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Differentiation of food spoilage yeast strains of the *Yarrowia* group by microsatellite polymerase chain reaction fingerprinting

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ABSTRACT

Yarrowia lipolytica is a frequently reported food spoilage yeast which occurs mainly in meat and milk products. Besides its detrimental role in the food spoilage it has many favourable properties. This yeast also has been developed as a production host for a large variety of biotechnological applications. Some species of the *Yarrowia* group cannot be differentiated by conventional methods based on phenotypic characteristics, so it is probable that not only *Yarrowia lipolytica*, but also other species of the *Yarrowia* group contribute to food spoilage or can have a beneficial role in the industry. A relatively fast, easy and low-cost but reliable method would be useful to differentiate and identify these species properly.

Microsatellite PCR fingerprinting method is commonly used for the discrimination of species or strains within species, it is also applied for the identification of yeast species and in many yeast diversity studies, however, its reliability can be queried.

The aim of this study was to assess the reliability of microsatellite PCR fingerprinting using $(GAC)_5$ primer in the differentiation and identification of the yeast strains of the *Yarrowia* group by clustering them using microsatellite-PCR fingerprinting, then identifying them by sequencing the D1/D2 regions of the LSU rDNA of one or a few selected representatives of each cluster.

Two hundred and nineteen yeast strains of the *Yarrowia* group were examined.

Using this molecular biological method, yeast strains of the *Yarrowia* group from raw meat, raw milk, cheese and cottage cheese were assigned to seven species The food spoilage yeast strains of the *Yarrowia* group can be successfully differentiated by using microsatellite PCR fingerprinting method using $(GAC)_5$ primer, even their identity, thus their diversity can be assessed.

Introduction

Yarrowia lipolytica is a frequently reported food spoilage yeast which occurs mainly in meat and milk products [2]. Besides its detrimental role in the food spoilage many studies report its favourable contribution to the ripening and quality of cheeses [3, 11, 20] and meat products [4, 16, 17, 19]. *Yarrowia lipolytica* has also been developed as a production host for a large variety of biotechnological applications, for example it has a high potential in the production of enzymes, organic acids, sugar alcohols,

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carotenoids and aroma compounds [5]. Some species of the *Yarrowia* group cannot be differentiated by conventional methods based on phenotypic characteristics [8, 9, 10], so it is probable that not only *Yarrowia lipolytica*, but also other species of the *Yarrowia* group contribute to food spoilage or can have a beneficial role in the industry. A relatively fast, easy and low-cost but reliable method would be useful to identify these species properly.

Microsatellites have emerged as the marker of molecular genetic applications and found highly efficient in the DNA fingerprinting analysis due to their abundant and uniform distribution throughout the genome, highly variable nature with regard to repeat number, show codominant inheritance, ease of transferability and reproducibility [12]. Microsatellites are potentially the most informative molecular markers with the advantage of easy and low-cost detection by PCR due to their high mutation rate [6]. The amplified products are separated by electrophoresis to detect the polymorphism in repeat length [12].
This fingerprinting method is commonly used for the discrimination of species or strains within species [1]. It is also used for the identification of yeast species, many yeast diversity studies are based on clustering the yeast isolates using microsatellite-PCR fingerprinting, followed by identification of one or a few selected representatives of each cluster by sequencing [18].

However, the reliability of this method can be queried. Ramirez-Castrillon and co-workers (2014) evaluated a standardized protocol using microsatellite PCR fingerprinting for the discrimination of wine associated yeasts. They applied this technique to 245 strains, and compared the results with the identification obtained by partial sequencing of the LSU rRNA gene, considered as the gold standard. It was revealed that unrelated species were clustered in the same group. They concluded that the yeast diversity inferred in several previous studies may have been underestimated.

The aim of this study was to assess the reliability of microsatellite PCR fingerprinting using $(GAC)_5$ primer in the differentiation and identification of the yeast strains of the *Yarrowia* group by clustering them using microsatellite-PCR fingerprinting, then identifying them by sequencing the D1/D2 regions of the LSU rDNA of one or a few selected representatives of each cluster.

Material and methods

Yeast isolates

Over a period of three years 219 strains presumed as members of the *Yarrowia* group based on physiological tests were isolated from raw meat (66 samples), raw milk (40 samples) and dairy products such as cheese (9 samples) and cottage cheese (18 samples). Most of the meat samples were obtained from a quality control laboratory, some of them were purchased from supermarkets. Milk samples were obtained from farms or were purchased from dairy shops, milk products were purchased from supermarkets or obtained from a quality control laboratory. All the samples originated from Hungary.
The isolation of the yeast strains was performed in three steps. First a three-step enrichment in a medium described by Nagy and co-workers (2013) was applied, followed by serial decimal dilutions and surface plating on Rose-Bengal chloramphenicol (RBC) agar. Finally cultures representing each colony type were isolated and purified by repeated streaking on glucose-peptone-yeast extract agar.

