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ORIGINAL ARTICLE

Microbiological and Molecular Identification (PCR-RFLP-ITS) of the Yeast From Carignan Grape Cultivated in Abdelmalek Ramdane (Wilaya of Mostaganem, Algeria)

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ABSTRACT
The grape is an exemplary fruit of the microbial diversity, it is considered as the habitat of multiple wild microorganisms (yeasts). In this study, we explored the divergence of the indigenous yeast flora in the vineyards of the Abdelmalek Ramdane region (Wilaya de Mostaganem, Algeria) by collecting grape samples (Carignan). Extraction of DNA as well as a PCR-ITS-RFLP has been developed for the ITS1-ADNR 5,8S-ITS2 region ; it is sensitive enough to detect the biodiversity of the different species of isolated yeasts. Then 5 species of yeast out of the 12 studied belonging to 3 different genera were characterized according to their molecular profiles. Thus, the strains studied were characterized with two restriction enzymes (Hinf I and HaeIII). Meanwhile, classical microscopic yeast identification studies were able to enhance the results. Among the yeast species identified : Candida intermedia, Candida magnoliae, Candida gropengiesseri, Pichia deserticola and Saccharomyces cerevisiae. According to the results obtained, the Carignan variety presents an excellent reservoir of yeasts Non saccharomyces. However, the tools of molecular biology have brought a notorious revolution in precise yeast identification tests. The PCR-RFLP-ITS is one of the most widely used methods of identification.
Key words : *Grape, Yeasts, Carignan, PCR-ITS-RFLP, Hinf I and HaeIII.*

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INTRODUCTION

Yeasts are naturally present on soils, plant surfaces, in particular grape berries, or in wine-growing areas [15, 4]. Their diffusion is ensured by the wind, insects and humans through their various interventions on the environment [9, 2].The microflora of grapes is made up of a wide variety of microorganisms distributed among yeasts, bacteria and molds. Their presence and number on the surface of the grapes are influenced by several factors such as the grape variety, climatic conditions during ripening, soil, cultivation practices, the geographical location of the vineyard and the age of the vines [7]. Carignan is a grape variety that forms the basis of the grape varieties in the vat vineyard. Regularly productive and not very demanding, it is poorly suited to the dry plains of the west where the heat of the summers does not allow it to ripen regularly, its berries which do not develop well there and remain small and dry. However,the high sugar content of the Cinsault grape must promote the growth of yeast, these microorganisms are an essential part of the food industry. They participate in the development of many food products (bread, dairy, brewery) and in the production of metabolites and enzymes (invertase, lactase, lipase and amylases) but also in the revaluation of agricultural and industrial waste. Biotechnologies and biomedical research largely exploit these microorganisms for the production of

molecules of medical interest. (ex : production of heterologous proteins, such as the hepatitis B vaccine) [14, 3]. The aim of this study is to isolate, characterize microscopically and identify the indigenous species of yeasts found in the grape variety (Carignan) grown in the region of Abdelmalek Ramdane (Mostaganem, Algeria) to develop and express the diversity of the phylogenetic heritage based on the molecular technique (PCR-RFLP-ITS). Our work is an initiative for the creation of a "Souchier" typical yeast in our country Algeria.

MATERIAL AND METHODS

Grape picking Source (Carignan) :

The samples are taken randomly in the month of September 2014 in the vineyards of the region of Abdelmalek Ramdane (Mostaganem, Algeria). We collected with sterile scissors, about 500 g healthy grapes, and it was collected in sterile bags. A laboratory arrival, they are scuffed and crushed to obtain a mash and ferment for one day at 25 ° C in order to increase the viability and quantityof the desired yeast [19].

Isolation, purification and conservation of cultures :

We carried a series of seedings by the method of the streaks on plates of agar cultures (YPG + Gentamicin) in order to have pure cultures. The operation is renewed by taking at random an isolated colony. This leads to obtaining a culture whose purity is estimated by microscopic observation. Then the purified strains are placed in a glycerol solution (sterile) with approximately 25% and stored in the freezer at -18° C.

