# MOLECULAR IDENTIFICATION OF WILD NON-*SACCHAROMYCES* YEASTS FROM WINE BY PCR-RFLP AND STRAIN DIFFERENTIATION BY RAPD

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**MOLECULAR IDENTIFICATION OF WILD NON-*SACCHAROMYCES* YEASTS FROM WINE BY PCR-RFLP AND STRAIN DIFFERENTIATION BY RAPD**

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# ABSTRACT

## MOLECULAR IDENTIFICATION OF WILD NON-*SACCHAROMYCES* YEASTS FROM WINE BY PCR-RFLP AND STRAIN DIFFERENTIATION BY RAPD

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Non-*Saccharomyces* yeasts are dominant during the early stages of alcoholic fermentation of grape must and play a role in the production of favorable organoleptic traits in the final wine. Identification of indigenous yeasts for using them as starter culture in a sequential inoculation along with *Saccharomyces cerevisiae* is an effective approach to produce wine with desirable characteristics. In this study, 18 non-*Saccharomyces* yeasts isolated at different maceration and fermentation times from traditional wine of five different grape types in Turkey were identified by PCR-RFLP (Polymerase Chain reaction- Restriction Fragment Lenghth Polymorphism) at genus and species level and then *Lachancea thermotolerans* (Lt), *Metschnikowia pulcherrima* (Mp), *Hanseniaspora uvarum* (Hu), *Hanseniaspora opuntiae* (Ho) species were selected for the genotypic differentiation of their strains using RAPD–PCR technique. Amplification of internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) using PCR allowed to identify some yeast isolates at the genus level. However, precise identification at genus and/or species level was possible by PCR-RFLP using appropriate enzymes. In this study, restriction digestion of rDNA ITS region using five endonucleases, *Hha*I (*Cfo*I), *Hae*III, *Hinf*I, *Dde*I and *Dra*I allowed identification of NS yeasts.

On the other hand, RAPD-PCR using one RAPD primer OPA-03, minisatellite primer M13 and, two microsatellite primers $(ATG)_5$, $(GTG)_5$, were useful for the differentiation of yeast population at the strain level.

Keywords: Non-*Saccharomyces*, starter culture, PCR-RFLP, RAPD-PCR, minisatellite, microsatellite

# ÖZ

## ŞARAPTAN İZOLE EDİLMİŞ YABANI *SACCHAROMYCES*-DIŞI MAYALARIN PCR-RFLP İLE MOLEKÜLER TANIMLANMASI VE RAPD İLE SUŞ FARKLILAŞTIRMASI

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*Saccharomyces*-dışı (Non-*Saccharomyces*) (NS) mayalar, üzüm şırasında alkol fermantasyonunun ilk aşamalarında baskındır, ve son aşamada şarapta uygun organoleptik özelliklerin oluşumunda rol oynar. NS mayaların, *Saccharomyces cerevisiae* ile birlikte sıralı aşılamada başlangıç kültürü olarak kullanılması için tanımlanması, arzu edilen özelliklere sahip şarap üretmek için etkili bir yaklaşımdır. Bu çalışmada, Türkiye'deki beş farklı üzüm türünün geleneksel şaraplarından farklı maserasyon ve fermantasyon sürelerinde izole edilen 18 Saccharomyces dışı maya, PCR-RFLP (Polymerase Chain reaction- Restriction Fragment Lenghth Polymorphism) yöntemi ile cins ve tür seviyesinde tanımlanmış ve daha sonra *Lachancea thermotolerans* (Lt), *Metschnikowia pulcherrima* (Mp), *Hanseniaspora uvarum* (Hu), *Hanseniaspora opuntiae* (Ho) türleri seçilerek, bu türlere ait suşların RAPD–PCR tekniği ile genotipik farklılıklar belirlenmiştir. PCR ile rDNA'nın internal transcribed spacer (ITS) bölgesinin (ITS1-5.8S-ITS2) amplifikasyonu maya izolatlarının bazılarında cins seviyesinde tanımlamaya izin vermiştir. Ancak, cins ve/veya tür seviyesinde kesin tanı, ITS bölgesinin uygun restriksiyon enzimler ile kesildigi PCR-RFLP yöntemi ile mümkün olmuştur. Bu çalısmada, beş farklı endonükleaz enziminin, *Hha*I (*Cfo*I), *Hae*III, *Hinf*I, *Dde*I and

