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IDENTIFICATION OF NEW YEAST ISOLATES FROM THE ŞTEFĂNEŞTI VINEYARD THROUGH PCR ITS-RFLP TECHNIQUE

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Abstract

The Ştefăneşti-Argeş wine region is known for its unique terroir and traditional practices in viticulture and winemaking. Yeasts play a significant role in shaping wines' characteristics and fermentation processes. The surface of grape berries is a natural habitat for various microorganisms, mainly represented by yeasts, lactic acid bacteria, and acetic acid bacteria, known as the epiphytic microbiota. The aim of this work was the isolate, identify, and characterize some yeast strains from different grape varieties: five internationally well-recognized varieties for white and red wines grown in the INCDBH Stefanesti vineyard. The isolates selected in pure culture proved to belong to both Saccharomyces and non-Saccharomyces genera, an aspect confirmed by microscopic and molecular analyses. For correct identification, the morphological characteristics were complemented by molecular analyses by PCR amplification of the ITS1-5.8S-ITS2 region.

Key words*: grapes, wine, local strains, PCR, Ştefăneşti vineyard.*

INTRODUCTION

Yeasts are eukaryotic, unicellular microorganisms belonging to two different phyla (*Ascomycota* and *Basidiomycota)* which reproduce asexually by mitosis and develop predominantly by vegetative budding or fusion (Kurtzman C.P. & Fell J.W., 1998). Yeasts are free-living organisms present in diverse terrestrial, aquatic, and marine environments on all continents, forming associations with numerous species of plants, fungi, and insects (Kurtzman et al., 2011; Chavez et al., 2024).

The study of yeast taxonomy has been an important objective for numerous research efforts, both nationally and internationally (Gayon et al., 2006; González, 2007) but, the reference work was done by Lodder (1970). Numerous yeast species play a very important role in various industries and human activities, such as the baker's yeast *Saccharomyces cerevisiae*, used in baking, brewing, winemaking, and biotechnology. The classification into *Saccharomyces* and *non-Saccharomyces* was established following various studies carried out on their diversity and the frequency of yeasts on the surface of grape bunches and wine production (Grangeteau et al., 2017; Abdo et al., 2020). The frequency and type of yeasts present on grapes are closely related to the health status of the grapes and the vegetative stage (phenophase) of berries development (Dumitrache et al., 2020). A lower frequency and diversity of yeasts was observed in unripe grapes compared to ripe grapes, which showed a significantly higher abundance of yeasts with an extensive variety of species and genera. (Barata et al., 2008; Grangeteau et al., 2017). *Non-Saccharomyces* yeasts grow better in the preliminary stages of the fermentation when the ethanol concentration is still low, being later replaced by *Saccharomyces*, which are more ethanol tolerant and competitive for growth in high sugar media (Fleet & Heard, 1993; Sabate et al., 2002). Traditionally, strain identification depends on morphological and physiological characteristics (Dumitrache et al., 2020). These classic methods require about 50-100 tests to accurately determine the species of most yeasts. (Lin & Fung, 1987; Sabate et al., 2002). The progress of biotechnology, together with molecular studies, has opened new opportunities and possibilities for the faster identification of numerous samples (Sabate et al., 2002). The analysis at the DNA level

allows the evaluation and identification of a larger sample of samples in a much shorter time. One of the most effective methods is RFLP (restriction fragment length polymorphism) using the ITS-5.8S region (Esteve-Zarzoso et al., 1999; Fernández-Espinar et al., 2000; Dumitrache et al., 2020).
This research aims to identify and characterize native yeast strains from the Ştefăneşti vineyard, with potential application in the process of producing wine.

