Peer Reviewed
Current Research on Biological Sciences
(CRBS) Vol.01,Issue.01,No:21-31,2016

MOLECULAR IDENTIFICATION WITH PCR_DELTA AND PCR_ITS_RFLP OF YEAST IN THE VINEYARDS OF THE PLAIN GHRISS, GRAPE VARIETIES (MERLOT, CABERNET AND CARIGNAN)

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Abstract:

Identification and isolation of yeasts can often be problematic. Our work aims to study the diversity of local native flora yeasts by collecting samples of different varieties of grapes (Merlot, Cabernet and Carignan) which are scattered over the vineyards of the plain of Ghriss (Mascara).The main purpose of this study is to develop new methods of detection of our yeast isolates after a realization of macroscopic and microscopic studies that investigate their morphological and cultural characteristics.A protocol for DNA extraction as well as two methods of molecular detection of fungi has targeted DNAr, a PCR-ITS-RFLP was developed for the region ITS 1-DNAr 5.8 S-ITS 2 and the target PCR-Delta Ty1 retrotransposons (Delta region). They are sensitive enough to detect the biodiversity of different yeast species isolated.The results were revealed considerable phenotypic divergence accompanying the molecular diversity and show that there is a strain variation.Despite this diversity, several cases of dominance and survival of strains were observed and ten genera were identified: *Pichia, Saccharomyces, Cryptococcus, Rhodotorula, Leucosporidiella, Brettanomyces, Candida, Clockera, Dekkera* and *Diplodia.*

Keywords: yeast, PCR, molecular diversity, Cabernet, Carignan, Merlot.

1. Introduction:

The wine grapes are one of the most diverse plants that are, and certainly the one that has done most men dream since ancient times (Dussert, 2012).It is a creeping shrub of the botanical family *Vitaceae* essentially *Vitis*. Its fruit or grapes are *Vitis vinifera* which is regarded as the second most cultivated fruit in the world (Blouin, 2007).This plant is mainly used to make wine from its fermented juice, but it also consumed as fruit, either fresh table grapes, either dry grapes. It also extracts the grape seed oil.As any food product, grape has an indigenous microflora.In the vineyard, the yeast populations are low and competition from mold. They develop on the surface of grapes and are a constituent of the bloom (thin powder layercovering the grape) and are specific to certain areas or villages (Beguin, 2008).

Most species are oxidative and they do not transform, or very little, the sugars in the grapes into alcohol. There are only very few fermenting yeast *Saccharomyces cerevisiae* type on the cuticle of the grapes. This yeast microflora varies significantly from one plot to another probably related to cultural practices and climatology of the vintage. Yeasts in nature live with the wine. Their disappearance in a field is due to intensive treatment that is inflicted on the vine.Several techniques have therefore been developed to discriminate and / or identifying microorganisms by analysis of the DNA or a portion thereof (Divol, 2004).Molecular methods based on the analysis of repeated units of the ribosomal DNA (rDNA) are the most widely adopted for the delimitation of yeast species (Ayoub, 2006).

In this research, we used the technique of Polymorphism Restriction Fragment (RFLP), which is the result of a combination of digestion of the high number of restriction enzyme cleavage sites and a single electrophoresis (Boulouis*et al.*, 2001). The rDNA 18 S or 26 S with or without the IGS (intergenic space) between the genes of the 18 S rDNA and 26 S are amplified with universal primers defined by aligning sequences available, the PCR product is then digested with restriction enzymes (Moussa Sassi, 2011).Different authors used 18 S regions or even regions NTS (Non- Transcribed Spacer) to identify yeast species by PCR-RFLP. However,the sequences 26 S rDNA or ITS (Internal Transcribed Spacer) are the most used in the identification of species by PCR-RFLP (Zott, 2009).PCR ITS is the region of the

DNA including the ITS 1 and 4 as well as the rRNA 5.8S gene is amplified by PCR (Janvier, 2007).PCR Delta is a technique based on PCR (polymerase chain reaction) amplification of regions of the genome located between the deltaselements, portions of DNA dispersed in variable number according to the yeast strain. *Saccharomyces cerevisiae* has only this delta as this technique is applied to it this species (Ayoub, 2006).

