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# OENOLOGICAL CHARACTERISTICS AND VINIFICATION RESULTS OF THE YEAST OF MALAGOUSIA GRAPE ISOLATED IN GREECE

Aikaterini KARAMPATEA[1,2]*, Argirios TSAKIRIS[1], Ioannis KOURKOUTAS[3]

[1]Department of Wine, Vine and Beverage Sciences, University of West Attica, Aigaleo 12243 Athens, Greece
[2]Department of Agricultural Biotechnology and Oenology, International Hellenic University, 66100 Drama, Greece
[3]Department of Molecular Biology and Genetics, Democritus University of Thrace, 68100 Alexandroupolis, Greece
*Corresponding author: katerina_karampatea@yahoo.gr

## ABSTRACT

Malagousia grapes, selected from 5 different PGI Greek zones, in 3 different growing seasons (2018-2019, 2019-2020, 2020-2021) were collected in the stage of technological maturity. Quantity of 25 Kg grapes fermented spontaneously in 30 l thermo regulated stain steel tanks, produced white wine. The indigenous yeast flora, isolated at three phases of the alcoholic fermentations, was studied. Different yeast species were isolated, purified and characterized. The restriction fragment length polymorphism of PCR-amplified fragments from the rDNA gene cluster (PCR RFLP of rDNA) has been used for the differentiation of yeast species. The standard identification procedure has been performed on representative strains that shared identical RFLP profiles showed great diversity of the yeast population. Including grape berries, must and fermented must, the following yeast species were identified: a number of Pichia and Candida species, Kloeckera apiculata, Cryptococcus curvatus, Metschnikowia pulcherrima, Kluyveromyces, as well as Saccharomyces cerevisiae and Saccharomyces stranieri. We performed microscopic, macroscopic and carbon assimilation tests by API 32C standardized system, biotechnological testing and hydrolase profiling obtained by the API-ZYM system. The most significant findings in population dynamics of yeasts in the spontaneous fermentations were bigger diversity of different species of Non-Saccharomyces in organic grapes and almost complete absence of non-Saccharomyces species, at least at grapes and at the beginning of the alcoholic fermentations at conventionally cultivated grapes, from all the examined PGI zones in all the three years of the study. The use and enhancement of indigenous yeasts is an increasing trend. Rapid identification of the yeast population is necessary for successful monitoring of the fermentation process and for obtaining a good wine quality as well as contributing to the optimization of Greek wine production.

**Keywords:** *Yeast strain selection, Spontaneous alcoholic fermentation, organic grapes.*

## INTRODUCTION

There are so many different factors contributing in the physicochemical and sensory profile of a wine. The variety, the terroir in general, and more specifically the microbial terroir can be a determining factor in regional variation among wine grapes (Alexandre 2020, Chalvantzi et al 2021). Vitivinicultural "terroir" is a concept which refers to an area in which collective knowledge of the interactions between the identifiable physical and biological environment and applied vitivinicultural practices develops, providing distinctive characteristics for the products originating from this area (OIV 2010).

The deeper understanding of the microbiology of the wine-making process is a consequence of the employment of phenotypic and molecular techniques in wine yeast characterization. Monitoring of induced fermentations gave an understanding of the evolution of the entire microfora during this process, making clear that wine quality is a consequence of the dynamics and composition of the micro-organisms involved in its production (Querol et al., 1992; Schuetz et al., 1993). More detailed scientific researches on the geographical distribution of wine yeast strains in entire areas became possible, while phylogenetic affinities and evolutionary scenarios were explored (Pramateftaki et al., 1999; Versavaud et al., 1995; Nadal et al., 1996).

In order to ensure the microbiological control of the alcoholic fermentation process, a very common winemaking practice is the inoculation of grape juice with selected cultures of Saccharomyces cerevisiae. In this way there is a better management of the alcoholic fermentation (Barre et al., 1984; Bisson et al., 2010; Pretorius, 2000; Ranieri and Pretorius, 2000; Fleet 2008). It is believed that a selected and inoculated strain of Saccharomyces cerevisiae, once inoculated correctly in a given population, will dominate the fermentation process, suppressing any indigenous species, leading to a wine having fewer possibilities of organoleptic deviations. However, in addition to Saccharomyces, there are other genera of yeast in winemaking, present in different stages either pre-fermentative or at the early beginning of winemaking and alcoholic fermentation. These yeasts are known as non-Saccharomyces' (NS) and have long been considered undesirable and are usually classified into 15 different yeast genera including Dekkera/Brettanomyces, Candida, Cryptococcus, Debaryomyces, Hanseniaspora/Kloeckera, Kluyveromyces, Metschnikowia, Rhodotorula, Pichia, Saccharomycodes, Schizosaccharomyces, Zygosaccharomyces, and Saccharomyces (Kurtzman et al., 1998; Raspor et al., 2006).