*Dra*I kullanıldığı ITS bölgesi restriksiyon kesimi NS mayaların tanımlanmasını sağlamıştır.

Diğer yandan, RAPD primeri OPA-03, minisatellite primer M13 ve, microsatellite primerleri $(ATG)_5$, $(GTG)_5$ aynı türe ait olan suşların (Lt, Hu, Ho, Mp türlerine ait suşlar) farklılaştırılmasında fayda sağlamıştır.

Anahtar Kelimeler: Non-*Saccharomyces*, başlangıç kültürü, PCR-RFLP, RAPD-PCR, minisatellite, microsatellite

To my  loving father

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## LIST OF ABBREVIATIONS

NS: Non-*Saccharomyces*

AF: Alcoholic Fermentation

RFLP: Restriction Fragment Length Polymorphism

RAPD: Random Amplification of Polymorphic DNA

Mp: *Metschnikowia pulcherrima*

Hu: *Hanseniaspora uvarum*

Ho: *Hanseniaspora opuntiae*

Lt: *Lachancea thermotolerans*

Hg: *Hanseniaspora guilliermondii*

Sb: *Starmerella bacillaris*

Rm: *Rhodotorula mucilaginosa*

Wa: *Wickerhamomyces anomalus*

Sa: *Solicoccozyma aeria*

DSMZ: German collection of microorganisms and cell cultures GmbH

CM: Cold Maceration

NM: Normal Maceration

O: Öküzgözü Grape Variety

E: Emir Grape Variety

K: Kalecik Karası Grape Variety

B: Boğazkere Grape Variety

D: Dimrit Grape Variety

# CHAPTER 1

# INTRODUCTION

## 1.1 Yeasts

Yeasts have been harnessed by humans for millennia to produce industrially important goods, such as foods, fermented beverages, probiotics, pharmaceuticals, enzymes, biofuels, antibiotics.

Fermentative yeasts can produce some secondary metabolite, essential for improving the organoleptic properties of alcoholic beverages, such as fuel alcohols (e.g. isoamyl alcohol), glycerol, organic acids (e.g. citrate, succinate, acetate), aldehydes (e.g. acetaldehyde) and esters, (e.g. ethyl acetate).

Under the different growth conditions, yeasts, specifically *S. cerevisiae,* have a unique ability in that they can switch from respiration (in the presence of oxygen) to fermentation (anaerobic conditions), and vice versa (Walker & White., 2005).

### 1.1.1 Morphology

Yeasts are unicellular eukaryote, classified mostly as ascomycete fungi that are reproduced asexually by budding or fission. A common brewer or baker yeast *Saccharomyces cerevisiae* is ellipsoid shaped with a 5–10 μm large diameter and a 5 μm small diameter (Figure 1.1).

The color, geometry, and texture of grown yeasts on agar represent a diverse morphology. Some yeasts are pigmented, for example, cream (e.g. *S. cerevisiae*); red (e.g. *Rhodotorula rubra*), yellow (e.g. *Cryptococcus laurentii*). With a peripheries or contours geometry (Walker & White., 2005).

