



ORIGINAL ARTICLE

PCR-based method for the rapid identification of astaxanthin-accumulating yeasts (*Phaffia* spp.)



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KEYWORDS

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Abstract It has been recently found that the natural distribution, habitat, and genetic diversity of astaxanthin-producing yeasts (*i.e.* *Phaffia rhodozyma*, synonym *Xanthophyllomyces dendrorhous*) is much greater than previously thought. *P. rhodozyma* is biotechnologically exploited due to its ability to produce the carotenoid pigment astaxanthin and thus, it is used as a natural source of this pigment for aquaculture. *P. rhodozyma* was also capable of synthesizing the potent UVB sunscreen mycosporine-glutaminol-glucoside (MGG). Therefore, further environmental studies are needed to elucidate its ecological aspects and detect new potential strains for the production of astaxanthin and MGG. However, obtaining new isolates of *P. rhodozyma* and related species is not always easy due to its low abundance and the presence of other sympatric and pigmented yeasts. In this work we report a successful development of a species-specific primer which has the ability to quickly and accurately detecting isolates representing all known lineages of the genus *Phaffia* (including novel species of the genus) and excluding closely related taxa. For this purpose, a primer of 20 nucleotides (called PhR) was designed to be used in combination with universal primers ITS3 and NL4 in a multiplex amplification. The proposed method has the sensitivity and specificity required for the precise detection of new isolates, and therefore represents an important tool for the environmental search for novel astaxanthin-producing yeasts.

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PALABRAS CLAVE

Astaxantina;
Micosporina;
Xanthophyllomyces;
Levaduras

Un método basado en la PCR para la identificación rápida de levaduras acumuladoras de astaxantina (*Phaffia* spp.)

Resumen Recientemente, se ha encontrado que la distribución natural, el hábitat y la diversidad genética de levaduras productoras de astaxantina (p. ej., *Phaffia rhodozyma*, sinónimo *Xanthophyllomyces dendrorhous*) son mucho mayores de lo que se pensaba. *P. rhodozyma* se

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explota biotecnológicamente debido a su capacidad para producir el pigmento carotenoide astaxantina y, por lo tanto, se utiliza como una fuente natural de este pigmento para la acuicultura. También se encontró que esta levadura es capaz de sintetizar el potente protector solar UVB micosporina-glutaminol-glucósido (MGG). Por lo tanto, más estudios ambientales para dilucidar sus aspectos ecológicos y detectar nuevas cepas potenciales productoras de astaxantina y MGG son necesarios. Sin embargo, la obtención de nuevos aislamientos de *P. rhodozyma* y especies relacionadas no siempre es fácil debido a su baja abundancia y a la presencia de otras levaduras simpátricas y pigmentadas. En este trabajo se describe el desarrollo exitoso de un cebador especie-específico que tiene la capacidad de detectar rápidamente y con precisión cepas representativas de todos los linajes del género *Phaffia* previamente reportados (incluyendo nuevas especies del género) y excluir especies estrechamente relacionadas. Para ello, se diseñó un cebador de 20 nucleótidos (denominado PhR) para ser utilizado en combinación con los cebadores universales ITS3 y NL4 en una amplificación multiplex. El método propuesto tiene la sensibilidad y la especificidad requerida para la detección precisa de nuevos aislamientos y, por lo tanto, representa una importante herramienta para la búsqueda ambiental de nuevas levaduras productoras de astaxantina.

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Introduction

Phaffia rhodozyma (also known as *Xanthophyllomyces dendrorhous*) is a basidiomycetous yeast, forming orange-red colonies and known worldwide since, until now, it is the only yeast capable of producing the carotenoid pigment astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione). This compound is of economic importance because it is the most expensive feed component for aquaculture and aviculture⁸. Moreover, *P. rhodozyma* is the only known carotenogenic yeast species that is able to vigorously ferment a number of sugars, including glucose, maltose, sucrose and raffinose⁹. Moreover, it has recently been discovered that this yeast has the ability to synthesize a UV-absorbing molecule called mycosporine-glutaminol-glucoside of applied interest¹⁴.

