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Identification of two yeast strains from oil-polluted environment by RFLP on ITS-5.8S rDNA and RAPD analyses

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Abstract

Two yeast strains DP3 and DP4, isolated from oil-polluted environment and previously studied using morpho-physiological tests, presented 91.40% similarity with Issatchenkia orientalis CBS5147 (DP3) and, respectively, 83.80% similarity with Rhodotorula glutinis CBS20T (DP4). For a more accurate characterization, we performed restriction of the amplified region of ITS1-5,8S-ITS2, and compared the results with those from laboratory reference yeast strains, MYCOBANK and similar experiments described in literature. Strain DP3 ITS-PCR and ARDRA profiles were almost identical with I. orientalis CMGB224 and I. orientalis CBS5147, while an obvious similarity was observed between the DP4 patterns and those from R. glutinis ICCF and R. glutinis CBS20T. For the RAPD studies, we used eight primers: OPA01, OPA03, OPA10, SOY, RP4-2, M13 for DP3, and M13, OPH15, OPH19 for DP4. Further dendograms were constructed using UPGAMA method from Quantity One (Bio-Rad) program and OPA03 profiles for DP3, DP4 and reference yeast strains. The dendrograms revealed a high degree of relatedness between DP3 and Issatchenkia orientalis CMGB224, DP4 and Rhodotorula glutinis ICCF.

Keywords: yeasts, oil-polluted environment, ITS-5.8S rDNA, RAPD, UPGAMA.

Introduction

Bioremediation represents the stimulation of biodegradation, as an alternative technology for restoring polluted environments [1]. Microorganisms (bacteria and yeasts) are subjects of many bioremediation studies, mainly due to their ability of assimilating a large range of compounds, some of the most important being represented by oil (petrolleum) components, such as hydrocarbons (n-alkanes, cycloalkanes), phenol and its derivates or polycyclic aromatic hydrocarbons.

Even though there are many data regarding the bacteria species used in biodegradation of oil spills, there is rather poor knowledge on yeasts involved in xenodegrading processes. Until now, the yeast species described as being able to use hydrocarbons as sole carbon source, belong especially to the genera *Candida* [2, 3], *Clavispora, Debaryomyces, Leucosporidium, Lodderomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Sporidiobolus, Sporobolomyces, Stephanoascus, Trichosporon* and *Yarrowia* [4]. Therefore, one of the main problems in developing new bioremediation strategies, is a better identification and taxonomical classification of new yeast strains with biodegrading abilities. Besides DNA sequencing, some of the most reliable and extensively used techniques for investigation of the molecular structure of the genetic material and for obtaining information

of taxonomical value, are the restriction analysis of the amplified rDNA (PCR-RFLP analysis of rDNA) and the random amplification of polymorphic DNA (RAPD).

The two yeast strains used in the present study, *DP3* and *DP4*, have been previously analysed using classical taxonomy techniques, guanine plus cytosine content determination, and ability of growing on n-alkanes as sole carbon sources [5]. For a more accurate characterization, we performed ARDRA analysis of the amplified region of ITS1-5,8S-ITS2 and we studied the RAPD patterns using eight primers. The results were correlated with those obtained or described in literature during similar experiments on reference yeast strains.

Material and Methods

Yeast strains and media

The two yeast strains *DP3* and *DP4* were isolated from oil-polluted environment from Pitesti area (Romania). Reference strains used: *Candida parapsilosis* CBS604 and *Saccharomyces cerevisiae* L5366 (Faculty of Natural Sciences, Comenius University, Slovakia), *Kluyveromyces lactis* CBS2359/152 (Institut de Genetique et Microbiologie, Universite Paris-Sud, France), *Candida boidinii* ICCF (National Institute for Chemical and Pharmaceutical Research and Development, Bucharest, Romania), *Rhodotorula glutinis* ICCF, *Yarrowia lipolytica* ICCF, *Candida krusei* CMGB94 (Center for Research, Consulting and Training in Microbiology, Genetics and Biotechnology, Faculty of Biology, University of Bucharest, Romania), *Candida guilliermondii* CMGB44, *Candida tropicalis* CMGB165, *Issatchenkia orientalis* CMGB224. Yeasts were maintained at -70^{o}C on yeast peptone glucose (YPG) medium (yeast extract 5 g/l, peptone 10 g/l, glucose 20 g/l) supplemented with 20% glycerol. Previous to taxonomical tests, yeast were cultured on YPG slants, and grown for 18 hours, at 30^{o}C