Microbiological identification :

Study of characters crop :

After incubating the cultures for 3 days at 25-28 ° C on agar medium (YPG + Gentamicin), macroscopic observation can describe the appearance of colonies (size, pigmentation, contour, viscosity ...) [12].

The cellular characteristics :

Microscopic observation to define the shape, arrangement and mode of cell division. These characteristics are observed on microscopic slides to fresh (40 x objective) [17].

Molecular identification

•DNA extraction :

The technique developed by [1] makes it possible to efficiently extract genomic DNA from yeasts in the case of liquid pure cultures but also cell suspensions collected on the surface of petri dishes. When isolated colonies of dishes are obtained, it is possible to extract the genomic DNA from the biomass present on the surface of the agar (YPG agar). When relatively fresh (maximum 15 days), a small amount of biomass is collected and treated by several extraction steps. The first step consists in mixing the collected biomass with 660 µl of 50% TE / SDS mixture (lysis buffer), then vortexing the mixture and incubating it at 65 ° C/5 minutes. To neutralize the medium, a volume of 340 µl of potassium acetate was added to the mixture, the latter is placed in the refrigerator for 30 minutes until the suspension became semi-solid. Then a second centrifugation was performed for 10 minutes and a volume of 750 µl of supernatant was mixed with 750 µl of isopropanol followed by a final centrifugation to precipitate DNA. Then, the collected DNA pellet was rinsed gently with approximately 120 µl of 95% ethanol, then suspended in a volume of 300 µl of TE1x (storage buffer) and incubated at 65 ° C for 15 minutes. Finally, the DNA was stored refrigerated 4°C until use (According to IFV Nantes, 2012).

•Amplifying the target region by the molecular techniques (PCR-RFLP-ITS) :

Amplification of the ITS region

The sequence ITS1 5.8S rRNA ITS2 present a conserved region in the majority of yeast species and the variable regions. The primersITS1 (5'-TCCGTAGGTGAACCTGCGG-3 ') and ITS4 (5' TCCTCCGCTTATTGATATGC-3') described as universal primers by [20]. The reaction mixture is summarized in Table 1.The amplification is carried out in the thermocycler (Biométra) according to the program described by [16].

Enzymatic digestion (According to Renouf, 2006)

In order to identify the different species of yeast. ITS PCR products are treated with restriction enzymes that recognize specific DNA sequences or motifs (the restriction site). Two enzymes were used in this study HinfI and HaeIII. The reaction mixture is summarized in Table 2 and Table 3. After restriction, the restriction profiles are obtained by electrophoresis (migration 120-130 for 30 minutes). The number and length of the fragments are compared with the size marker according to (IFV).

Electrophoresis on agarose gel

The purification of a double-stranded DNA fragment (ITS PCR products) is carried out by agarose gel electrophoresis, which consists of a mixture of 1.5% agarose and 1X TBE buffer , The gel is mixed with

ethidium bromide (20µl BET / 200ml TBE) and hot-dipped in the electrophoresis tank. 20 µl of the mixture contained in each PCR microtube, mixed with 5 µl of loading buffer containing bromophenol blue, are deposited on the wells. Fragments of DNA of known molecular mass called markers are also deposited: they make it possible to correlate the migration distance of the DNA fragments to their size (in base pairs). Then, applying a voltage of 120V / 30 minutes until the migration of the fragments [5].The nucleotide different bands resulting from the electrophoresis are shown on the gel under UV light (254 nm) and photographed with a UV camera.

RESULTS AND DISCUSSION

Isolation and purification by successive subcultures made after collection of yeasts in September 2014 from the Carignan grapes grown in the ABDELMALEK RAMDANE area (Wilaya of Mostaganem). This allowed us to get a collection of 12 yeast isolates.

Study cultivation of isolated yeasts :

Macroscopic examination of cultures "levuriennes" after incubation at 25 ° C for 4-5 days shows generally well isolated colonies are whitish, cream or sometimes yellowish opaque and irregular outline, they have an intense smell. Microscopic observation has allowed us to identify cell shape of isolated isolates and vegetative reproduction modes. Of the 12 individual isolates, 11 are spherical or cylindrical, elongated or short form and are budding as vegetative reproduction mode. The remaining isolate is characterized by an oval shape and has a monopolar budding, it has a smell of yeast. The uniformity of cells confirms the purification of isolates studied. According to [12], yeasts are in the form of independent free single cell or combined in pairs with characteristic morphology for example: spherical, ovoid, cylindrical, apiculé, bottled, pyramidal.