The natural distribution and habitat of *P. rhodozyma* are much broader than previously thought. Initially, genetically similar isolates of this yeast were found in the Northern Hemisphere in association with slime exudates of trees, being obtained from Japan, Canada, Russia, Italy, Germany and USA^{3,22,27,30}. A novel and genetically distinct *Phaffia* population was later isolated in the Southern Hemisphere, from the sugary stromata of the *Cyttaria hariotti* fungus, a parasite of the *Nothofagus* trees in Argentina¹⁵. An even more genetically divergent strain was described in Chile, which might represent a novel species although a single strain is known²⁹. More recently, David-Palma et al.² investigated the association of *Phaffia* with *Nothofagus-Cyttaria* in Australasia, the other region of the world where *Nothofagus* are endemic, and discovered an even higher *Phaffia* diversity, including two endemic and markedly divergent lineages which represent putative new species, both with the capability to produce astaxanthin and MGG (unpublished results). In this work it was suggested that *Phaffia* adaptation to different tree hosts/niches has driven population structure, thus, it can be anticipated that novel lineages of this yeast might

be yet undiscovered. Proof of this fact are the recent isolates found by Yurkov et al.³³, and Contreras et al.¹ from soil and Antarctic-related environments, respectively. In this scenario and due to the biotechnological relevance of this genus, specific culture media and biochemical techniques that allow a more favorable isolation and simpler identification of *P. rhodozyma* were reported in previous works^{2,13,26}. This is particularly important considering that the environmental isolation of *P. rhodozyma* is not always easy due to its low abundance and because it is easily misidentified with other sympatric and carotenoid-accumulating yeasts of the genera *Rhodotorula*, *Cystofilobasidium* and *Dioszegia*¹⁶. Not many yeast species are able to produce carotenoid pigments and these are distributed among a few lineages¹⁸. Those species accumulating mainly xanthophyll-like carotenoids that produce orange-type colonies (*Cystofilobasidium* and *Dioszegia*) have higher probabilities of misidentification with astaxanthin-producing yeasts like *Phaffia*. Other sympatric species such as many *Rhodotorula* spp. or few *Cryptococcus* spp. can produce salmon-pink or intense red colonies depending on the relative level of accumulation of less polar carotenoids like torulene, torularhodine and/or β -carotene¹⁸.

The objective of this work was to provide a rapid and accurate PCR-based detection method for astaxanthin-accumulating yeast isolates from environmental samples.

Materials and methods

Yeast strains

Seven representative strains of the different *Phaffia* spp. clades described in David-Palma et al.² were used in this study (Table 1). Strains ZP938 and ZP875 were kindly provided by Dr. J. Sampaio (UNL, Portugal). The *Phaffia*-related

Table 1 List of yeast strains tested with *P. rhodozyma* specific primers

Species	Strain	Lineage ^a	Origin	Substrate	Reference
<i>Phaffia rhodozyma</i> (<i>Xanthophyllomyces dendrorhous</i>)	CRUB 1149	A	Bariloche, Argentina	Water sample near <i>C. hariatii</i> on <i>N. pumilio</i>	15
	ZP 874	B	Tasmania, Australia	<i>C. gunnii</i> on <i>N. cunninghamii</i>	2
	CBS 5905	C1 and B (hybrid strain)	Kyoto, Japan	Exudate of <i>Fagus crenata</i>	Type strain of <i>P. rhodozyma</i> ¹⁷
	CBS 7918 ^T	C2	Moscow, Russia	Exudate of <i>Betula verrucosa</i>	Type strain of <i>X. dendrorhous</i> ⁶
	ATCC 24229	D	Hiroshima, Japan	Exudate of <i>Cornus brachypoda</i>	17
<i>Phaffia</i> sp. I	ZP 938	E	Queensland, Australia	Leaves of <i>N. mooreii</i>	2
<i>Phaffia</i> sp. II	ZP 875	F	Tasmania, Australia	<i>C. gunnii</i> on <i>N. cunninghamii</i>	2
<i>Phaffia</i> sp.	TSN-67	n.a.	Baden-Württemberg, Germany	Soil adjacent to <i>Picea abies</i> and <i>Fagus sylvatica</i>	33
<i>Cystofilobasidium capitatum</i>	CBS 7420	n.a.	France	<i>Larus marinus</i> (great black-backed gull)	20
<i>Cystofilobasidium macerans</i>	CBS 2206	n.a.	Unknown	Dew-retted flax straw	12

C., *Cyttaria*; N., *Nothofagus*; n.a., Not applicable.