Genomic DNA isolation and purification

Genomic DNA isolation and purification was performed using as starting point the method described by Ausubel et al. [6]. Yeast strains were cultivated for 18 h on YPG medium, centrifuged and the cell pellet was resuspended in 375 μl TEG (25 mM Tris, 10 mM EDTA, 50 mM glucose, pH 8). Cell walls were lysed with 5 μl β-mercaptoethanol (incubation 30 min at 37^{o}C) and zymolyase 20T in final concentration of 0.4 mg/ml for strain *DP3* with incubation 60 minutes at 37^{o}C and 0.8 mg/ml for *DP4* with incubation 90 minutes at 37^{o}C/-20 ^{o}C /37 ^{o}C. For total cell lysis, protoplasts were resuspended in 300 μl TEG and 33 μl 10% SDS. Deproteinization was performed by protease K treatment (200 μg/ml, incubation 30 min at 37^{o}C) and 2.5 M KCl. After centrifugation, the supernatant was mixed with fenol:chloroform:isoamylic alcohol (25:24:1 v/v/v), followed by RNase A treatment (100 μg/ml, incubation 30 min at 37^{o}C). After a new deproteinization with chloroform:isoamylic alcohol (24:1 v/v), DNA was precipitated with isopropilic alcohol at room temperature and resuspended in 40 μl TE (10 mM Tris, 1 mM EDTA, pH 8.0).

PCR-RFLP analysis of ITS-5.8S rDNA

The ITS1-5,8S-ITS2 region was amplified in a PTC150-MiniCycler^{TM} (MJ RESEARCH) using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers. In a total volume of 50 μl PCR mixture, we used 1 μl genomic DNA, 1,2 μM of each primer (ITS1 and ITS4), 5U RedTaq polymerase (SIGMA) and 0.2 mM of each dNTP. PCR conditions were as follows: initial denaturation 3 minutes at 95^{o}C, 35 cycles of denaturating 1 minute at 95^{o}C, annealing 2 minutes at 50^{o}C, extension 1 minute at 72^{o}C, and a final extension for 10 minutes at 72^{o}C.

The PCR products were digested with restriction endonucleases *Cfo* I (5'-GCG/C-3'), *Dde* I (5'-C/TNAG-3'), *Hae* III (5'-GG/CC-3'), *Hinf* I (5'-G/ANTC-3') and *Hpa* II (5'-C/CGG-

3') (10U/μl, PROMEGA). The amplicons and the restriction fragments were separated by agarose gel electrophoresis using 2% agarose and TBE 0.5X at 3 V/cm. After electrophoresis, gels were stained with ethidium bromide, visualized under UV light (UV-VIS Spectrophotometer) and digitalized.

Additional comparative analysis of amplicons and restriction fragments was done using online data from international reference yeast strains retrieved from MYCOBANK (http://www.mycobank.org/DefaultPage.aspx) and the BioloMICS software (www.bio-aware.com).

RAPD assay

RAPD was performed in a PTC150-MiniCycler^{TM}MJ RESEARCH, in a total reaction volume of 25 μl containing: 1 μl genomic DNA, 4 μM primer, 1U Taq polymerase (SIGMA) and 0.4 mM of each dNTP. The amplification program was 3 minutes at 94^{o}C, 45 cycles of 1 minute at 94^{o}C, 1 minute at 36^{o}C and 2 minutes at 72^{o}C, and a final extension of 7 minutes at 72^{o}C. We used eight primers: SOY (5'-AGGTCACTGA-3'), RP4-2 (5'-CACATGCTTC-3'), M13 (5'-AGGGTGGCGGTTCT-3'), OPA01 (5'-CAGGCCCTTC-3'), OPA03 (5'-AGTCAGCCAC-3'), OPA10 (5'-GTGATCGCAG-3'), OPH15 (5'-AATGGCGCAG-3') and OPH19 (5'-CTGACCAGCC-3'). The RAPD fragments were analyzed in 1.2 % agarose gels in TBE 0.5X; electrophoresis was run for 3 hours at 3 V/cm.