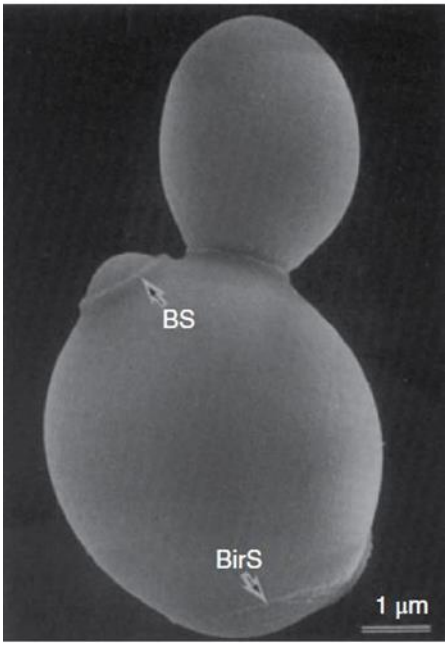


Figure 1.1. Photomicrograph of a typical yeast cell (×10,000). BS, Bud scar; BirS, birth scar
(Osumi, 1998)

### 1.1.2 Cytology

Like all eukaryotes, yeasts cell contains subcellular structures including, a nucleus, cytoskeleton, endoplasmic reticulum (ER), mitochondria and microbodies, Golgie apparatus, and similar to plants a vacuole and a firm cell wall surrounding the plasma membrane (Kurtzman et al., 2011).

### 1.1.3 Taxonomy of Wine Yeasts

More than 100 genera and 700 species of yeasts have been known, however, a few numbers among them are isolated from the wine environment, including, *Saccharomyces*, *Candida*, *Hanseniaspora*, *Pichia*, *Metschnikowia*, *Torulaspora*, *Saccharomycodes*, *Brettanomyces*, *Rhodotorula*, *Dekkera*, *Cryptococcus*, *Debaryomyces*, *Kluyveromyces*, *Schizosaccharomyces* and *Zygosaccharomyces* (Swiegers et al., 2005).

For thousands of years, *Saccharomyces cerevisiae* has thought to as the only important yeast in the biotechnological process for the production of food and beverage. Nevertheless, the non-conventional yeasts or non-*Saccharomyces* yeasts have recently become increasingly important (Spencer et al., 2002).

### 1.1.4 Non-*Saccharomyces* Yeasts

In the past, non-*Saccharomyces* yeasts were assumed to participate in stuck or sluggish fermentations and to produce undesirable flavor. However, nowadays, their positive role in producing acceptable aromatic compound in wine such as esters, higher alcohols, and acids and organoleptic characteristic that is missing in *S. cerevisiae* has been reported. (Viana, et al., 2011; Di Maro et al., 2007; Medina et al., 2012).

Non-*Saccharomyces* also have been demonstrated an antagonistic mechanism that can be used as biofungicide for eliminating spoilage microorganisms, such as lactic acid bacteria or *Brettanomyces bruxellensis* (Capozzi et al., 2015; Oro et al., 2014; Garofalo et al., 2016).

Recently, the capacity of native non-*Saccharomyces* species as starter culture in a sequential inoculation with *S. cerevisiae* during wine-making has been drawing attention. Therefore, isolation and identification of the grape related strains are important (Banilas et al., 2016; Mateus et al., 2020).

## 1.2 Identification of Yeasts

### 1.2.1 Phenotypic Identification

Conventional identification of yeast species has been more based on phenotypic methods including, assimilation and fermentation tests that detect the ability of yeasts to utilize substrates as the only source of carbon or nitrogen, and

morphological traits (Esteve-Zarzoso et al., 2001; Pincus et al., 2007) such as, colony appearance (filamentation, pigment), the presence of specialized structures like true hyphae, arthroconidia, and different cell shape (spherical or oval cells) (Pincus et al., 2007). These methods are mostly done through the cultivation of unknown yeasts on a variety of selective media such as lysine agar, crystal violet medium, actidione medium, and MYGP+copper medium (Pham et al., 2011).