^a Following David-Palma et al.².

strain TSN-67 (astaxanthin and MGG non-producing, unpublished results) recently reported by Yurkov et al.³³ was kindly provided by the author (A. Yurkov) and was tested together with two species of the genus *Cystofilobasidium*. The latter species were included as controls due to their phylogenetic proximity to *P. rhodozyma* (also belonging to the order Cystofilobasidiales), habitat co-occurrence and similar appearance of their colonies.

Primer design

Phaffia spp. specific primer was designed based on concatenated sequences of internal transcribed spacers (ITS) and D1D2 partial sequences of 20 *P. rhodozyma* strains (available at GenBank by May, 2011) and 5 *Cystofilobasidium* spp. Using the NCBI primer BLAST tool and then checked using Primer3. Both widely used ITS3 and NL4 and new *Phaffia* spp. specific primers are shown with full detail in Table 2.

Phylogenetic analysis

rRNA gene sequences corresponding to the internal transcribed spacers 1 and 2 (ITS 1 and ITS 2), 5.8S rRNA of all the strains depicted in Table 1 were obtained from NCBI. DNA sequences alignment and analyses were performed using MEGA 5.0²⁴. Alignments were carried out using CLUSTALW²⁵ and were edited manually when required. Phylogenetic analysis using Neighbor Joining were performed in MEGA 5.0, using the Kimura 2-parameter model as substitution model.

The reliability of the NJ trees was assessed by bootstrap analysis including 1000 replications.

Cell and molecular methods

Cell growth was performed on YMA agar plates (3 g/l yeast extract, 3 g/l malt extract, 5 g/l bactopectone, 10 g/l glucose and 15 g/l agar), DNA was extracted using the protocol described previously in Libkind et al.¹¹ and purified using chloroform:isoamyl alcohol reagent (24:1, v/v). DNA was quantified using Shimadzu UV-1800 spectrophotometer and Multiplex PCR assays were performed in a Labnet MultiGene Gradient thermocycler, under the following conditions: denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C 30s, 55 °C 1 min, 72 °C 1 min, and a final extension at 72 °C for 7 min. PCR amplification was performed in a total volume of 25 µl, containing about 50 ng of total DNA, 1× GoTaq PCR buffer (Promega), 200 µM each dNTP (GE Healthcare), 1 U Taq polymerase (GoTaq, Promega) and 0.3 µM each primer. Gel electrophoresis was performed on 1% agarose in 0.5× TBE, 90 V for 30 min.

A 100 bp DNA size ladder (Highway) plus a lambda DNA digested with HindIII (Promega) were used as a molecular marker.

Results

A single primer of 20 nucleotides called PhR (Table 2) was designed to be used in combination with the universal primers ITS3³¹ and NL4¹⁰ to perform a multiplex

Table 2 Set of primers used in this work

Primer	Sequence	Reference
ITS3	5'-GCA TCG ATG AAG AAC GCA GC-3'	White et al. ³¹
PhR	5'-GAC TTG TAC ACA GGC CGG CA-3'	This work
NL4	5'-GGT CCG TGT TTC AAG ACG G-3'	Kurtzman and Robnett ¹⁰

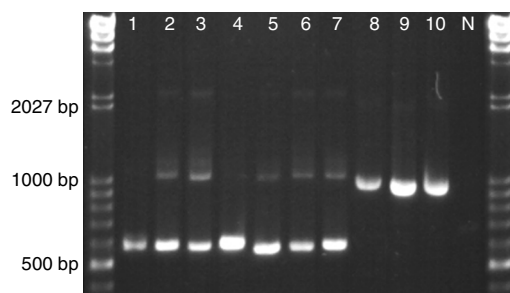


Figure 1 Yeast strains tested with multiplex PhR, ITS3 and NL4. 1–7 *P. rhodozyma*: (1) CBS 5905 (Lineage C1/B); (2) CBS 7918^T (Lineage C2); (3) CRUB 1149 (Lineage A); (4) ATCC 24229 (Lineage D); (5) ZP 938 (Lineage E); (6) ZP 875 (Lineage F); (7) ZP 874 (Lineage B); (8) TSN-67; (9) *C. capitatum* CBS 7420; (10) *C. macerans* CBS 2206; N – negative control (no template).

