Molecular identification of yeast using amplification and sequencing of ITS1-5.8S-ITS2 rDNA region

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Molecular identification of yeast using amplification and sequencing of ITS1-5.8S-ITS2 rDNA region. *The advancement of molecular techniques for analysis of various parts of the yeast genome have alowed for a more accurate identification of yeast species. A collection of 35 yeast species, identified by morphological and biochemichal methods was subjected to molecular analysis by PCR amplification and sequencing of ITS1-5.8S-ITS2 rDNA gene, which is known for its high species-related varibility. The results confirmed only 37.1% of the species identified by classical methods, which demonstrates the importance of the molecular techniques as a reliable methodology for identificaiton of yeast.*

Key words: *Yeast, PCR, ITS4, ITS5, Sequencing, BLAST analysis*

INTRODUCTION

There are a variety of molecular techniques for identification of yeast species developed in recent years. Some of those techniques are restriction fragment length polymorphism (RFLP) of internal transcribe spacer (ITS) of ribosomal DNA and its sequencing [1, 2, 3, 4, 7], random amplification of polymorphic DNA (RAPD) [3, 8], determination of chromosome polymorphism by pulsed-field gel electrophoresis (PFGE) [7], as well as denaturing gradient gel electrophoresis (DGGE) and sequencing of 26S rDNA gene [5]. RFLP analysis and sequencing of ITS is an effective approach for identification of yeast species within a genus because of the high level of interspecific sequence variability of ITS. RAPD is useful for comparison and resolution of types of microorganisms within a species. Capece et al. (2010) used RAPD for preliminary discrimination of over 300 isolates of indigenous *S.cerevisiae* isolated from Nero d'Avola wine fermentation. PFGE is another common method for determination of intraspecific variability by examining the patterns of chromosomal polymorphism that occur within a species. Jeyaram et al. (2008) used PFGE to determine chromosomal variations between *S.cerevisiae* strains isolated from traditional Indian rice wine starter called "Hamei". DGGE is used to identify different species present in a single DNA sample that is directly isolated from a particular matrix. Greppi et al. (2013) identified *Dekkera bruxellensis* and *Debaryomyces hansenii* in a DNA sample directly extracted from traditional West African fermented foods Oge, Mawe, Gowe, and Tchoukoutou.

The aim of this study is to apply PCR amplification and sequencing of the ITS rDNA region for identification of a total of 35 yeast species and compare the results with those obtained by classical methods.

MATERIALS AND METHODS

A collection of 35 yeast species, identified by morphological and biochemical methods, was obtained from the Department of Biotechnology and the Department of Technology of Wine and Beer in the University of Food Technologies. The cultures were maintained on slant malt agar. Prior to PCR analysis the cells were activated at 30°C in a shaker for 24 h at 220 rpm in nutrient medium containing (g/dm^3): Sucrose-60; Yeast Extract – 2.5; KH_2PO_4 – 6.0; K_2HPO_4 – 7.0; $MgSO_4$ – 0.5. The activated cultures were transferred on petri dishes with identical medium containing 3% agar and incubated for 24h at 30°C.

Amplification of the ITS1-5.8S-ITS2 region was carried out by transferring biomass from a fresh colony with the tip of a sterile toothpick into a PCR tube containing 10 µl sterile deionized water. 40 µl PCR reaction mix was added to the cell suspension. The PCR reaction mix contained 1 µM of primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), 1 µM of primer ITS5 (5'-GGAAGTAAAAGTGCTAACAAGG-3'), (Metabion, Germany),1

mM dNTPs, 1 x PCR buffer (Thermo Scientific, USA), 0.8 U Taq polymerase (Thermo Scientific, USA). The reaction was carried out in a PCR 2720 Thermal Cycler (Biosystems, Germany) using the following program: cell lysis and DNA extraction at 95 °C for 15 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 57 °C for 30 s, extention 72 °C for 1 min, and final extention at 72 °C for 10 min. PCR products were visualized with 2% agarose gel stained with SafeView (NBS Biologicals, UK) at 100V for 50 min, using VWR Mini Electrophoreis System (VWR, Germany) and MiniBis Pro (DNR Bio-Imaging Systems, Israel) for gel visualization. The DNA marker used was GeneRuler 1kb plus (Thermo Scientific, USA). The fragment sizes were calculated using image editing software ImageJ64 (freeware).