2. Materials and Methods:

Yeast strains: Isolation and purification by successive subcultures made after collection of yeasts in September 2011 from 3 grape varieties (Merlot, Cabernet and Carignan) implanted in the Ghriss plain. This allowed us to get a collection of 19 yeast strains.Before starting the molecular identification, the isolates were subjected to microscopic examination to determine their shape, budding,cultural characters, physiological and morphological as well as descriptions ofthe aptitude to sexual reproduction and review of the filamentisationaccording to the method of Bedossa(2010).

DNA extraction*:* preparing the yeast genome has been performed using 630 µl of a buffer mixture of TE / SDS supplemented with 340µl of potassium acetate which allowed to neutralize the medium before the addition of isopropanol 700µl and obtaining DNA under pelleted after a maximum centrifugation for 10 minrinsing the pellet with cold ethanol before drying using the method of Renouf (2006) with simple modification.

PCR -Delta*:* To perform this technique, two kinds of primers are commonly used whose sequences according to Legras (2003) are the following:
Delta 12: 5 ' TCAACAATGGAATCCCAAC 3'.
Delta 21: 5 ' CATCTTAACACCGTATATGA 3'.
The delta mix is indispensable for the realization of this technique using: 8µl Taq polymerase, 56µl buffer, 403µl distilled water, 25µl$MgCl_2$ and 2.5 µl of the two previously- mentioned primers.

PCR -ITS – RFLP*:* requires two primers whose sequences are the following according Połomska (2007):
ITS 1:5'-TCC GTA GGT GAA CCT GCG G- 3 '.
ITS 4:5'-TCC TCC GCT TAT TGA TAT GC-3 '.
Another mix was used in this method prepared with : 1200µl of distilled water,16µl of 10 X Buffer, 64µl dNTP, 10.8µl of ITS 1, 10.8µl of ITS 4, 80µlofMgCl2, 2% DMSO (31 µl), 24 µl of Taq polymerase and amplified DNA.

Electrophoresis was carried out on Agarose gel 1.5%, which was prepared from 3 g of agar resorphor more with stirring on a hotAgarosewas dissolved in 1X TBE (TBE 10X diluted 1:10 ; Tris 107.8 g / l, boric acid 55 g / l and 7.44 g / l EDTA) (Perron, 2004) .

At the onset of boiling, stirring is stopped. Ethidium bromide 200µl is added to the Agarose just before solidification in order to visualize the DNA on ultraviolet radiation (256 nm).

Following amplification of the DNA extracted, it was added bromophenol blue. Then, the samples were put in the wells of solid gel (prepared above). At this stage, the gel was placed in the electrophoresis tank containing TBE solution.

3. Results and Discussion:

Microscopic examination revealed various characteristics ofplate until the color became transparent. Theyeasts isolated Merlot, Cabernet and Carignanciting morphology where most were ovoid,spherical or without filamentisation sporulation for a few isolates.

Molecular study was represented by the PCR-Delta that identified the *S. cerevisiae*, the white electrophoresis, white ITS and ITS - RFLP using two restriction enzymes the *Hinf*1 and *Taq1*. Comparison of strains with referenced strains displayed by IFV Nantes allowed the selection of some strains but not definitively confirm the results. This was explained by the use of a small number of restriction enzymes and the DNA fragmentation.
From the table above, the amplified DNA has a size from 480 to 900 bp may vary depending on the isolate. After using two restriction enzymes, it was between 2 and 4 fragments at the end of digestion.The number of fragments obtained depends on the total DNA and position of the restriction site of each yeast genome.
The second variety Cabernet, the PCR- delta was negative no strain has the *sacchoromyces* profile cerevisiae. The isolates rather kind of *Brettanomyces, Candida* and *Kloeckera.*These results are not valid for all strains since digestion by *Hinf1*and *Taq1* failed for the last strains as is clear in the table above and Figure 4.

While the results of Carignan grape proved negative except for the extraction of DNA and so is amplified.This was confirmed by electrophoresis in white and almost identical size DNA amplified all of this variety, which has not allowed us to identify or even to suspect any yeast strain.