Dendrograms based on the RAPD profiles obtained with OPA03 primer were constructed using the UPGAMA method from the computer program Quantity One (BIO-RAD).

Results and Discussions

PCR-RFLP analysis of ITS-5.8S rDNA

In yeast taxonomy, enzymatic digestion of internal transcribed spacer regions of rDNA amplicons obtained by PCR allowed interspecific differentiation for many yeast species belonging to *Candida, Saccharomyces, Kluyveromyces, Pichia* and *Zygosaccharomyces* genera [7, 8, 9].

In a first place, a comparative analysis was done using BioloMICS online software from MYCOBANK and the results from morpho-physiological tests on *DP3* and *DP4*. The polyphasic identification revealed 91.40% similarity between *DP3* and *Issatchenkia orientalis* CBS5147, respectively, 83.80% similarity between *DP4* and *Rhodotorula glutinis* CBS20T. In a second step, genomic DNA isolated from *DP3* and *DP4* as well as from laboratory reference yeast strains belonging to *I. orientalis* and *R. glutinis*, was used for amplification of ITS-5.8S rDNA. Since the preliminary taxonomical studies indicated a possible relation between *DP3* and *Candida parapsilosis* or *Candida krusei*, strains belonging to these species were also used during this experiment. The amplicons were then digested using five endonucleases and the restriction fragments number and size were compared with those obtained from our reference strains and the data from MYCOBANK (Table 1).

For strain *DP3*, the amplicon of 520 bp formed two fragments with *Hae* III and *Hpa* II, three with *Cfo* I and *Hinf* I, while no restriction product was observed for *Dde* I. Highly similar restriction profiles were obtained for *I. orientalis* CMGB224 and *I. orientalis* CBS5147, similar to experiments described elsewhere [10, 11, 12]. Although the amplicon size of *DP3* approaches that of *C. parapsilosis* CBS604 and *C. krusei* CMGB94, the restriction profiles were clearly different, fact that leaves *I. orientalis* as first possible best taxonomical match.

Table 1. Amplicon and restriction fragments size for ITS1-5.8S-ITS2 PCR analysis of the *DP3*, *DP4* and reference yeast strains

Strain	Amplicon (bp)	Restriction fragments (bp)				
		***Cfo* I**	***Dde* I**	***Hae* III**	***Hinf* I**	***Hpa* II**
DP3	520	240, 180, 80	520	390, 90	220, 160, 140	250, 250
***I. orientalis* CMGB224**	520	210, 170, 80	520	400, 100	210, 150, 150	260, 260
***I. orientalis* CBS5147**	509	204, 178, 69, 52, 6	509	381, 90, 38	218, 154, 137	260, 249
***C. parapsilosis* CBS 604**	550	300, 250	550	410, 115	260, 290	550
***C. krusei* CMGB94**	500	160, 100, 80, 70	270, 160	340, 90	220, 280	250, 150, 50
DP4	650	320, 180, 120	380, 150, 100	400, 220	360, 220, 80	520, 110
***R. glutinis* ICCF**	650	300, 230, 80	380,130, 80	400, 220	340, 220, 80	550, 90
***R. glutinis* CBS20T**	638	320, 318	410, 134, 90	425, 213	232, 146, 115, 85, 60	532, 106

An obvious similarity was observed between the *DP4* patterns and those from *Rhodotorula glutinis* ICCF, with slight differences for *Cfo* I and *Hinf* I on *Rhodotorula glutinis* CBS 20T, such cases being already described in literature [13, 14].

RAPD analysis

For the last two decades, the RAPD technique has been successfully used for interspecific and intraspecific studies on *Candida* [15, 16, 17], *Hanseniaspora* [18], *Issatchenkia* [*19*], *Pichia* [20] and *Rhodotorula* [21]. The mechanisms of PCR-RAPD are not yet fully understood and the resulting patterns can be influenced by a series of factors, such as the primers set, Taq polymerase, amplification parameters, template concentration and RNA contamination degree, and even by the type of thermocyler used in the experiment [22]. As a consequence, artefacts may occur leading to different results in parallel experiments using the same primers. In order to eliminate these artefacts and to reduce their effects on accurate polymorphism analysis, scientists proposed various approaches such as use of similarity coefficients between sets of RAPD bands present in two samples [23], establishing a set of reference parameters for all the experiments when using certain primers [24], or correlating RAPD results with analysis of ARDRA on ITS-PCR amplicons or PFGE profiles [25, 26].