The aim of this work was to study yeasts population diversity of indigenous yeasts isolated from grapes and must collected from 5 different viticulture regions of Greece, focusing on Malagousia grape, in order to study their oenological characteristics.

Malagousia has been characterized as the Cinderella of the Greek vineyard (Kourakou 2016). The variety is mentioned for the first time in the book Oenological (1888) by Othon Roussopoulos, with a cultivation area in the prefecture of Etoloakarnania. Most Greek varieties are considered local varieties as

they are associated with their place of origin. However, the same does not happen with Malagousia, because when the first PDO zones (1970) were legislated, there were no modern / organized wineries in its cultivation area. Malagousia is therefore not connected to a specific place. Malagousia is authorized to be cultivated in all 11 viticultural departments and more specifically is a recommended variety in 43 regional zones of Greece, while is only authorized in the remaining 21 of the total 64 regional zones(Official Gazette of the Hellenic Republic 3276 / B / 2017). Malagousia is an early-maturing variety, its harvest takes place the second fortnight of August. It is a vigorous growth variety with large, cylindrical, sometimes winged, dense grapes, with medium to large-sized rails, spherical to oval in shape and medium-thick rind, slender flowering and cricket green to golden yellow. The flesh is soft, sweet, moderately juicy and slightly aromatic. It is relatively drought resistant, very sensitive to mildew, downy mildew and botrytis.


Figure 1. Greek wine map, New wines of Greece, EDOAO Athens Greece

## MATERIALS AND METHODS

**2.1 Sampling sites:** In Greece there are 120 delimited zones with protected geographical indication. We collected Malagousia grapes in the following wine producing zones: Drama (PGI Drama), Kavala (PGI Pangeon), Chalkidiki (PGI Chalkidiki), Thessaloniki (PGI Thessaloniki), Fthiotida (PGI Atalanti Valley). The mode of culture was either conventional or biological and the configuration of the vineyards was linear.

**2.2. Grape sampling:** Grapes samples were collected in the stage of technological maturity in 3 different growing seasons (2018-2019, 2019-2020 and 2020-2021). 25 Kg of grape berries Malagousia variety were collected into sterile plastic bags. Samples were kept cold and transferred to the laboratory within 12 h.

**2.3. Microbial analysis of grapes and fermented musts:** Directly fresh grapes or defrosted were destemmed and crushed with hands. The grape mass obtained, quantity of 25 l for each batch, was fermented spontaneously at 25°C (constant temperature). Once placing the grape mass in the tank, diammonium phosphate (20gr/hl) was added, followed by good homogenization. All the experimental

spontaneous fermentations took place in 30l stain-steel thermo regulated tanks, exact copies of professional winemaking tanks. Before all the treatments hands, tanks and other equipment had been washed and disinfected with alcohol. The vinifications took place to the premises of Laboratory of Marketing and New Products Development of the Department of Agricultural Biotechnology and Oenology of the International Hellenic University in Drama.

Figure 2. Malagousia grape

Figure 3. Spontaneous fermentations in 30 l tanks

**2.4 Isolation of yeast strains from spontaneous fermentations:** The fermentations were carried out spontaneously for the whole grape mass, without separating the must from the marcs. The alcoholic fermentation was conducted at 25°C. The yeasts were isolated by taking wine samples from each tank during fermentation (beginning of the AF (12–14 Baumé), middle (6 Baumé) and end (<1 Baumé). Aliquots (0.1 mL each) of several decimal dilutions in 0.1% peptone-water were spread onto YPD Nutrient Agar (Sigma-Aldrich, USA) that had been treated with streptomycin sulfate (250 mg /l)(Fisher Scientific Belgium). Plates were incubated at 25°C for 5 days (Berber et al., 2017, Renouf et al., 2005, OIV 2017). Plates containing between 30 and 300 colonies were examined according to their macroscopic features to be re-isolated on YPD agar. A number of representative colonies were isolated and purified: 250 yeast isolates were stored at 4°C on YPD (glucose, peptone, yeast extract, chloramphenicol, biphenyl, agar-agar H2O qsp) and used for further analysis. Yeast isolates were preserved on YPD agar slants, stored at 4oC and subcultured every 2 months. The cultures were also kept at -20$^{o}$C with 20%v/v glycerol as a cryoprotectant agent (Monaco et al., 2014).