Physiological identification:Microscopic observation brings up cells in multiple forms: ovoid, elongated, spherical. Yeasts have several types of budding (monopolar, bipolar and multipolar) as well as sexual spores. Generally, microbiology yeasts, the study was done on live cells, it allowedto the visualization of the cellular morphology of the strains.The morphological appearance of the cells isolated from three grape varieties was identical to the microscopic observations found by confirming that whereas they were ovoid, multipolar budding fermentation early elongation of the cells was observed. Some budding cells were observed, but the size of the mother cell and daughter greatly reduced (Barbin, 2006).Studied morphological characters were influenced by several criteria quote:

- The heterothallism: sporulation can take place only if there was a meeting types sexed compatible (Mating Type).
- The composition of the medium: to sporulate, *S. cerevisiae* needs a source of fermentable carbon, a limiting nitrogen content and good aeration (Bourgeois *et al.*, 1996).
- The reduction of the dissolved oxygen concentration can stimulate the formation of spores in some species of *Hanseniaspora*.
- The temperature: most spore-forming species sporulate between 18 and 25 °C. Values of 12 to 15 °C are favorable for *Metschnikowia* (Larpent, 1997).
- It should be mentioned that the time required for sporulation of a culture varies widely between species and between strains of the same species. We must therefore follow up for several weeks (Thuriaux, 2004).
- For the Filamentisation, there is some yeast which is able to form mycelia and other not.

As underlined by several authors, the morphology of microorganisms was subjected to variations imposed depending on the species, its characteristics and its growing conditions. This polymorphism helps make difficult their detection and identification simply by microscopic observation.In the case of the microbial ecosystem of the grapes, the crop independent analysisnot reveals an interspecific diversity greater than the analysis using step insulation on boxes. It is likely that, given the importance and heterogeneity number of species present only the major species are detected in both cases (Renouf, 2006).

Molecular identification*:*Molecular techniques in this study were based on the study of the genome of yeasts by amplification of the ITS1 5.8 -ITS S 2 and transposon Ty1 (Delta).Analysis of the results showed that yeast strains probably corresponded to the genera *Cryptococcus, Candida, Diplodia, Saccharomyces, Rhodotorula, Crytococcus, Leucosporidiella, Pichia* and *Dekkera* for isolated strains of Merlot and genera *Brettanomyces, Candida* and *Kloeckera* for stem Cabernet.

The advent of molecular biology has to understand that the methods of identification of microorganisms based on the phenotypes of different individuals were not always reliable because the results could vary over time.If we compare this work with other efforts, we can conclude that the results were almost identical to the works cited below.

Berata and coll. (2007) worked on *Ascomycetous* yeast species: recovered from grapes damaged by honeydew and sour rot where they found similar results to those we found with a polymorphism detected with an abundance of *Candida*strain.Isolation of indigenous yeasts wine may have several drawbacks since the determination of *Brettanomyces* species may be disturbed by the presence of *Saccharomyces cerevisiae* (Oxenham, 2007). The yeast ecosystem was predominantly colonized by non- *Saccharomyces* yeastsduring the early stages of the vinification. The population of non –*Saccharomyces*remains rather stable during the fermentation and increases to reach its maximum populationin the first half of the fermentation, and then decreases towards the end of the fermentation (Zott, 2009).This complied with our work.The third part of Zott's work was to develop a quick and <<Independent culture>>, the real-time PCR, to detect and quantify some non-*Saccharomyces* species. We have developed this effect primers Specific to quantify directly the musts or wines *Candida* species *zemplinina, Issatchenkia orientalis, Metschnikowia pulcherrima* and *Torulaspora delbrueckii*,but also *Hanseniaspora* (*uvarum* and *guilliermondii*) and *Saccharomyces* (Barbin, 2006). Working on native yeasts of different varieties of grapes using methods of PCR DGGE showed mostly *Saccharomyces cerevisiae* as it encountered problems in identifying strains as we met.

On the other hand, the re- invigoration was only possible on some lots and not concernedas fermentative yeasts (*Saccharomyces cerevisiae* or *Zygosaccharomyces sp*.) andsometimes oxidative yeasts (*Rhodotorula mucilaginosa*), identifications have beenperformed by PCR-RFLP targeting the region ITS1-5,8S rRNA - ITS2 (Divol, 2004)

In theory, if we work with pure strains, PCR product of the digestion shouldgenerate a fragment marked terminal, since the digestion is complete (Janvier, 2007).

However, the results appeared negative:

- Delta: for all the isolated yeast strains.
- digestion by *Taq* 1: Cabernet (Cb2 'Cb4, Cb4' Cb5 and Cb5 ')Carignan (Cr2, Cr3, Cr4 and Cr5)

- White electrophoresis: Carignan (Cr2, Cr3, Cr4 and Cr5).