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ORIGINAL PAPER
ISSN: 2416-0008

Members of the *Yarrowia* group were selected based on the results of physiological tests obtained by using API ID 32 C tests (bioMérieux) and by examining their glucose-fermentation, nitrate- and hexadecane-assimilation [15].
In this experiment, the type strains of the *Yarrowia* group were also used as reference.

DNA extraction
DNA was extracted from the strains by using Qiagen DNA extraction kit (Qiagen 69106, Switzerland), according to manufacturer's instructions.

Microsatellite PCR fingerprinting
A microsatellite primer $(GAC)_5$ was used in PCR amplification reactions from the DNA extracted from the strains. For amplification the following conditions were applied: initial denaturation at 95 ˚C for 3 minutes followed up by 30 cycles of denaturation (96 ˚C, 30 seconds), annealing (59 ˚C, 35 seconds) and extension (73 ˚C, 55 seconds). Amplification was finished with a final extension at 72 ˚C for 30 seconds and cooling at 12 ˚C. The PCR products were separated by horizontal agarose gel electrophoresis on 1.4% agarose gel, in 1X TAE buffer (0.04 M Tris-acetate, 0.00l M EDTA - pH 8.0) at 5 V/cm and stained with ethidium bromide (7×10^{-5} µg/µl). The resulting DNA fragments and the molecular size marker (Thermo Scientific, Generuler 100bp SM0321) were visualized by UV transillumination.

Amplification and sequencing of the D1/D2 region of the LSU rRNA gene
The D1/D2 domain of the large subunit (26S) rDNA from selected strains was sequenced as described by Kurtzman & Robnett (1998). The amplicons were sequenced by commercial sequencing facilities (Bay Zoltán Nonprofit Ltd. for Applied Research, Hungary; or Biomi Ltd., Hungary). Sequence similarity searches were performed against the GenBank sequence database using the BLAST 2.2.28 database search program [21].

Results and discussions

PCR fingerprinting
The resulting PCR products were separated by using horizontal agarose gel electrophoresis and stained with ethidium bromide then visualized by UV transillumination. They showed clear-cut polymorphism.
Strains were assigned to groups based on PCR fingerprinting, using the $(GAC)_5$ microsatellite primer and the above mentioned conditions. The 13 type strains showed 13 different fingerprints. The examined 219 strains, selected as members of the *Yarrowia* group based on their physiological characteristics formed seven groups by showing 7 different fingerprints. The identity of all of these strains could be assessed by comparing their fingerprints to the fingerprints of the type strains.

Amplification and sequencing of the D1/D2 region
To confirm the identity of the strains sequencing of the D1/D2 domain of the LSU rRNA gene was necessary. Selected members of each group were identified by amplifying and sequencing the D1/D2 region of the ribosomal RNA's large subunit coding gene.
Using this method, yeast strains of the *Yarrowia* group from raw meat, raw milk, cheese and cottage cheese were assigned to seven species. Four of them (*Y. lipolytica, Yarrowia deformans, Candida galli* and *Candida alimentaria*) are earlier described species, three of them proved to be novel ones and had been described as *Y. divulgata* [13], *Y. porcina and Y. bubula* [14]. Using conventional methods based on phenotypic characteristics probably many strains would

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ORIGINAL PAPER
ISSN: 2416-0008

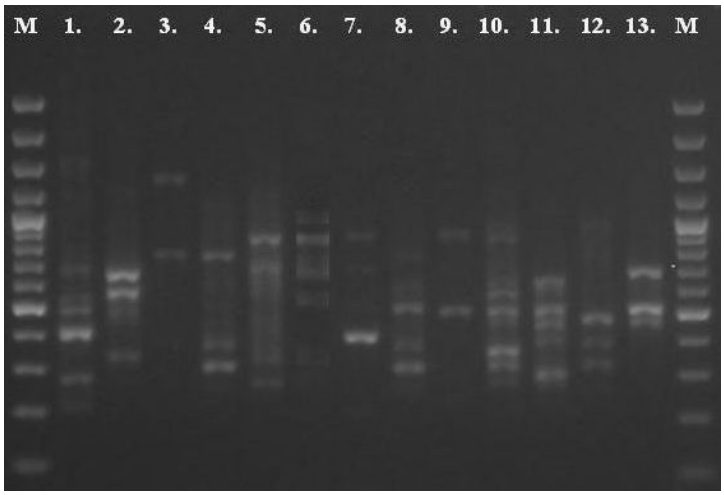

Fig 1. PCR fingerprints with the microsatellite primer $(GAC)_5$ from the type strains of the *Yarrowia* group.
M: Size marker (Thermo Scientific, Generuler 100bp SM0321); 1. *Y. lipolytica* NCAIM Y.00591^T, 2. *Y. deformans* NCAIM Y.02005^T, 3. *C. galli* NCAIM Y.01482^T, 4. *C. alimentaria* JCM 10151^T, 5. *C. hispaniensis* NRRL4-5580^T, 6. *C. hollandica* CBS4855^T, 7. *C. oslonensis* CBS10146^T, 8. C. *phangngensis* CBS10407^T, 9. *Y. yakushimensis* JCM12782^T, 10. *Y. divulgata* F6-17^T, 11. *Y. porcina* NCAIM Y.02100^T, 12. *Y. bubula* NCAIM Y.01998^T, 13. *Y. keelungensis* CBS11062^T.