The primers used in experiments with *DP3* (OPA01, OPA03, OPA10, SOY, RP4-2, M13) and *DP4* (M13, OPH15, OPH19), were chosen according to the ARDRA results and to similar works mentioned above. Analysing the profiles from Figure 1 and Figure 2, we obtained a total of 15 bands for *DP3* and 20 bands for *DP4*, counting only the distinct and higher intensity ones. The molecular weights ranged from aproximately 100bp (*DP3* amplified with M13) and 200bp (*DP4* amplified with OPH15) to 1500bp for *DP3* with OPA-10 and *DP4* with OPH15. The greatest number of amplicons were obtained for *DP3* when using OPA03, M13 and OPA10, while three of the primers (OPA01, SOY and RP4-2) yield singular amplification fragments. For strain *DP4*, while OPH19 resulted in a great number of amplicons, M13 and OPA03 (Figure 4) seem to provide a clearer pattern for further taxonomical studies.

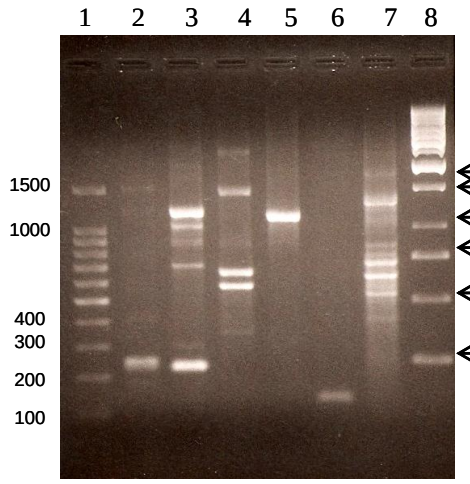

Figure 1. RAPD profiles obtained with different primers using DNA isolated from strain *DP3* (lanes: 1-100bp DNA ladder Promega; 2-OPA01; 3-OPA03; 4-OPA10; 5-SOY; 6-RP4-2; 7-M13, 8-GeneRuler 1kb DNA Ladder Fermentas)

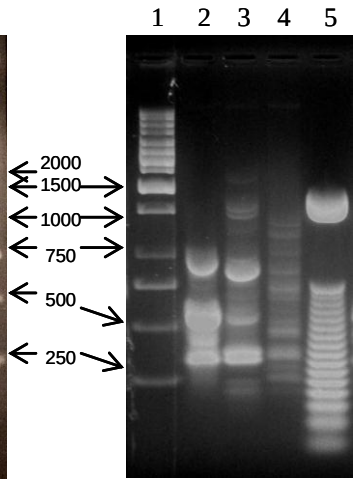

Figure 2. RAPD profiles obtained with different primers using DNA isolated from strain *DP4* (lanes: 1-GeneRuler 1kb DNA Ladder Fermentas; 2-M13; 3-OPH15; 4-OPH19; 5-50bp DNA Step Ladder Promega)

In order to obtain a better phylogenetic position for *DP3* and *DP4* strains, we used the OPA03 patterns obtained from a wide spectrum of yeast species (*C. tropicalis / parapsilosis / krusei / guillermondii / boidinii; S. cerevisiae, Y. lipolytica, K. lactis*) (Figure 3), to construct a dendrogram based on UPGAMA method.

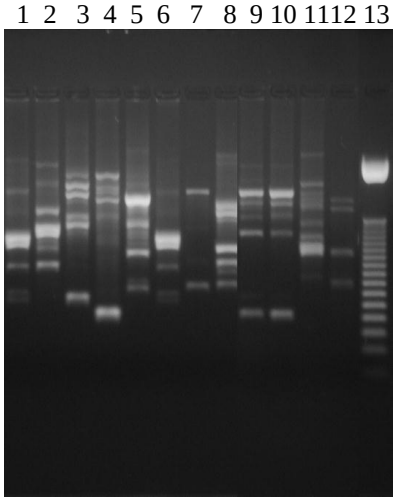

Figure 3. RAPD profiles obtained with OPA03 primer for: 1-*C. boidinii* ICCF; 2-*C. krusei* CMGB94; 3-*C. guilliermondii* CMGB44; 4-*C. tropicalis* CMGB165; 5-*C. parapsilosis* CBS604; 6-*K. lactis* CBS2359/152; 7-*Y. lipolytica* ICCF; 8-*S. cerevisiae* L5366; 9- *I. orientalis* CMGB224;10-*DP3*; 11-*DP6*; 12-*DP4*;. 13- 50bp DNA Step Ladder (Promega)