The PCR products were sent for sequencing to MacroGen Europe. The nucleotide sequences were compared to the database GenBank (http://www.ncbi.nlm.nih.gov/blast/), using the BLAST program.

RESULTS AND DISCUSSION

A collection of 35 yeast species, which had previously been identified by classical methods, was subjected to identification by PCR amplification of the ITS1-5.8S-ITS2 rDNA region with ITS4 and ITS5 primers. The PCR products were further subjected to sequencing and BLAST analysis. The size of the resulting amplicons ranged between 450 bp (*P.fermentans*) and 880 bp (*S.cerevisiae*) both shown on figure 1.

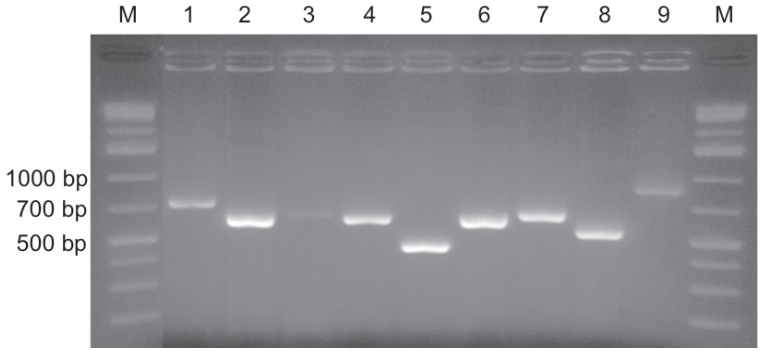

Figure 1. Amplicons of ITS1-5.8S-ITS2 rDNA region of yeast. M-molecular marker, 1 - *Kluyveromyces marxianus*, 2 - *Wickerhamomyces anomalus*, 3,4,6 - *Rhodotorula mucilaginosa*, 5 - *Pichia fermentans*, 7 - *Debaryomyces hansenii*, 8 - *Candida tropicalis*, 9 - *Saccharomyces cerevisiae*

The results from the PCR analysis and the subsequent BLAST analysis are shown in table 1. All of the species were identified with 95% identity or greater with the exception of *C.tropicalis* Y27, which was most closely related to *S.cerevisiae* YJM1389 with 80% identity, whereas the sequence obtained from *Pichia alcoholophila* 3601 was matched to *P.membranifaciens* CBS 214 with 88% identity. This result could be attributed to novel sequences present in those two species. The size of the obtained amplicons corresponded with those published in the literature for the respective species [1,2,3,4,5].

The most numerous species in the collection were *S.cerevisiae* representing 28.6% of the total species, *R.mucilaginosa* 22.9%, and *K.marxianus* 11.4%. The rest of the species in the collections are members of the genera *Candida*, *Clavispora, Debaryomyces, Meyerozyma*, *Pichia*, and *Wickerhamomyces*. It should be noted that there have been some changes in the taxonomic nomenclature in recent years. Thus, *Hansenula anomala* and *Pichia anomala* are now *Wickerhamomyces anomalus* and *Candida guilliermondii* is changed to *Meyerozyma guilliermondii* [9].