Molecular study of isolated yeasts :

DNA extraction :

DNA extraction has allowed us to observe a small white mass rushed to the microtube bottom, then it was inoculated in a buffer prior TE1x and must keep to the time of use.

PCR-RFLP-ITS :

A total of 12 isolates of yeasts isolated from the Carignan grape juice were analyzed. To identify these yeasts, the region of the rRNA repeat unit comprises two non-coding regions referred to as internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene were amplified and digested by two enzymes of restriction (HinfI and HaeIII). The profiles obtained from each isolate were compared with reference strains in the determination of the IFV Guide (2012). The results of this study are summarized in Table 3. The size of the PCR products and restriction fragments of the major species identified in this study are shown in Table 4.

The species of yeasts isolated from the grape (Carignan) :

12 yeast isolates were identified as belonging to 3 genera and 5 different species : *Pichia* (06 strains), *Candida* (05 strains), *Saccharomyces* (01 strain) (Table 3). These different kinds of yeast are well documented in the literature as present on grapes and the start of alcoholic fermentation [36,1]. The review of the results indicates that 5 selected yeast strains (strain 1, 2, 6, 7,5) belong to the genus *Candida* (Table 5), according to the determination of IFV guide (2012), while the strains (1) and (2) were identified as belonging to the species *Candida intermedia* and *Candida magnoliae* respectively, while the strains (strain 6, 7,12) were identified as belonging to the species *Candida gropengiesseri*. Furthermore, the combination of the two results (morphological and molecular studies) indicated that the strain (3) belongs to the species *Saccharomyces cerevisiae.* The molecular profile of six yeast strains (strain 4, 5, 8, 9, 10,11) indicates that they look like the species *Pichia deserticola,* according to the determination of IFV guide (2012).

The yeast species present on the surface of grape berries are significantly limited in number, on the other hand, carignan grape must is very rich in non-Saccharomyces yeasts as evidenced by the results obtained. Tableau.

Pichia desrticola is the predominantly isolated species (6 strains), followed by other species belonging to the genus *Candida* (5 strains), and finally a single kind of *Saccharomyces cerevisiae* was isolated from the must. The presence of yeasts non-*Saccharomyces* in grape must has been widely documented [8, 18], they are known to be most often associated with grape fermentations, including the genera *Pichia, Torulaspora, Candida* and *Hanseniaspora* predominate, and to a lesser extent by the kinds of *Kluveromyces* and *Metschikowia*. In addition, the variability of species within the same genus has also been reported, especially in the genus *Candida* (*C.intermedia, C.magnoliae* and *C.gropengiesseri*).

The grape must (Carignan) is an excellent reservoir of Non-Saccharomyces yeasts. These yeast species were represented by the species *C.intermedia*, *C.magnoliae, Pichia deserticola* and *C.gropengiesseri*, then

that only one strain (3) was identified as *Saccharomyces cerevisiae*. The growth and / or the persistence of the different species of non-Saccharomyces yeasts in the fermentations is probably determined by different sensitivities to temperature and ethanol as well as some other factors [8]. In addition, the lower fermentation temperatures (between 10 and 20 ° C) have been shown largely favorable for the growth and / or persistence of *Pichia* species and *Candida* [11], this probably because of the tolerance of these ethanol yeast produced at lower temperatures [10]. In the same meaning and according to [6], the species belonging to the genera *Pichia* and *Candida* show growth rates comparable to that of *Saccharomyces cerevisiae* at low temperature (10 ° C).

It is commonly accepted that the start of fermentation is achieved by non-Saccharomyces yeasts, fermentative but not very resistant to ethanol (such as *Pichia sp*, *Candida sp Hanseniospora Uvarum*, and *Torula sp*). These yeasts will quickly be inhibited by their own ethanol production while yeasts more resistant to ethanol develop such as *Saccharomyces cerevisiae*.