**2.5. Molecular analysis:** Genomic DNA was isolated from cultures using a commercial kit, Genomic DNA from tissue (Macherey-Nagel, USA), according to the manufacture protocol/ support protocol for yeast 01/2017, Rev.17. Finally, 50 µL of a mixture containing 5–30 ng/µL of genomic DNA was obtained. Internal transcribed spacers (ITS) (ITS1 and ITS2) and 5.8S rDNA gene regions were amplified using specific primers ITS1 (5_-TCCGTAGGTGAA CCTGCGG-3_)

and ITS4 (5_-TCCTCCGCTTATTG ATATGC-3_) (White et al., 1990). DNA amplification was carried out in the final volume of 25 µL containing 0.2 mM of dNTP (Invitek, Germany), 1.25 µL of each primer (100 pmol/µL) (KapaBiosystems, USA), PCR reaction buffer (1X) and 0.1 µmol/min of Taq DNA polymerase(KAPA2G Robus) (Kapa Biosystems, USA). PCR conditions were as follows: initial denaturation cycle at 95°C for 5 min followed by 35 cycles of amplification, denaturation at 95°C for 15 sec, annealing at 55°C for 15 sec, and extension at 72°C for 30 sec; final extension at 72°C for 10 min. The amplification reaction was carried out in a PCR MiniOpticon (Biorad, France). PCR fragments were separated and detected by electrophoresis on agarose gel (1.5 mass %) in TBE buffer (1X) at 120 V for 2.5 h, Sub-Cell GT Agarose Gel Electrophoresis Systems (Biorad, France). The gel was stained with Gel Red (10 mg/L), visualized under UV light (Gel Doc EZ Imager, Biorad France) and documented by the Image Lab Software (Biorad, France). For RFLP, PCR products were purified by ethanol precipitation and digested by restriction endonucleases HaeIII, Hinf I, TaqI, AluI, MseI, HhaI, and HpaII (Takara, Japan) following the manufacturer's instructions. The restriction fragments were separated on agarose gel (1.5 mass %) under the same conditions as the amplified products. Representative samples, grouped by PCR-RFLP of ITS regions.

**2.6. Phenotypic identification:** Fermentative vigor without and with sulphites were measured according to Caridi et al., 2002: flasks containing 100 ml of sterile white must (20°Brix, pH 3.20, filtered by steri-cup vacuum filtration system, Millipore, Billerica, MA, USA), with and without $SO_2$ (100 mg/l) and covered with 10 ml of sterile liquid paraffin to prevent evaporation, were inoculated in triplicate with 5 ml of 48 h pre-cultures of each isolated yeast and incubated at 25°C. Fermentative vigor was measured as weight loss caused by $CO_2$ production (g $CO_2$ per 100 ml) after 2 and 7 days. Reducing sugars, ethanol, glycerol and volatile acidity were measured in must prepared as described above (without $SO_2$) using a Winematic apparatus (Gibertini, Italy) after 15 days of fermentation. We estimated hydrogen sulfide production on Biggy agar (Oxoid) recording the biomass color after 48 h at 25°C (Comitini et al., 2011). Beta-glucosidase production was assayed as in Strauss et al., 2001 onto selective medium containing 6,7 g /l Yeast Nitrogen Base (Difco, Detroit, MI, USA), 5 g/l arbutin (Sigma-Aldrich, Saint Louis, MO, USA), 0.2g/l ammonium ferric citrate and 20 g/l agar (pH 5.0).

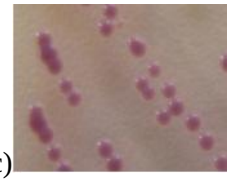
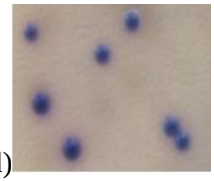
a)  b)  c)  d) 

Figure 4. Typical cultures of different yeasts in Chromagar culture medium. a)Candida albicans, b) Kluyveromyces thermotolerans c) Hanseniaspora/Kloeckera d)Saccharomyces cerevisiae

Chromagar (Chromagar France) is a chromogenic differential culture medium facilitates the isolation and presumptive identification of certain clinically important yeast species, especially when in the YPD medium the differentiation is not visible (Ainscough et al., 1998; Koehler et al., 1999; Romeo et al., 2011). The identification system ID 32C (Bio-Merieux, SA, Marcy-l'Etoile, France) was used for the carbon assimilation tests. ID 32 C is a standardized system for the identification of yeasts, which uses 32 miniaturized assimilation tests (dehydrated carbohydrate substrate) and a database.