Table 1. Molecular identification of yeast species and correlation to their previous identification by classical methods

Yeast Collection	PCR Product (bp)	PCR Identification	Nearest neighbour	Identity (%)
Saccharomyces cerevisiae A-30	880	*Saccharomyces cerevisiae*	*S.cerevisiae* ySR127	100
Saccharomyces cerevisiae P1-26	880	*Saccharomyces cerevisiae*	*S.cerevisiae* YJM456	100
Saccharomyces cerevisiae K-32	880	*Saccharomyces cerevisiae*	*S.cerevisiae* ySR127	100
Saccharomyces cerevisiae Pilsen G	880	*Saccharomyces cerevisiae*	*S.cerevisiae* M32	99
Rhodotorula glutinis 3101	640	*Rhodotorula mucilaginosa*	*R.mucilaginosa* ANT12-058	100
Torulaspora cantarelli 1011	700	*Candida cantarellii*	*C.cantarellii* BCRC21613	100
Zygosaccharomyces marxianus 1561	740	*Kluyveromyces marxianus*	*K.marxianus* NBRC 1777	100
Saccharomyces ellipsoideus 0232	880	*Saccharomyces cerevisiae*	*S.cerevisiae* YJM689	100
Candida lambica Y30	740	*Kluyveromyces marxianus*	*K.marxianus* DKMU3-1042	100
Hansenula anomala 3370	610	*Wickerhamomyces anomalus*	*W.anomalus* LMICRO189	100
Rhodotorula mucilaginosa 3062	640	*Rhodotorula mucilaginosa*	*R.mucilaginosa* ANT12-058	100
Rhodotorula mucilaginosa 3064	640	*Rhodotorula mucilaginosa*	*R.mucilaginosa* ANT12-058	100
Saccharomyces ellipsoideus 0227	880	*Saccharomyces cerevisiae*	*S.cerevisiae* YJM450	100
Candida albicans 2637	550	*Clavispora lusitaniae*	*C.lusitaniae* PMM08-861-DL	99
Rhodotorula mucilaginosa 3039	640	*Rhodotorula mucilaginosa*	*R.mucilaginosa* PMM08-3684L	99
Rhodotorula rubra 3035	640	*Rhodotorula mucilaginosa*	*R.mucilaginosa* AUMC 7782	99
Candida pulcherrima 2253	880	*Saccharomyces cerevisiae*	*S.cerevisiae* BY-1	97
Candida tropicalis Y27	880	*Saccharomyces cerevisiae*	*S.cerevisiae* YJM1389	80
Saccharomyces cerevisiae PAK	880	*Saccharomyces cerevisiae*	*S.cerevisiae* YJM1307	100
Zygosaccharomyces marxianus 1562	740	*Kluyveromyces marxianus*	*K.marxianus* NBRC 1777	100
Candida glabrata FGB	450	*Pichia fermentans*	*P.fermentans* ATCC 10651	99
Hansenula anomala 3311	610	*Wickerhamomyces anomalus*	*W.anomalus* LMICRO189	100
Rhodotorula glutinis 3001	640	*Rhodotorula mucilaginosa*	*R.mucilaginosa* PMM08-3684L	100

Rhodotorula rubra 3036	640	*Rhodotorula mucilaginosa*	*R.mucilaginosa* PMM08-3684L	100
Torulopsis lactis 2701	575	*Pichia jadinii*	*P.jadinii* CBS 621	100
Candida pseudotropicalis 1201	650	*Debaryomyces hansenii*	*D.hansenii* ylx-13	100
Candida tropicalis 1501	550	*Candida tropicalis*	UOA/HCPF 12742	100
Candida scotii 1601	500	*Candida blankii*	*C.blankii* PMM09-206L	98
Saccharomyces cerevisiae 2101	880	*Saccharomyces cerevisiae*	*S.cerevisiae* YC9.13	95
Candida utilis 1401	500	*Candida blankii*	*C.blankii* PMM09-753L	98
Candida melibiosica 1701	625	*Meyerozyma guilliermondii*	*M.guilliermondii* PY-14	100
Pichia alcoholophila 3601	500	*Pichia membranifaciens*	*P.membranifaciens* CBS 214	88
Candida pelliculosa 1801	610	*Wickerhamomyces anomalus*	*W.anomalus* LMICRO150	100
Kluyveromyces lactis 3611	740	*Kluyveromyces marxianus*	*K.marxianus* NBRC 1777	100
Rhodotorula rubra 5501	640	*Rhodotorula mucilaginosa*	*R.mucilaginosa* PMM08-1216L	99