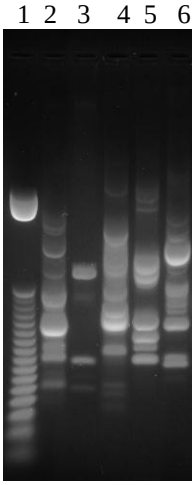

Figure 4. RAPD profiles obtained with OPA03 primer for: 1- 50bp DNA Step Ladder (Promega); 2-*DP4*; 3-*R. glutinis* ICCF; 4-*DP6*; 5-*S. cerevisiae* L5366; 6-*C. parapsilosis* CBS604.

Analyzing this dendrogram (Figure 5), strain *DP3* appears to be closely related to *I. orientalis* CMGB224 (81%), fact strongly supported also by the PCR-RFLP analysis of ITS-5.8S rDNA. In what concerns strain *DP4*, this first dendrogram revealed a 53% similarity

with *S. cerevisiae* L5366, the next closest branch being formed by *C. parapsilosis* CBS604 and another yeast strain isolated from oil-polluted environment (*DP6*) probably belonging also to *Candida* genus [unpublished data].

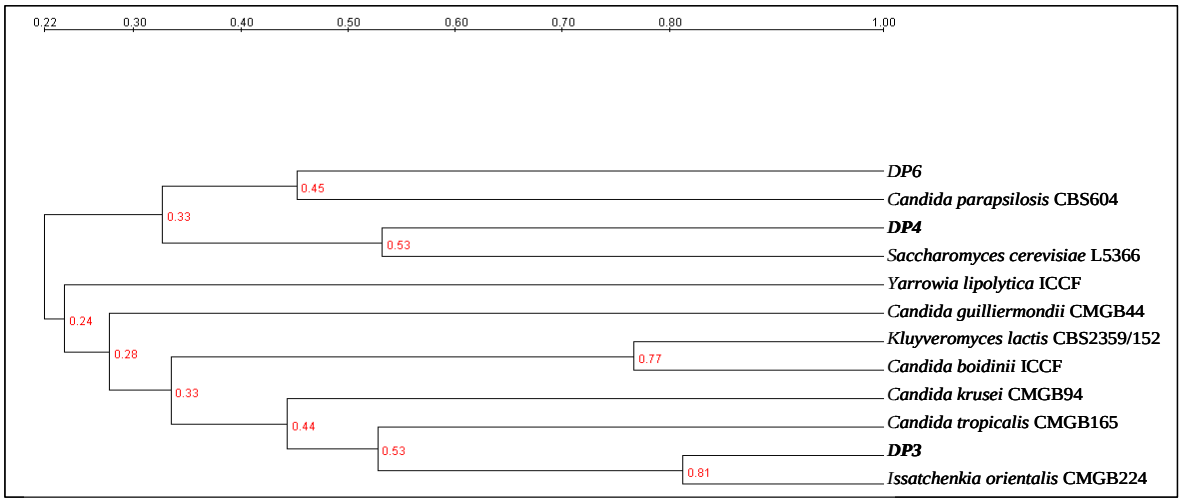

Figure 5. UPGAMA dendrogram for *DP3*, *DP4* and reference yeast strains, obtained from RAPD profiles with OPA03 primer

Since morpho-physiological tests and PCR-RFLP of ITS-5.8S rDNA results suggest the belonging of *DP4* to *R. glutinis*, a second RAPD experiment has been performed using only the closest related species (*C. parapsilosis* CBS604, *DP6*, *S. cerevisiae* L5366), introducing also the reference *R. glutinis* ICCF (Figure 4). The influence of the various parameters over the RAPD patterns, mentioned often in the literature, was observed when Figure 3 and 4 were compared, representing different experiments, with the same parameters except for the used genomic DNA isolates. Nevertheless, the two experiments are entirely compatible, since fragments from the Figure 4 can be found in Figure 3. Also, a clear resemblance appeared between *DP4* and *R. glutinis* ICCF patterns (Figure 4 lane 2 and 3).