have been misidentified as *Y. lipolytica.* Strains of the *Yarrowia* group could be successfully differentiated by using microsatellite PCR using $(GAC)_5$ primer. They formed seven clusters corresponding to the seven species identified by amplifying and sequencing the gene coding the D1/D2 region of the large subunit ribosomal RNA from some selected strains of each group.

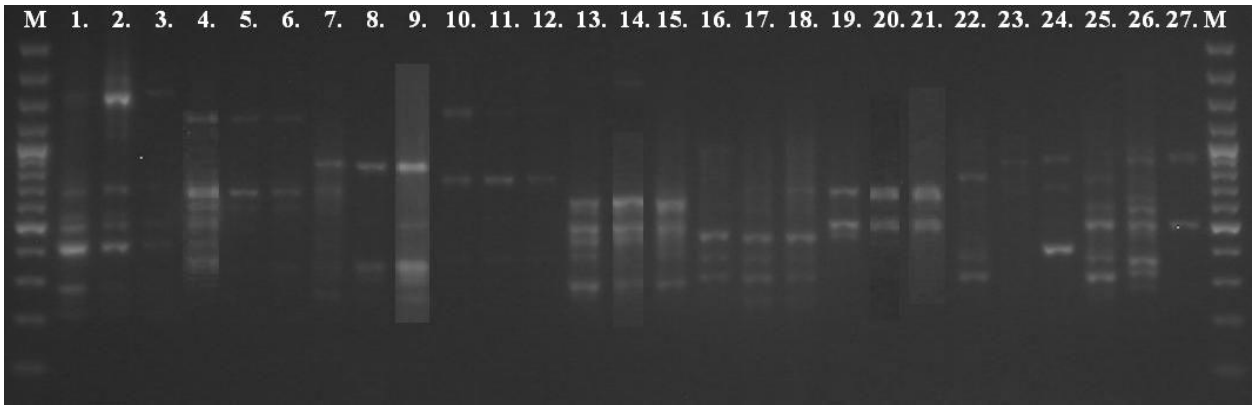

Fig 2. PCR fingerprints with the microsatellite primer $(GAC)_5$ from a few strains of the species of the *Yarrowia* group showing that this method is suitable to distinguish them.
M: Size marker (Thermo Scientific, Generuler 100bp SM0321); 1. *Y. lipolytica* NCAIM Y.00591^T, 2. *Y. lipolytica* 148/4, 3. *Y. lipolytica* 854/4, 4. *Y. deformans* NCAIM Y.02005^T, 5. *Y. deformans* 440/2, 6. *Y. deformans* 435/1, 7. *C. alimentaria* JCM 10151^T, 8. *C. alimentaria* 440/1, 9. *C. alimentaria* 450/5, 10. *C. galli* NCAIM Y.01482^T, 11. *C. galli* 437/3, 12. *C. galli* 435/4, 13. *Y. divulgata* F6-17^T, 14. *Y. divulgata* 864/2, 15. *Y. divulgata* 862/1, 16. *Y. porcina* NCAIM Y.02100^T, 17. *Y. porcina* 855/1, 18. *Y. porcina* 863/1, 19. *Y. bubula* NCAIM Y.01998^T, 20. *Y. bubula* 855/3, 21. 863/1, 22. *C. hispaniensis* NRRL4-5580^T, 23. *C. hollandica* CBS4855^T, 24. *C. oslonensis* CBS10146^T, 25. C. *phangngensis* CBS10407^T, 26. *Y. yakushimensis* JCM12782^T, 27. *Y. keelungensis* CBS11062^T.T: type strain.

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Conclusions

Microsatellite PCR can be an effective fingerprinting method for the differentiation, in some cases even for the identification of yeast strains. It can be useful to detect food spoilage yeasts, or even in tracing back the origin of spoilage outbreaks. However before using it the reliability of this method should be tested on the yeast species and strains wished to detect.

The food spoilage yeast strains of the *Yarrowia* group can be successfully differentiated by using microsatellite PCR fingerprinting method using $(GAC)_5$ primer, even their identity, thus their diversity can be assessed. However, additional sequencing is necessary for further evaluation the species specificity of the method.

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