The molecular identification confirmed 37.1% of the species and 68.6% of the genera identified with classical methods. This demonstrates the great difference in accuracy between classical and molecular methods for yeast identification. Alvarez-Martin et. al. also reported major differences in the results obtained by phenotypic and molecular identification of yeast isolated from dairy products [1]. It is noticeable that among the studied species, *S.cerevisiae* and *R.mucilaginosa* were most successfully identified by classical methods. That could be due to the common use of *S.cerevisiae* in the food and beverage industry, which makes it a well known microorganism in terms of morphological and biochemical characteristics. The distinct orange/red color of the colonies formed by the *Rhodotorula* genus facilitates to some extent the identification of its species. However, table 1 clearly shows that *R.mucilaginosa* can be confused with *R.rubra* or *R.glutinis* by classical methods.

CONCLUSION

A total of 35 yeast species of yeast were subjected to PCR amplification and sequencing of the ITS1-5.8S-ITS2 rDNA gene. The results differed from those obtained by classical means as 37.1% of the species were confirmed by the molecular analysis. The present study demonstrates the high degree of accuracy and reliability of molecular identificaiton of yeast compared to morphological and biochemical methods. This is the reason why the molecular techniques are the most preffered procedures by research teams for identificatoin of microorganisms.

REFERENCES

[1] Alvarez-Martin, P., A.B. Florez, T.M. Lopez-Diaz, B. Mayo. Phenotypic and molecular identification of yeast species associated with Spanish blue-veined Cabrales cheese. International Dairy Journal 17, 2007, 961–967.

[2] Arroyo-Lopez, F.N., M.C. Duran-Quintana, J.L. Ruiz-Barba, A. Querol, A. Garrido-Fernandez. Use of molecular methods for the identification of yeast associated with table olives. Food Microbiology 23, 2006, 791–796.

[3] Capece, A., R. Romaniello, G. Siesto, R. Pietrafesa, C. Massari, C. Poeta, P. Romano. Selection of indigenous Saccharomyces cerevisiae strains for Nero d'Avola wine and evaluation of selected starter implantation in pilot fermentation. International Journal of Food Microbiology 144, 2010, 187–192.

[4] Capece, A., G. Siesto, C. Poeta, R. Pietrafesa, P. Romano. Indigenous yeast population from Georgian aged wines produced by traditional “Kakhetian” method. Food Microbiology 36, 2013, 447-455.

[5] Greppi, A., K. Rantsiou, W. Padonou, J. Hounhouigan, L. Jespersen, M. Jakobsen, L. Cocolin. Determination of yeast diversity in ogi, mawè, gowé and tchoukoutou by using culture-dependent and -independent methods. International Journal of Food Microbiology 165, 2013, 84–88

[6] Greppi, A., K. Rantsiou, W. Padonou, J. Hounhouigan, L. Jespersen, M. Jakobsen, L. Cocolin,. Yeast dynamics during spontaneous fermentation of mawè and tchoukoutou, two traditional products from Benin. International Journal of Food Microbiology 165, 2013, 200–207.

[7] Jeyaram, K., W. Mohendro Singh, A. Capece, P. Romano. Molecular identification of yeast species associated with ‘Hamei’ — A traditional starter used for rice wine production in Manipur, India. International Journal of Food Microbiology 124, 2008, 115–125.

[8] Lopandic, K., S. Zelger, L.K. Banszky, F. Eliskases-Lechner, H. Prillinger. Identification of yeasts associated with milk products using traditional and molecular techniques. Food Microbiology 23, 2006, 341–350.

[9] Shneider, J., O. Rupp, E. Trost, S. Jeanicke, V. Passoth, A. Goesmann, A. Tauch, K. Brinkrolf. Genome sequence of Wickerhamomyces anomalus DSM 6766 reveals genetic basis of biotechnologically important antimicrobial activities. FEMS Yeast Research 12, 2012, 382–386.

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