MATERIALS AND METHODS

Grape samples and yeast isolation

The grapes were harvested aseptically from the INCDBH Stefanesti-Arges vineyard. Were selected some grape varieties very appreciated for the production of white and red wines ('Fetească regală'-FR, 'Tămâioasă românească'-TR, 'Sauvignon'-Sauv, 'Cabernet sauvignon'-CS, 'Burgund' - Burg.).
The procedure for isolating microorganisms, regardless of their nature, involves applying a series of techniques designed to facilitate the sampling and separation of the microorganisms of interest from their natural environment, followed by their inoculation in specific culture media under controlled laboratory conditions (*in vitro*).
Healthy grapes were crushed in a sterile mortar and mixed a the pestle, and the resulting mixture was allowed to stand for approximately 25 minutes under laminar flow to facilitate samples infusion.
The must samples were processed using serial decimal dilutions, according to the Domerq (1956) methodology. The crushed extracts were inoculated with automatic pipettes on Petri dishes with Potato-Dextrose-Agar (PDA/ch) and Yeast Extract-Peptone-Dextrose (YPD/ch), both culture media supplemented with chloramphenicol to inhibit bacterial growth. After inoculation, Petri dishes were incubated for 48-72 hours at 27°C in an incubator controlled by the thermostat (CL 53, Pol-EKO).

Morphological characterization

Yeast strains were isolated by cultivating them on a selective medium YPD and YPG at 27°C for 48-72 hours. The strains that were newly isolated were morphologically characterized using the "OPTIKA" binocular digital microscope, and the visualization of the obtained results was possible using the B-290TB tablet following the Pintilie (2011) protocol for yeast analysis.
Yeasts were then classified based on colony characteristics and microscopic observations according to the methodologies updated by Kurtzman et al. (2011) and Boekhout et al. (2016).

DNA isolation

DNA extraction from yeast isolates was performed using the ZR Fungal/Bacterial MiniPrep™ kit (Zymo Research, USA) following the producer's protocol.
The extracted total DNA samples were checked for their quantity and quality using a BioPhotometer plus spectrophotometer (Eppendorf, USA). Extracted DNA was stored at -20°C before processing.

PCR technique for amplifying the ITS region

The ITS1-5.8S-ITS2 region was obtained by amplifying each yeast isolate using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')
universal primers as described by White et al. (1990).
PCR reactions were performed using the Mango Taq™ DNA polymerase kit from Meridian Bioline in a total volume of 50 µl.
The methodology of these reactions was based on the protocols used by Dumitrache et al. (2020) and Manolescu et al. (2022), in similar research. The amplification process was performed with the Techne TC-512 thermal cycler.

Restriction digestion

According to the ITS-RFLP methodology described by Esteve-Zarzoso et al. (1999), the digestion of the ITS-PCR products was performed with the restriction enzymes HhaI, HaeIII and HinfI (Thermo Scientific, USA). Each digestion reaction had a total volume of 20 µl, containing 2 µl of 10X digestion buffer, 1 U of the corresponding enzyme, 10 µl of PCR product, and ultrapure water to reach the final volume.
The enzymes were incubated at 37°C for 90 minutes, following the instructions for preparing the reaction mixture.

Electrophoresis and DNA products

After ITS-PCR amplification and digested PCR products, for migration in agarose gel (1% and 2% with TAE buffer and stained with ethidium bromide). Electrophoretic profiles were visualized under UV light using the Gene Flash Syngene Bio Imaging system.

A Quick-Load Purple 100 bp DNA ladder was used to determine the sizes of separated DNA fragments.

According to Esteve-Zarzoso et al. (1999), Sabate et al. (2002), and Baffi et al. (2010), restriction fragment profiles were analyzed for the electrophoretic patterns of different yeast species.

RESULTS AND DISCUSSIONS

Morphological characterization

The microbial contamination associated with freshly squeezed must be estimated at 10^6 CFU/ml. The semi-selective PDA/ch medium facilitated the growth of yeasts, molds, and some chloramphenicol-resistant bacteria (Figure 1-left), so too few yeasts could be isolated in pure culture.

The first strains with morphology similar to yeasts were observed on the YPG/ch selective culture medium, thus obtaining the first pure cultures (Figure 1 - center). This method led to the isolation of five different strains of yeast (Figure 1 - right).

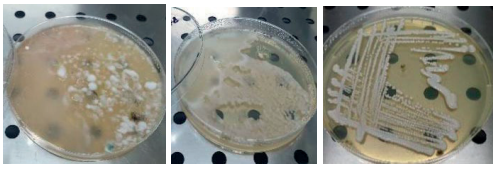

Figure 1. Yeast cultures at various stages of isolation and purification; **left.** in mixed cultures on PDA/ch; **center.** cultures with distinct colonies on YPD/ch and **right.** pure cultures of yeast on YPG medium

This approach is considered more efficient than the direct isolation of yeasts from intact grapes Valero et al. (2007); Manolescu et al. (2022). The reduced presence of fermentative *Saccharomyces* on intact grape skins, in contrast to the higher abundance of other microbial colonizers like non-Saccharomyces yeasts and filamentous fungal spores, likely accounts for this difference (Ükelgi, 2011).