Since the results were negative, then the causes and probabilities are numerous:

- Concentration or quality of the sample: Excess DNA can reduce the effectiveness of the amplification reaction and even too low concentrations give poor quality sequences.
- PCR products of more than 2000 bp could be difficult to see, the DNA concentration becomes a limiting factor.
- Concentration of the primer binding site not present or poor primer used, primer degraded or failure of hybridization: An excess of primers or too low concentrations give lower quality amplicons.
- Absence of the target region in the gene of the strain studied.

Further, other bands were difficult to detect which limit their interpretation.

- ITS White: Carignan (Cr2, Cr3, Cr4 and Cr5)
- Digestion Hinf: Carignan (Cr2, Cr3, Cr4 and Cr5)

The low intensity of these bands can be explained by:

- Insufficient concentration of the sample.
- Degradation of the DNA during extraction.
- Switchgear fault (Non availability of suitable UV reader, electrophoresis not suitable relevant experience and a poorly functioning power generator ... etc.)

Renouf (2006)explained by the fact thatinthe first case, without culture, this is explained by a competition between the DNA templates during the first amplification cycles: the probability of amplification and therefore detection of a sequence is most important for the majority species. At long PCR, the cooperatives of the amplification process widens the gap between the sequences initially the majority and minority so that in the end, only the former are detectable. In the second case, that is the step of culturing which via dilutions imposed single colonies, evades minority species. To access past the enrichment step is needed.

4. Conclusion:

It is necessary to declare that a strong relationship exists between the phenotypic diversity and the molecular diversity.There is no doubt that both are responsible for the phenotypic variation, evolution and the molecular diversity of strains.Though the phenotypic characterization was important and allowed to the identification of many microorganisms.It nevertheless proved insufficient for classification.The isolates were identified to genus and species based on their genetic characteristics that are universally applicable and possible to explore the polymorphism at different levels (comparison between strains, species, gender, etc.). They are based on the study of a gene (locus) of several genes (multilocus) of a DNA fragment defined (ITS-1 rDNA ITS 5.8 S 2, Delta) or of total DNA, depending on the intended purpose.

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Table 1: size in bp of restriction fragment isolated from yeast species of Merlot.

strain	Total DNA	Number and size of the fragments obtained after restriction		Results Identification(According to the IFV table)
		Hinf I	*Taq* I	
M2b	600	(250 to 300) x 2	100 to 200 200 to 300 100 <100	**NL00726/RAFS 8-5** **NL00726/Nash 8-6**
M1	500 to 600	(200 to 300) x 2	(200 to 300) x2 100	***Cryptococcus laurentii*** ***Candida rugopelliculosa*** ***Diplodia sarmentorum***
M2	800 to 900	350 to 400 150 to 200	100 to 200 300 to 400 <100	***Saccharomyces uvarum*** ***Saccharomyces cerevisiae***
M2'	500 to 600	(150 to 250) x2 <100	(200 to 300) x2 <100	***Rhodotorula graminis*** ***Dipoldia sarmentorum***
M3'	500 to 600	180 to 220 <100	200 x2 <100	***Leucosporidiella fragaria*** ***Cryptococcus laurentii***
M3	500	100 to 200 <100	(150 to 250) x2 <100	***Pichia membranefaciens*** ***Candida edax***
M1b	480 to 550	(100 to 200) x2	(200 to 300) x2 100	***Dipoldia sarmentorum*** ***Candida edax*** ***Cryptococcus laurentii***
M3p	500 to 550	(100 to 200) x2 <100	(200 to 300) x2 100 to 200 <100	***Rhodotorula minuta*** ***Cryptococcus laurentii*** ***Dekkera naardenensis***

Table 2: size in bp of restriction fragment isolated from yeast species ofcabernet.

strain	Total DNA	Number and size of the fragments obtained after restriction		Results Identification(According to the IFV table)
		Hinf I	*Taq* I	
Cb1	450 to 550	(180 to 250) x2	(200 to 300) x2 150	***Brettanomyces*** (souche **GC3**) ***Brettanomyces intermedius***
Cb2	450 to 550	(200 to 250) x2	250 to 300 150 to 200	***Kloeckera flurescens*** ***Brettanomyces intermedius*** ***Brettanomyces*** (souche **GC3**) ***Candida vini***
Cb4	400 to 500	400 to 500 ?	Résultat négatif	/
Cb5	400 to 500		Résultat négatif	/

Table 3: size in bp of restriction fragment isolated from yeast species of Carignan.