amplification reaction that leads to the rapid detection of astaxanthin-producing yeasts of the genus *Phaffia*. The ability of this method to detect all proposed lineages of *Phaffia* sp. and at the same time retain the sufficient specificity to avoid false positives was evaluated. For this purpose, an up-to-date set of eight representative strains of all known *Phaffia* and *Phaffia*-related lineages were assayed, including the *Phaffia* sp. TSN-67 isolate reported by Yurkov et al.³³ from soil and representative strains of the seven different lineages recently described by David-Palma et al.². Additionally, *Cystofilobasidium capitatum* and *Cystofilobasidium macerans* were included due to their phylogenetic proximity and, since they inhabit similar substrates and grow in morphologically similar colonies, they can be frequently confused with *Phaffia*^{4,5,22,27,28}.

The reverse PhR primer was designed to anneal in the conserved D1D2 domains of the 26S rRNA gene and to generate a 643 bp amplicon when it is used together with the universal forward primer ITS3. The universal reverse primer NL4 was included for amplification control since it will produce 1155 bp in any eukaryotic microorganism. When tested in our set of astaxanthin-producing strains we observed the specific *Phaffia* band (~640 bp) for all *P. rhodozyma* lineages and even for strains of group E and F, which represent novel species of the genus *Phaffia* (Fig. 1). In most of these cases, the control band (~1150 bp) was also present though displaying a weak signal.

The *Phaffia* sp. strain TSN-67³³ was negative for the specific primer. Two nucleotide differences were detected in the 3' end of the specific primer for this strain explaining the lack of amplification (Table 3), which is in accordance with its relative distant phylogenetic relationship with the *Phaffia* clade (Fig. 2). Based on our phylogenetic analysis, the strain belongs to the order Cystofilobasidiales

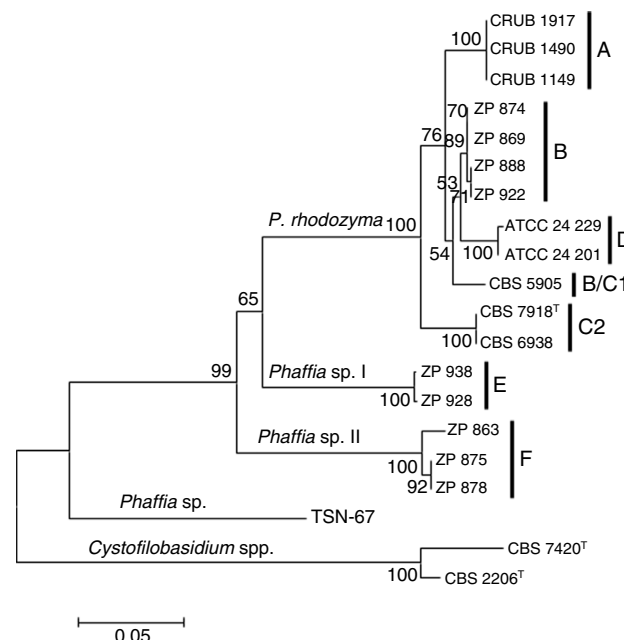


Figure 2 Neighbor joining phylogenetic tree of *Phaffia*, based on internal transcribed spacer sequences. Bootstrap values (1000 replicates) and lineages are indicated. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The outgroup is constituted by *Cystofilobasidium capitatum* (CBS 7420) and *Cystofilobasidium macerans* (CBS 2206).

(Tremellomycetes, Agaricomycotina), occupying a basal position and having *P. rhodozyma* as the closest match, but showing more than fifty substitutions in the ITS region.

