.

3. Results

The primer pair designed for *D. hansenii* was based on the *S. cerevisiae* *PAD1/YDR538W* gene sequence (<http://www.yeastgenome.org>). Clustal Omega (McWilliam et al., 2013) was used for primer alignment. The target sites of the primers are sequences flanking a putative homologous region of the *PAD1* gene. This region presents a homology of 69% with the *PAD1* gene in *S. cerevisiae*. Subsequently, the suitability of the primers was confirmed by PCR amplification on the yeast strains listed in Table 1. We have tested the DhPadF/DhPadR primers in three different thermocycles and the results did not depend on the PCR machine. It is important to note that at every annealing temperature assayed, a positive result was obtained only with *D. hansenii*. By contrast, the closest outgroup, *D. fabryi*, gave negative results. The best result was obtained after 30 cycles and with an annealing temperature of 67 °C. A clear single fragment about 400 bp was produced by all *D. hansenii* strains. The remaining species of *Debaryomyces*, as well as other yeast species commonly found in foodstuffs, were not amplified with the *D. hansenii* specific primers (Table 1, Fig. 1). In addition, the specificity of the PCR assay developed in this work was tested on i) yeast strains previously identified in our laboratory by RFLP of the IGS region (Quirós et al., 2006; Romero et al., 2005), and ii) on other isolates from several contaminated or spoiled foods identified by PCR-RFLP-5.8S ITS in this work, marked in Table 2 with an ^a. All the *D. hansenii* strains gave positive results with amplicons of 400 bp (Table 2) with two exceptions; *D. hansenii* EPDI6 and *D. hansenii* Yaa, identified by PCR-RFLP-5.8S ITS. To confirm the identification the D1/D2 domain of 26SrDNA has been

sequenced. The results confirmed that these two strains do not belong to *D. hansenii* and should instead be included in the species *D. fabryi*. Neither *D. fabryi* nor *D. subglobosus* profiles are included in the yeast-id data base which complicates their identification. Fig. 2 shows the identical profiles obtained by *Hae*III PCR-RFLP-5.8S ITS for *D. hansenii*, *D. fabryi* and *D. subglobosus* for the strains EPDI6 and Yaa. Identical profiles for all strains were also obtained with *Cfo*I and *Hin*fI (data not shown).

4. Discussion

In some laboratories, common techniques such as the RFLPs of the 5.8S ITS rDNA region are a routine practice (Padilla et al., 2014). In this work we confirm that these RFLPs produce the same profiles in *D. hansenii*, *D. fabryi* and *D. subglobosus* and therefore lead to misidentifications.

Previously, in our laboratory we have been able to identify *D. hansenii* and to discriminate among *Debaryomyces* species (Quirós et al., 2006; Romero et al., 2005) through PCR-RFLP of the IGS region (rDNA), which, although precise, is more expensive and time consuming than a rapid identification with species-specific primers.

The putative *PAD1* homologous sequence of *D. hansenii* species, especially in the primer regions, is quite different across available yeast species. Namely, when the sequence on which the primers were designed was introduced in the nucleotide-BLAST it only detected itself on the genomic copy, LOCUSXM_46154. Unfortunately, close outgroups are not available.

The primers DhPADF/ DhPADR developed in this assay produced a clear single fragment of 400 bp in all of the *D. hansenii* strains tested; no false negatives were detected. These primers always identify *D. hansenii* irrespective of the temperature used. Nevertheless, the optimal temperature for this new assay is 67 °C (a restrictive temperature) because at this temperature we obtained more defined bands. On the other hand, no false positives were found in the other 22 species from Culture Collections such as *Torulaspora*, *Hanseniaspora*, *Zygosaccharomyces* that are commonly found in foods or from other origins (Tables 1 and 2 and Fig. 1). In fact, it was remarkable to verify that no amplification was obtained in *D. fabryi* (formerly *D. hansenii*

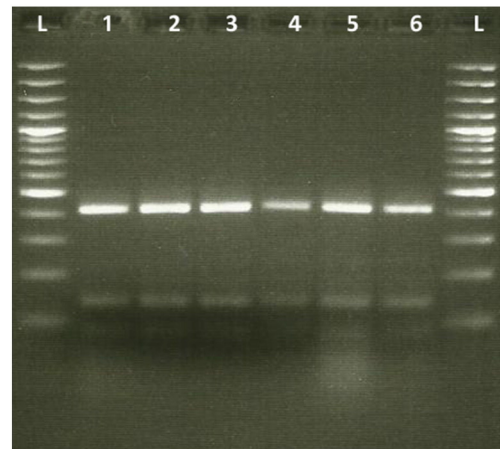


Fig. 2. ITS-PCR RFLP profiles of *Debaryomyces* strains digested with restriction enzyme *Hae*III. L: 100 bp ladder. Lane 1: *D. fabryi* EPDI6, Lane 2: *D. hansenii* EPEC1.3, Lane 3: *D. fabryi* Yaa, Lane 4: *D. subglobosus* CBS 792^T, Lane 5: *D. hansenii* CECT11369^T, and Lane 6: *D. fabryi* CECT11370^T.

var *fabryi*), *D. subglobosus* (formerly *D. hansenii* var *fabryi*) as well as in other yeast species formerly included in the genus *Debaryomyces* (Kurtzman and Fell, 1998). The revision of the genus addressed recently (Kurtzman et al., 2011) makes it necessary to review the identity of many strains in private collections. Common methods may lead to mistakes and for modest laboratories sequencing a large number of strains can be expensive and slow. Also, it is important when the search is directed at one species, in this case *D. hansenii*, which may be a small fraction of all isolated yeasts.