strain	Total DNA	Number and size of the fragments obtained after restriction		Results Identification(According to the IFV table)
		Hinf I	*Taq* I	
Cr2	500	/	/	/
Cr3	500	400 à 500 ?	/	/
Cr4	500	400 à 500 ?	/	/
Cr5	500 à 550	400 à 500 ?	/	/

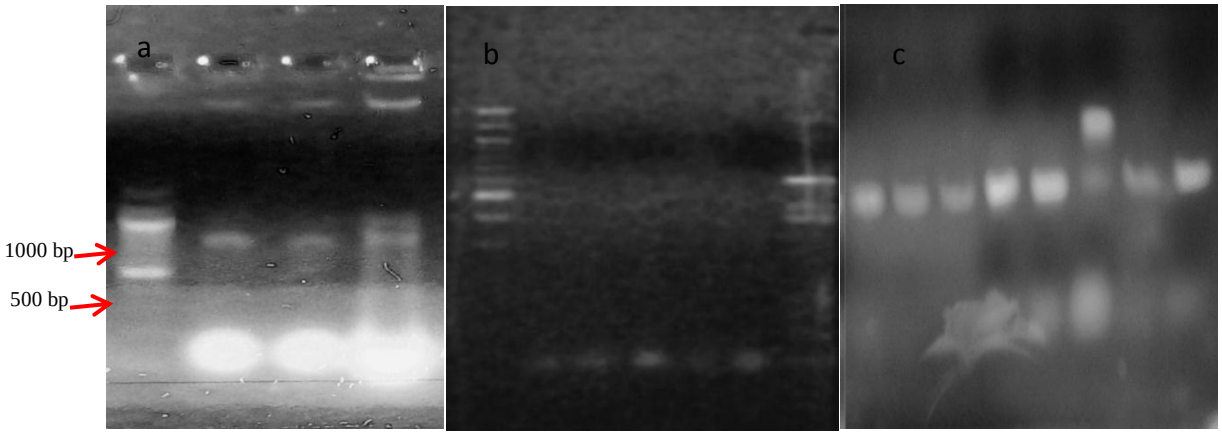

Figure 1: (a) white electrophoresis, (b): migration of the amplified delta region (Tr. Ty1) and (c): ITS without restriction from isolated strains of Merlot.
M: marker X, sc: *Saccharomyces cerevisiae*

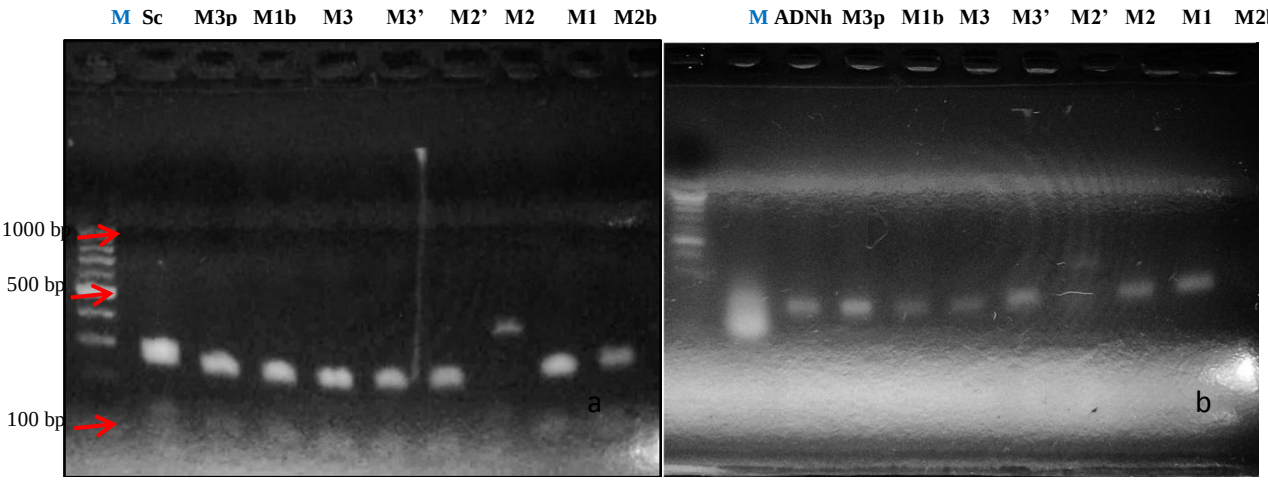

Figure 2:(a)Taq 1 restriction, (b) hin 1 restriction of isolated strains of Merlot.
M: marker X, sc: *Saccharomyces cerevisiae*, ADNh : DNA without restriction.