Microscopy studies showed that all yeast strains used in the identification procedure were from pure cultures. The morphological aspect of the cells varied from strain to strain, with different shapes and sizes (Figure 2). Some yeast isolates showed a cream pigmentation, and others red-brown, with smooth or opaque surfaces, and with ovoid cell morphology until apiculation (lemon shape). The spheroidal and ellipsoidal shape cells were also observed for other isolated strains with a milky white to light brown (buttery) appearance with regular borders. The yeast cell size for each isolate is presented in Table 1.

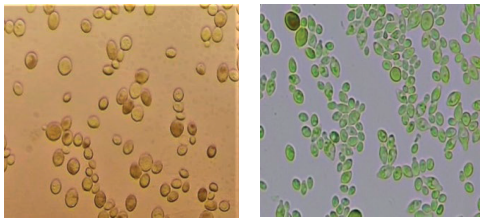

Figure 2. Microscopic observations of yeast cells derived from pure cultures (**left** - *Saccharomyces cerevisiae* and **right** - *Hanseniaspora uvarum)*

Table 1. Average dimensions of yeast cells grown on the PDA/ch medium after three days of incubation

No. code	Yeast Isolate Code	Cell Dimensions	
		Average Length (L) µm	Average Width (l) µm
D1	Isolate 1 FR	5,60±0,46	5,27±0,31
D2	Izolate 2 TR	5,34±0,37	4,92±0,29
D3	Izolate 3 Burg.	6,30±0,36	5,01±0,33
D4	Izolate 4 CS	5,68±0,32	4,30±0,35
D5	Izolate 5 Sauv.	5,60±0,31	4,86±0,34

Based on our results and microscopic characteristics of the cells, the following yeast species were estimated for the five analyzed isolates: Isolate 1 FR (D1), with dimensions of 5.60 µm x 5.27 µm, corresponds to *Saccharomyces cerevisiae* (Figure 2 - left); Isolate 2 TR (D2), with dimensions of 5.34 µm x 4.92 µm, is associated with *Pichia pastoris*; Isolated 3 Burg. (D3), which measures 6.30 µm x 5.01 µm, aligns with *Hanseniaspora uvarum* (Figure 2 - right); Isolate 4 CS (D4), measuring 5.68 µm x 4.30 µm, can be identified as *Kluyveromyces lactis;* and finally Isolate 5 Sauv (D5), measuring 5.60 µm x 4.86 µm, is associated with *Debaryomyces hansenii*.

With classic microscopy techniques, sizes from 4 µm for haploid cells to 6 µm for diploid cells were highlighted, these results being by those obtained by Milo & Phillips (2015). The observations regarding these morphological dimensions are recorded in the case of single cells, but also for mother cells that have a single bud, as in the case of yeasts from the Saccharomyces genus.
This identification is frequently supplemented by molecular biology analyses involving digestion with restriction enzymes to detect the ITS1-5,8S -ITS2 region, and to determine the polymorphism among strains (White et al., 1990; Esteve-Zarzoso et al., 1999; Sabate şi colab., 2002; Baffi et al., 2010).

DNA concentration and purity

Following the DNA extraction, the obtained results showed that the applied method was efficient in obtaining a DNA extract with a concentration of 7.1 to 15.8 ng/µl, and of good purity for PCR reactions. These results are presented in table 2.

Table 2. DNA concentration and purity

Isolate code	Concentration <ng/µl>	Rapport A260/A280	Rapport A260/A230
D1 – FR	15,7	1,73	0,81
D2 – TR	12,9	1,80	0,99
D3 – Burg.	15,0	1,76	0,48
D4 – CS	7,1	1,77	0,55
D5 – Sauv.	15,8	1,66	0,00

Analyzing the A260/A280 ratio, which provides information on RNA contamination in the final DNA solutions, it was certain that the obtained genetic material exhibited very good purity. The A260/A280 values ranged between 1.66 and 1.80, indicating high-quality DNA, despite relatively low concentrations in ng/µl.