The identification of yeast species using traditional techniques (biochemical, morphological, and physiological tests) is influenced by culture conditions. Therefore, it is laborious, unreliable, and unable to discriminate between strains of one species. Approximately 50–100 tests are necessary to identify most yeasts to species level reliably (Sabate et al., 2002; Las Heras-Vazquez et al., 2003).

There are other techniques for identification of yeasts through analyzing the total protein of the cell and fatty acids by using gas chromatography, however, as they are based on the physiological characteristics of the yeasts, the reproducibility of these methods is controversial (Esteve-Zarzoso et al., 1998).

### 1.2.2 Genotypic Identification

Molecular techniques provide alternative approaches that are rapid and reliable, enabling the study of a large number of samples (Arroyo-López et al., 2006). Theses techniques are widely used for the identification and /or genotyping of species and strains (Echeverrigaray et al., 2000). Among the molecular methods, PCR-based techniques are simple and quick for species identification and differentiation at strain level (Hierro et al., 2004).

Table 1.1. Molecular characterization technique used for identification or genotyping microorganisms in different studies.

| **Molecular technique** | **Reference** |
|---|---|
| PFGE, RAPD-PCR, Microsatellite | (Kállai et al., 2019) |
| TRtRNA | (Banilas et al., 2016) |
| RFLP | (Bautista-Gallego et al., 2011) |
| PFGE, PCR-PFLP, mtDNA | (Bernardi et al., 2008) |
| PFGE, PCR-PFLP | (Christine et al., 2007) |
| RFLP | (Nisiotou and Nychas, 2007) |
| RAPD-PCR, PFGE, SAU-PCR | (Cocolin et al., 2004) |
| PFGE, PCR-RFLP, SAU-PCR | (Gil-Lamaignere et al., 2003) |
| RAPD-PCR, PFGE, mtDNA | (Fernández-Espinar et al., 2001) |
| RAPD-PCR, Microsatellite, RFLP | (Couto et al., 1996) |

#### 1.2.2.1 Molecular Methods for Identification of Species

Molecular methods based on ribosomal genes (5.8S, 18S and 26S) variability are the most used techniques for the identification of yeast species (Arroyo-López et al., 2006). Some of these methods are as follow;

- Sequencing the 5.8S-ITS Region of rDNA
- Sequencing the D1/D2 Domain of rDNA
- Restriction Fragment Length Polymorphism (RFLP)

#### 1.2.2.1.1 Sequencing the 5.8S-ITS Region of rDNA

Recently, ribosomal genes have the potential for molecular taxonomic and phylogenetic studies. The ITS region, including the 5.8S rRNA gene, displays low intraspecific variability, and high interspecific polymorphism and two surrounding non-coding regions ITS1 and ITS2 are very variable, therefore, sequencing the region is a useful method for differentiation of yeast species (Pham et al., 2011; Arroyo-López et al., 2006; Martin & Rygiewicz., 2005; Lewis., 1996; Madani et al., 2004; Van Der Aa Kühle & Jespersen., 2003).

The universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-GCATATCAATAAGCGGAGGA-3′) can amplify the ITS1 and ITS2 sequences located between two coding sequences of small subunit (SSU) and large subunit (LSU) (Leaw et al., 2006).

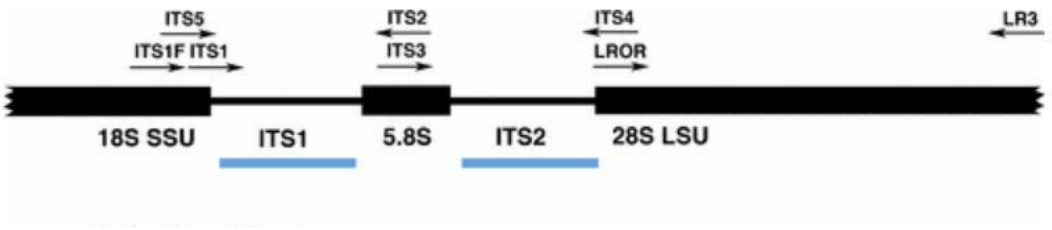


Figure 1.2. Primers for amplification of the ITS region
(Raja et al., 2017)

#### 1.2.2.1.2 Sequencing the D1/D2 Domain of rDNA

D1–D2 domains of 28S large subunit can be amplified by Primers NL1 (5′ GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′). The PCR products are then sequenced, and the species are identified by comparison of the results with the databases on the BLAST tool.