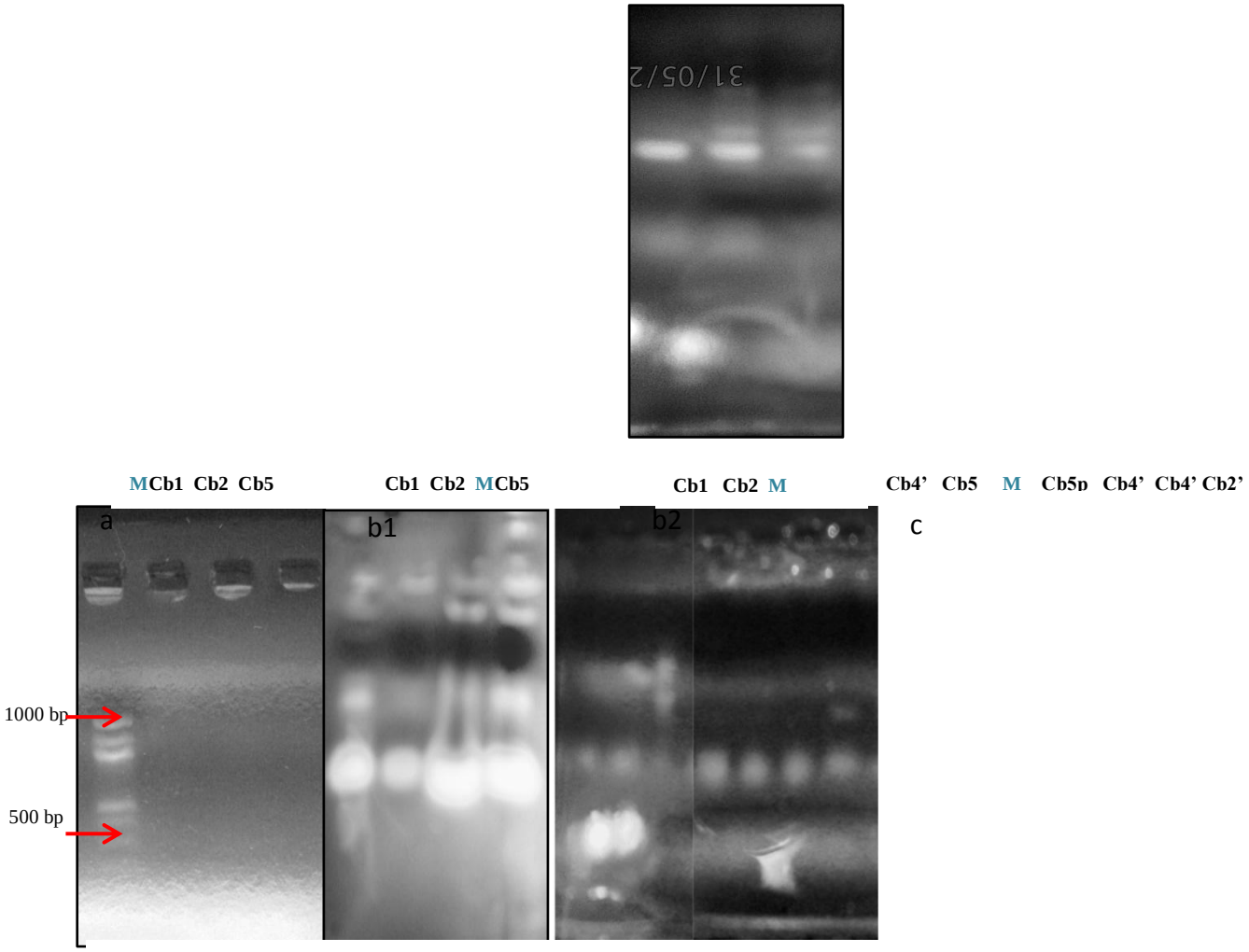

Figure 3:(a)migration of the amplified delta region,(b1; b2) electrophoresis white (c): ITS without restrictionfrom isolated strains of Cabernet.M: marker X.