## RESULTS AND DISCUSSION

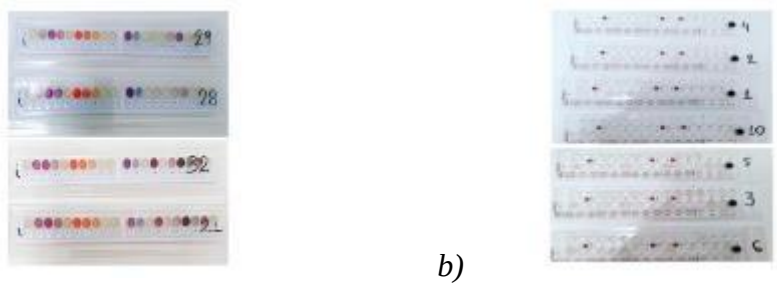
**3.1. Yeast population:** The 'NS' isolates investigated were mostly Pichia and Candida. More specifically 31% Pichia, 17%Candida lusitanea, 10% Metschnikowia, 10% Cryptococcus curvatus, 7% Kloeckera apiculata, 7% Candida colliculosa, 7% Candida globosa and 7% Kluyveromyces thermotolerans. Candida species were mostly found in grapes and at the first day of the alcoholic fermentation, while Metschnikowia species were detected only at mi-fermentation and Pichia species were found at all the stages of the alcoholic fermentations. In organically grown grapes, Candida strains in a given area (PGI Pangeon) were isolated in the grapes of the same vineyard for three consecutive years. The same was observed for Pichia strains which seem to succeed Candida strains in fermentation, as for the same grapes they were isolated in sampling in the middle of the alcoholic fermentation also for three consecutive years. Metschnicowia strains, in another area (PGI Chalkidiki), were located in the middle of the alcoholic fermentation for two consecutive years at grape samples from two different vineyards of the same zone in proximity. In another area (PGI Thessaloniki) greater heterogeneity was observed as each year different strains were identified (Cryptococcus, Kloeckera apiculata, Pichia). In conventionally grown grapes, in grape samples of almost all zones, Saccharomyces strains were isolated during all years of observation. However, this picture is reversed as in the isolates at the middle of the fermentation there was identification of Metschnicowia strains (PGI Drama), Pichia two consecutive years (PGI Atalanti Valley) and Candida colliculosa two consecutive years (PGI Pangeon). At the end of the experimental spontaneous fermentations of conventionally cultivated grapes, in almost all five zones Saccharomyces strains were isolated in all three years of our study. Beside the impact of common oenological practices during prefermentation stage (clarification, temperature, sulphite and starter yeast addition) on the growth of 'NS' yeasts (Albertin et al., 2014), it seems that the culture mode is also influencing the 'NS' population composition. However, there also other factors: climate, viticultural practices that could determine the population dynamics differ from year to year. (Alexandre 2020, Chalvantzi et al 2021).

Table 1. Distribution of yeast species (%) during must fermentation and sampling stage (BF beginning of fermentation, MF middle of fermentation, EF end of fermentation