Other species Non-Saccharomyces as *Candida zemplinina*, *Torulaspora delbrueckii and Hanseniaspora spp* are also an important part of the diversity of the community of the bay and are present during the fermentation, in particular during the stages pre fermentative [21].

Table 1 : The composition of the reaction mixture used for PCR-ITS according to (IFV)

Products	Volume (µl)	Volume (µl)
H2O	1220	1230
Tampon 10X	160	160
dNTP	64	64
ITS1	12,8 (40pmol /reaction)	12,8
ITS4	12,8 (40pmol / reaction)	12,8
Mgcl2	80	80
DMSO	31(2%)	31(2%)

Table 2 : The restriction by the Hinf I.

Products	Volume en µl
Tampon 10X –Mix III	2,5
BSA	0,2
Enzyme Hinf I (10U/µl)	1

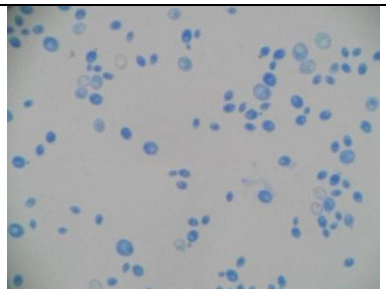
The reaction mixture is incubated at 37° C for 4 hours.

Table 3 : The restriction by the Hae III.

Products	Volume en µl
Tampon 10X –Mix III	2,5
BSA	0,2
Enzyme Hae III (10U/µl)	1

The reaction mixture is incubated at 37° C for 4 hours.

Table 4 : Example of macroscopic and microscopic observations in two isolated strains.

Strain	Macroscopic observation	Microscopic observation
(1)	 Creamy colonies of white, medium sized, smooth surface and have a yeasty smell of beer.	 The vegetative form is spherical. The mode of reproduction is monopolar by budding.

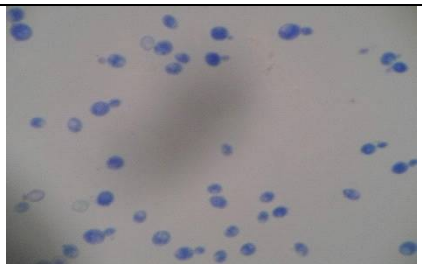
(2)	 Creamy colonies with a yellowish surface, small in size, smooth and with an intense yeast odor.	 The vegetative form is ovoid. The mode of reproduction is monopolar by budding.

Table 5 : Frequency of yeast species isolated from the grape " Carignan "

Isolation source	Species	Frequency of Isolation (Number of strains)
Carignan grape variety	*Candida intermedia*	01
	Candida magnoliae	01
	Saccharomyces cerevisiae	01
	Pichia deserticola	06
	Candida gropengiesseri	03

Table 6 : Size in bp of the PCR products and the restriction fragments obtained with two different endonucleases (Hinf I and HaeIII) of the major species identified in this study.

Species	Amplified product (pb)	Restriction fragments (pb)	
		Hinf I	HaeIII
Candida intermedia (Strain 1)	400	210/190	400
Candida magnoliae (Strain 2)	450	220/190	290/150
Saccharomyces cerevisiae (Strain 3)	850	370/360/110	320/230/170/120
Pichia deserticola (Strains 4, 5, 8, 9, 10,11)	450	250/210	280/100/80
Candida gropengiesseri (Strains 6,7, 12)	410	190	290/150