PCR amplification and ITS-RFLP profiling of the ITS1-5.8S-ITS2 region

The ITS1-5.8S-ITS2 region was amplified via PCR, and the resulting products exhibited five distinct bands of varying sizes: 380 bp, 650 bp, 740 bp, 750 bp, and 840 bp, corresponding to the five isolates (Figure 3). According to White et al. (1990); Arlorio et al. (1999), and Esteve-Zarzoso et al. (1999), these band sizes are characteristic of isolates from the genus *Saccharomyces, Pichia*, *Hanseniaspora*, *Kluyveromyces*, and *Debaryomyces*.
The genetic variability of the yeast isolates introduced into the study was analyzed using 3 restriction enzymes: *Hae*III, *Hinf*I and *Hha*I. By using each of these, in distinct reactions.

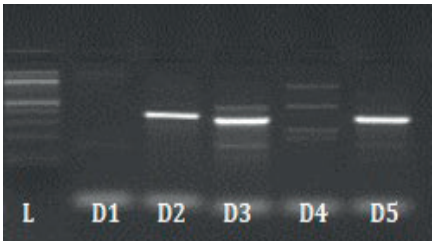

Figure 3. Electrophoretic profile of the ITS1-5.8S-ITS2 region of yeasts obtained using the ITS 1/4 primer set
Legend: **L = DNA ladder; **D 1÷5** = code for isolated yeasts*

The genetic variability of the yeast isolates included in the study was investigated using three restriction enzymes: *HaeIII, HinfI* and *HhaI*.
The electrophoretic profiles obtained in distinct reactions for each of these enzymes revealed differences between the analyzed yeast strains. The sizes of the PCR products and restriction fragments are summarized in Table 3.

Table 3. RFLP analysis for the identification of isolated yeasts

Strains identification	Analyzed strains	Produced by PCR (pb)	Obtained restriction fragments (pb)		
			HaeIII	***HinfI***	***HhaI***
Saccharomyces cerevisiae	D1	840	320 + 230 + 180 + 130	360 + 120	360 + 330+130
Pichia pastoris	D2	380	380	360	250+130
Hanseniaspora uvarum	D3	750	750	320+310+105	350 + 200 + 180
Kluyveromyces lactis	D4	740	655 + 80	290+ 180+ 120+80+65	360 + 230+140
Debaryomyces hansenii	D5	650	420+ 150+90	325 + 325	300+250

The comparative analyzes of the electrophoretic profiles obtained for each yeast isolate showed the differences at the molecular level among them Based on this analysis,

isolates that showed different restriction profiles were grouped into different categories according to their profiles for ITS1-5.8S-ITS2 region, previously described in reference studies (White et al., 1990; Esteve-Zarzoso et al., 1999; Sabate et al., 2002; Baffi et al., 2010). The five yeasts strains newly isolated from grapes belong to the among the species listed in Table 3.

Saccharomyces cerevisiae, the yeast predominantly involved in alcoholic fermentation, was isolated from the grapes of a single white wine variety, 'Fetească regală'. According to specialized literature, S. cerevisiae is not frequently detected on grapes (Combina et al., 2005), this observation being a notable one. Its presence was rarely detected during ripening or on the surface of damaged grapes (Renouf et al., 2007; Dumitrache et al., 2020). The other four strains belong to the *non-Saccharomyces* group of yeasts. For this reason, our studies will continue aiming to identify the role of these yeasts in the wine fermentation process.

CONCLUSIONS

The study successfully isolated and characterized five distinct yeast strains from grape samples, integrating both morphological and molecular techniques. Microscopic analysis revealed diverse cell shapes and sizes, aiding initial species identification. ITS1-5.8S-ITS2 region amplification by PCR, coupled with restriction enzyme analysis using *HaeIII, HinfI*, and *HhaI*, confirmed species such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hanseniaspora uvarum*, *Kluyveromyces lactis*, and *Debaryomyces hansenii*.

This combined approach not only demonstrated the effectiveness of yeast identification but also its importance for winemaking. The identification of fermentative *Saccharomyces* species alongside *non-Saccharomyces* yeasts, which can influence the aromatic complexity of wine, emphasizes the relevance of yeast biodiversity in viticulture and enology. The study reinforces the role of molecular techniques in the precise identification of microbial communities, important for wine fermentation and for improving the special aroma and taste of winemaking products.

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