| **Yeast species** | **PGI Drama 2018** | | | **PGI Drama 2019** | | | **PGI Drama 2020** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **BF** | **MF** | **EF** | **BF** | **MF** | **EF** | **BF** | **MF** | **EF** |
| Saccharomyces | 100 | 50 | 100 | 100 | 55 | 100 | 100 | 35 | 100 |
| Metschnicowia | | 50 | | | 45 | | | 65 | |
| **Yeast species** | **PGI Pangeon 2018** | | | **PGI Pangeon 2019** | | | **PGI Pangeon 2020** | | |
| | **BF** | **MF** | **EF** | **BF** | **MF** | **EF** | **BF** | **MF** | **EF** |
| Candida | 90 | 30 | | 95 | 35 | | 95 | 25 | |
| Pichia | 5 | 70 | 70 | | 60 | 65 | | 73 | 70 |
| **Yeast species** | **PGI Thessaloniki 2018** | | | **PGI Thessaloniki 2019** | | | **PGI Thessaloniki 2020** | | |
| | **BF** | **MF** | **EF** | **BF** | **MF** | **EF** | **BF** | **MF** | **EF** |
| Cryptococcus | | 25 | 30 | 20 | 35 | 45 | 35 | 10 | 15 |
| Kloeckera apiculata | 78 | 25 | 30 | 25 | 40 | 25 | 30 | 50 | 45 |
| Pichia | 15 | 50 | 35 | 50 | 20 | 15 | 35 | 40 | 40 |
| **Yeast species** | **PGI Chalkidiki 2018** | | | **PGI Chalkidiki 2019** | | | **PGI Chalkidiki 2020** | | |
| | **BF** | **MF** | **EF** | **BF** | **MF** | **EF** | **BF** | **MF** | **EF** |
| Metschnicowia | 10 | 40 | 20 | | 80 | 55 | 20 | 85 | 45 |
| Candida | 90 | 50 | 50 | 80 | 10 | 15 | 80 | 10 | 40 |
| **Yeast species** | **PGI Atalanti Valley 2018** | | | **PGI Atalanti Valley 2019** | | | **PGI Atalanti Valley 2020** | | |
| | **BF** | **MF** | **EF** | **BF** | **MF** | **EF** | **BF** | **MF** | **EF** |
| Saccharomyces | 100 | 80 | 100 | 100 | 45 | 100 | 100 | 30 | 100 |
| Pichia | | 15 | | | 55 | | | 68 | |

### 3.2. Oenological characterization of the yeast species

Enzymatic profile: The enzymes α-fucosidase, esterase, esterase/lipase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin aminopeptidase, phosphohydrolase, galactosidase were detected in all different strains. Both N-acetyl β-glucosaminidase and α-fucosidase activity were detected in all 'NS' yeasts except Pichia strains. Although each strain had a unique enzyme pattern, the mean enzyme activity was esterase, esterase / lipase, lipase with that of esterase / lipase slightly larger. The action of N-acetyl β-glucosaminidase shows maximum enzymatic activity in almost all strains except three in which does not appear at all or almost not at all. Low β-galactosidase activity is shown in all strains. Also β-glucosidase has generally low production with the exception of two Candida strains which show activity of 30 nmoles. The following enzymes were not detected in any of the tested strains: alkaline phosphatase, α-chymotrypsin, α-glucosidase (Canal-Llaubères, 1993).



*a)* *b)*

Figure 5. a) API ZYM – Semiquantitation of enzymatic activities b) API ID 32 C Identification system for yeasts

Table 2. Number of Non Saccharomyces (NS) species isolates

| *PGI Region* | *Vintage* | *Nombre of Non Saccharomyces (NS) species isolates* | |
|---|---|---|---|
| | | *Conventional Grapes/Must* | *Organic Grapes/Must* |
| *Drama* | *2018* | *0/15* | |
| | *2019* | *5/10* | |
| | *2020* | *0/14* | |
| *Pangeon* | *2018* | *1/17* | *10/12* |
| | *2019* | *5/17* | *15/15* |
| | *2020* | *2/13* | *10/20* |
| *Thessaloniki* | *2018* | | *5/18* |
| | *2019* | | *2/10* |
| | *2020* | | *4/15* |
| *Chalkidiki* | *2018* | | *9/12* |
| | *2019* | | *11/14* |
| | *2020* | | *8/12* |
| *Atalanti Valley* | *2018* | *0/10* | |
| | *2019* | *2/12* | |
| | *2020* | *0/12* | |
| | | | |
| *Total* | | *89* | |
| *Total of yeast isolates* | | *248* | |

## CONCLUSION

To summarize this study provides more information regarding yeast communities on the conventionally cultivated and organic Malagousia grapes and musts from 5 different PGI Greek zones in 3 consecutive years. In the present study we applied modern molecular techniques that are suitable for rapid identification of Saccharomyces and Non Saccharomyces strains and further testing of various strains for their oenological properties. Several physiological characteristics of the yeasts used in this study are suitable for rapid selection of the different yeast strains that could be applied in the winemaking process. Inoculation or co-inoculation of

selected strains with suitable technological properties in the wine fermentation process could give in wines better organoleptic characteristics and enhance the quality of Malagousia wines.

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**Author Contributions:** A. Karampatea had the whole supervision of this work, making the analysis and writing sections of this paper, A. Tsakiris and I. Kourkoutas contributed in the interpretation of results and to writing.

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