The digitalized gel images of the amplification profiles from Figure 4, were subjected to the Quantity One (Bio-Rad) program. Observing the resulted UPGAMA dendrogram (Figure 6), the branch *DP6* - *C. parapsilosis* CBS604 was unchanged, *DP4* - *R. glutinis* ICCF form another branch, while *S. cerevisiae* L5366 locates separately. The variation of the similarity percentages compared to Figure 5, is most probably determined by the fact that the dendrograms are based on two different experiments. Also, we estimate that if more reference strains had been used, the position of *S. cerevisiae* L5366 would have been be reflected more accurately.

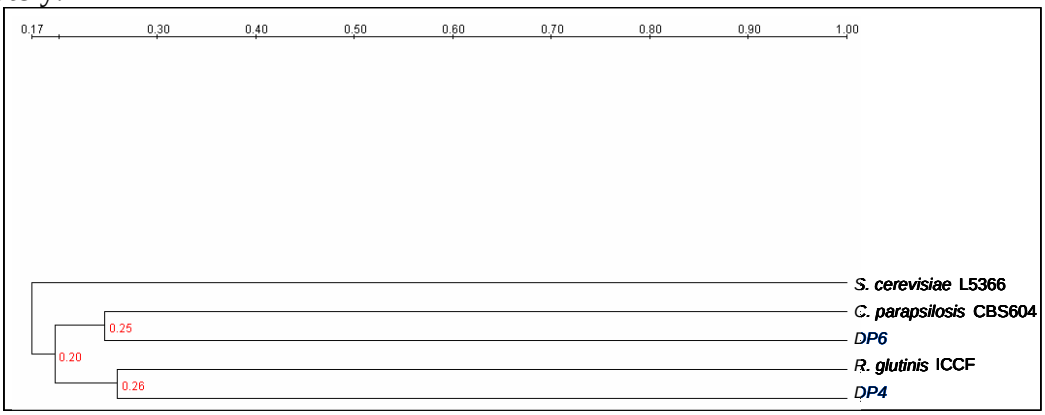

Figure 6. UPGAMA dendrogram for *DP4*, *R. glutinis* ICCF and most related reference yeast strains, obtained from RAPD profiles with OPA03 primer

Conclusions

The two yeast strains used in the present study, *DP3* and *DP4*, isolated from oil-polluted environment from Pitesti area (Romania) and previously tested using classical taxonomy techniques, were subject to polyphasic identification using BioloMICS online software from MYCOBANK. The analysis revealed a 91.40% similarity with *I. orientalis* CBS5147 (for *DP3*), respectively, 83.80% similarity with *R. glutinis* CBS20T (for *DP4*).

For a more accurate characterization, we performed restriction on the amplified region of ITS1-5,8S-ITS2 and RAPD analysis, and we compared the results with those obtained from laboratory reference yeast strains, MYCOBANK and similar experiments described in literature.

Strain *DP3* showed similar ITS-PCR and restriction profiles with *Issatchenkia orientalis* CMGB224 and *Issatchenkia orientalis* CBS5147, while for *DP4* matched *Rhodotorula glutinis* ICCF and *Rhodotorula glutinis* CBS20T.

For the RAPD studies, we used eight primers: OPA01, OPA03, OPA10, SOY, RP4-2, M13 for *DP3*, and M13, OPH15, OPH19 for *DP4*. The greatest number of amplicons were obtained for *DP3* with OPA03, M13, OPA10, and for *DP4*, with OPH19. Clear differences were observed for the patterns obtained with OPA03 and M13 primers.

The dendrograms constructed using UPGAMA method from Quantity One (Bio-Rad) program, on OPA03 profiles from our isolated and reference yeast strains, revealed the same relatedness between *DP3* and *Issatchenkia orientalis* CMGB224, and between *DP4* and *Rhodotorula glutinis* ICCF.

The PCR-RFLP of ITS-5.8S rDNA and RAPD analysis results corroborated with the previous polyphasic identification, suggest as most probable the belonging of *DP3* strain to *Issatchenkia orientalis* and *DP4* to *Rhodotorula glutinis*.

Nevertheless, for an accurate identification further molecular analysis and/or sequencing experiments need to be done.

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