As mentioned in the Introduction, RFLPs of the 5.8S-ITS rDNA region provided excellent results for other species, but did not differentiate between *D. hansenii* and *D. fabryi*, as we confirmed in work previously reported by Cano-Garcia et al. (2013) for *D. fabryi* and for other *Debaryomyces* species (Martorell et al., 2005). In fact, two misidentifications have been detected with this new method; EPDI6 and Yaa have now been confirmed by sequencing as *D. fabryi* strains. Little is known

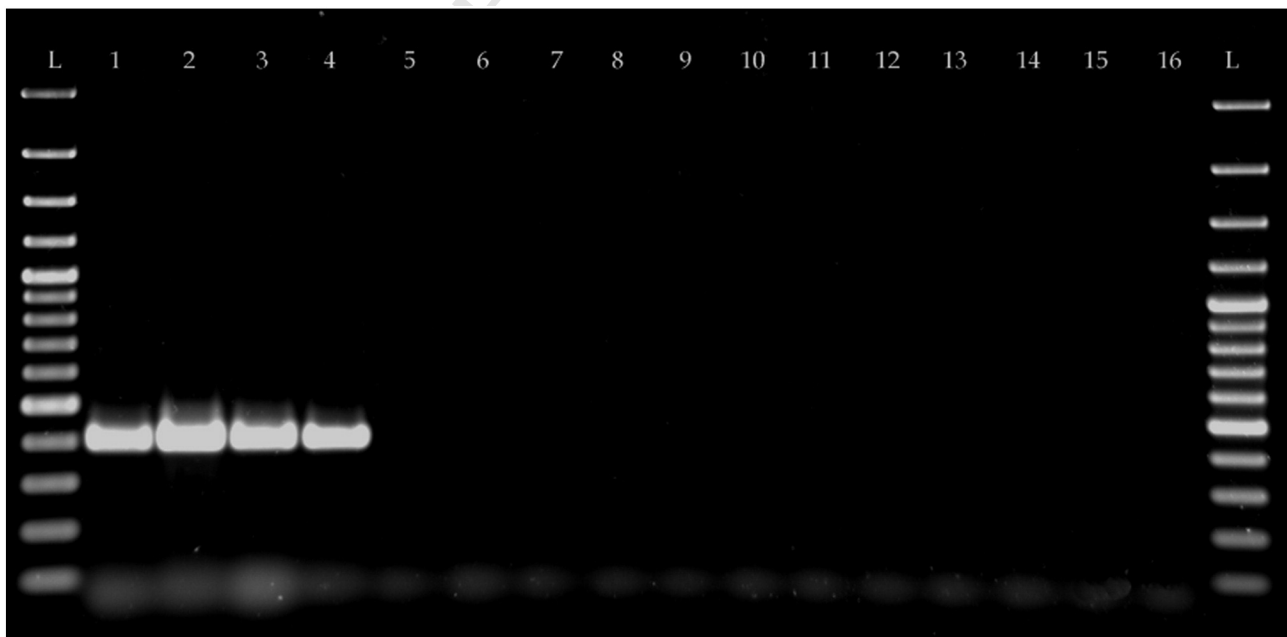


Fig. 1. Amplification results obtained with primer pair DhPADF/DhPADR. L: 100 bp ladder. Lanes 1–4: *Debaryomyces hansenii* CECT 10414, CBS 1792, CBS 164, CBS 1102, respectively. Lanes 5–16: *D. fabryi* CECT 11365, *D. maramus* CBS 4264, *Zygosaccharomyces bailii* CECT 1898^T, *Z. fermentati* CECT 11056^T, *Meyerozyma guilliermondii* Mi1, *Yarrowia lipolytica* CECT 10363, *Candida cretensis* CECT 12038, *Hanseniaspora uvarum* CECT 11105, *Torulaspora delbrueckii* CBS 1146^T, *Kluyveromyces lactis* CECT 10361, *Issatchenkia orientalis* PR3 and negative control, respectively.

about the ecology of *D. fabryi*, the majority of the isolates come from clinical sources, but around 40% come from foods (Kurtzman et al., 2011; Wrent et al., 2014). Our strains (EPD16 and Yaa) contribute to increasing this number.

Furthermore, the primers also recognized *D. hansenii* CBS 766, the identity of which was questioned by Gente et al. (2007) because the primer design by them failed to amplify in this strain.

The primers developed in this work can be used directly on colonies with 100% success rate. This would save users considerable time. However, we recommend the DNA extraction method described by Lööke et al. (2011) because the extracted DNA can also be stored and used for future PCR amplifications. No enzymes are required or extreme temperatures, and in 15 min the DNA extracted is suitable for PCR amplification of a large variety of yeasts.

Routine identification should be fast and reasonably priced. The assay here proposed is a rapid and affordable method that enables the identification of *D. hansenii* among all of the strains isolated from different foodstuffs and, in addition, institutional or individual culture collections might use this assay to achieve a rapid confirmation or re-identification of *D. hansenii* strains.

Acknowledgments

The authors thank the CECT (Spanish Type Culture Collection) for the free access provided to the yeast-Id database. Research by Eva Rivas has been supported by a PICATA predoctoral fellowship (CEI Campus Moncloa, UCM-UPM, Madrid, Spain). Research by Elena Gil de Prado has been supported by a FPU predoctoral fellowship by the Spanish Ministry of Education, Culture and Sports.

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