Carotenogenic species of the genus *Cystofilobasidium* were negative for the test since the specific band was absent due to the presence of 3 or more nucleotide substitutions mostly in the 3' end. An environmental strain of *Dioszegia* spp., which was also tested, was negative (data not shown). *In silico* analysis of the remaining 5 *Cystofilobasidium* species, all *Dioszegia* known species and representative species of pigmented yeasts of the genus *Rhodotorula*, *Rhodospiridium* and *Cystobasidium* (ex-*Rhodotorula*) showed that at least 3 nucleotide differences were present in all cases (Table 3). *Cystofilobasidium* species were those showing the lower number of substitutions while *Dioszegia* species differed in 4 or 5 substitutions. With the exception of *Cystobasidium minutum* (Cystobasidiales) that had only 3 substitutions, the red yeasts of *Rhodotorula* and *Rhodospiridium* had 6 or more substitutions.

Table 3 PhR primer specificity based on the nucleotide sequence of the annealing region

Specie	Sequence	Number of mismatches
<i>P. rhodozyma</i>	5'-GAC TTG TAC ACA GGC CGG CA-3'	0
TSN-67	5'-GAC TTG TAC ACA GGC CGA CG-3'	2
<i>Cystofilobasidium</i> spp. ^a	5'-GAC TTG TAC ACA GGC CAA CG-3'	3
<i>Cystobasidium minutum</i> ^b	5'-GAC TCA TAC ACG GGC CGG CA-3'	3
<i>Dioszegia</i> spp. ^c	5'-GAC TTA GAC ACG GTC CGG CA-3'	4

^a All species of *Cystofilobasidium* have at least 3 substitutions.

^b ex *Rhodotorula minuta*³².

^c All known species of *Dioszegia* have at least 4 substitutions (tested *in silico*).

Discussion

Based on our results, the specific primer PhR, coupled with the universal primers ITS3 and NL4, has the required sensitivity and specificity for proper detection of relevant astaxanthin-accumulating yeasts of the genus *Phaffia*, here represented by all seven lineages recently described by David-Palma et al.². Despite the wide genetic diversity of this group of yeasts, the test detected all desired strains, differentiating them from other co-occurring, also carotenogenic and closely related yeast species, such as members of the genus *Cystofilobasidium* (Fig. 1).

The specificity of the method for astaxanthin producing strains is based on the complete homology of the PhR primer to the 5' portion of the D1D2 domains of the large ribosomal subunit (26S), and the presence of 2 or more nucleotide differences in the 3' end for distantly related strains (TSN-67) and *Cystofilobasidium* spp.

In a previous report, a new and innovative strategy for improving *P. rhodozyma* recovery rate in environmental samples was obtained²⁶, which was successfully employed in the work of David-Palma et al.². A rapid identification method based on the simultaneous presence of astaxanthin and mycosporines was also described as a helpful tool for the screening of *P. rhodozyma* in a large set of new isolates. Although useful, this biochemical test is not as precise and reliable as DNA-based methods, hence we developed here a new molecular strategy (multiplex PCR reaction) for the accurate and rapid detection of astaxanthin-producing yeasts among environmental isolates or even for a rapid identity check of laboratory or production strains. In this regard, several successful developments with similar strategies for the molecular identification of *Saccharomyces* spp. and *Zygosaccharomyces* spp. have been reported^{7,19,21,23}.

The use of three primers in a multiplex reaction has the advantage of still obtaining an amplicon in a negative sample, therefore, the results are unambiguous and cannot be attributable to a failure in the amplification reaction. The cost, complexity and time required for the test is low enough to be performed in most microbiology labs in a routine manner and could be even expanded to perform a colony PCR assay. Our own experience shows rRNA gene amplification (such as the one implied here) of *Phaffia* strains and other basidiomycetous yeasts can be easily achieved using the direct colony PCR method described by Espinar et al.³.

Ecological and biodiversity studies in new environments have led to the description of highly divergent astaxanthin-producing yeasts that were hitherto unknown.

The biotechnological relevance of these new genetic lineages remains to be studied; however, the fact that other genetically different strains of *Phaffia* might be out there has been proved. Contreras et al.¹ recently obtained novel strains of *P. rhodozyma* from Antarctic environments, one of which showed improved astaxanthin production with respect to the average yields of wild strains. The methods described earlier^{13,26} in combination with the molecular method presented here should represent excellent tools for the successful hunting of more astaxanthin-accumulating yeasts in the wild.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this investigation.

Confidentiality of data. The authors declare that no patient data appears in this article.

Right to privacy and informed consent. The authors declare that no patient data appears in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

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