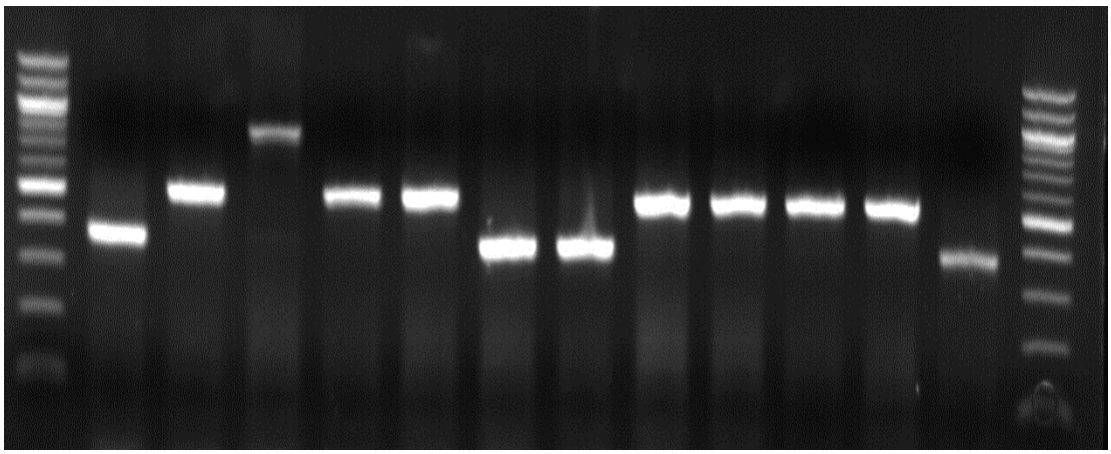


Fig. 1 : Viewing the amplified region (rRNA 5,8S ITSI-ITSII) in 12 isolates. Lanes M correspond to molecular size standards (100-bp DNA ladder from IFV)

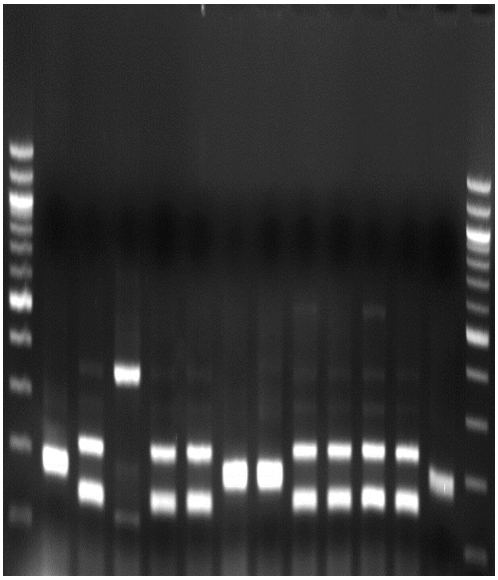


Fig. 2 : Viewing the region (rRNA 5, 8S ITSI-ITSII) digested by Hinf I in 12 isolates.

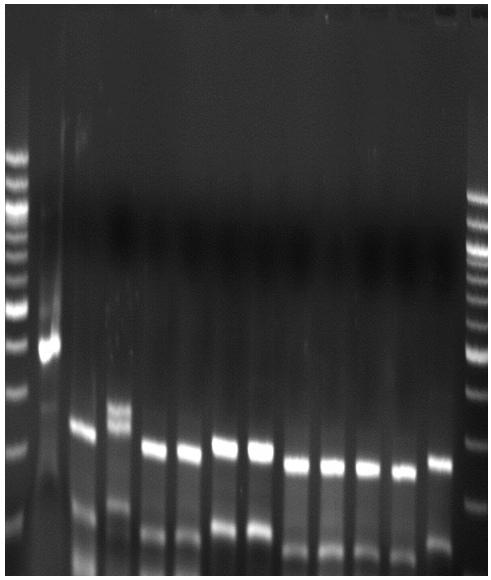


Fig. 3 : Viewing the region (rRNA 5, 8S ITSI-ITSII) digested by HaeIII in 12 isolates.

This study is based on the evaluation of a grape variety grown in Algeria (Carignan) .A genetic approach has been developed in this work and has achieved an identification of 12 isolates belonging to 3 genera and 5 different species: *Pichia, Candida* and *Saccharomyces*. This variety is an excellent tank of Non Saccharomyces yeasts as evidenced by the results. In terms of our study, we strongly encourage investigations to characterize biotechnologically these identified strains, and to encourage all studies concerned with the identification and characterization of other grape varieties in all regions of Algeria.
However, yeasts open up avenues of research for the future. Constantly improved, production systems make it possible to envisage the production of very different proteins, for the human or veterinary pharmacy. The final objective, drawn in the short and long term, is set in the development of the yeasts identified and then selected to serve mainly the agro-food domains (bread, dairy, brewery...) and new biotechnologies.

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