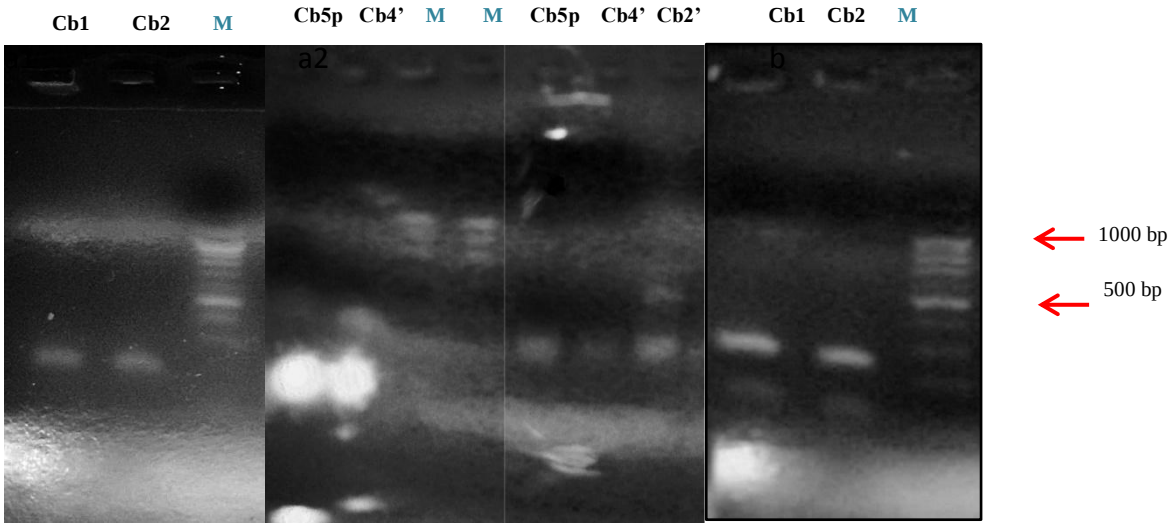

Figure 4:(a1, a2)hinf 1 restriction, (b) taq 1 restriction of isolated strains of cabernet. M : marker X.

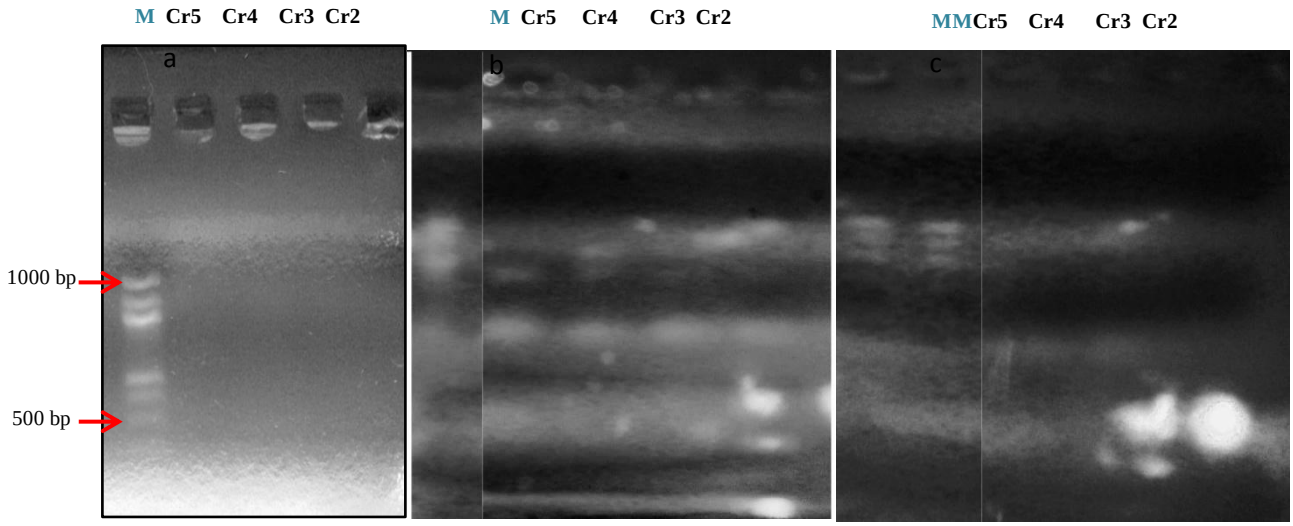

Figure 5:(a)migration of the amplified delta region (Tr. Ty1), (b): ITS without restriction and (c): hinf 1 restriction from isolated strains of carignan. M : marker X.