Recently, sequence analysis has been developed as a molecular technique for species-level discrimination of most ascomycetous yeasts and medically necessary zygomycetes (Dagar et al., 2011; Abliz, et al., 2004; Leaw et al., 2006).

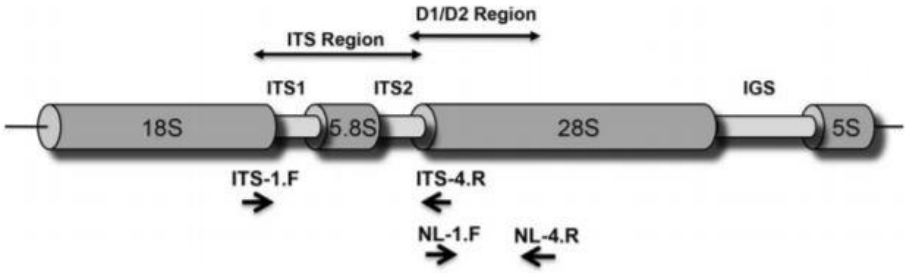


Figure 1.3. The location of D1/D2 variable domains inside the 28S ribosomal subunit, and ITS region flanking 5.8S subunit and related primers (Romanelli et al., 2014)

#### 1.2.2.1.3 Restriction Fragment Length Polymorphism (RFLP)

RFLP is a molecular technique applied for characterization of yeast species based on the restriction digestion of rDNA internal transcribed spacer (ITS) region consisting of the 5.8 rRNA gene which generates a specific restriction pattern for each species (Pham et al., 2011; ZHANG et al., 2010). The restriction patterns of a great number of yeasts that generated with endonucleases are available at www.yeast-id.com. Since 1999, the RFLP method has been used as a simple and rapid technique for the identification of yeast species associated with fermented foods and beverages (Jeyaram et al., 2008). Nisiotou and Nychas (2007) assessed the yeast microflora of healthy and Botrytis-infected grapes using the RFLP technique resulting in the identification of six genera and nine species. Bautista-Gallego et al. (2011), used RFLP for genetic identification of yeast isolates from industrial green table olive. Restriction endonucleases *Hae*Ⅲ, *Hinf*I and *Cfo*I were used for the evaluation of yeast population during the fermentation of cracked olive oil by Alves et al. (2012). They have reported great diversity at the beginning of fermentation (Alves et al., 2012). Wang and Liu. (2013), could identify six

different wine species by using RFLP technique with *Hae*III, *Hinf*I, *Dde*I and *Mbo*II enzymes.

#### 1.2.2.2 Molecular Methods for Genotyping of Strains

Besides species identification, several methods have been developed for intraspecies identification. Such reliable techniques are essential to finding the origin of spoilage yeasts, the source of contamination and the critical points associated with infectious yeasts (Martorell et al., 2006). Some of the molecular techniques that are used for characterization of yeasts at the subspecies level are as follow;

- Pulsed-Field Gel Electrophoresis (PFGE)
- Random Amplified Polymorphic DNA (RAPD)
- Mini and micro-satellite DNA fingerprinting

##### 1.2.2.2.1 Pulsed-field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is a technique with high taxonomic potential. This method is used to separate chromosome-sized and large fragments of DNA molecules in agarose gel (Mitterdorfer et al., 2002).

##### 1.2.2.2.2 Random Amplified Polymorphic DNA (RAPD)

RAPD analysis using arbitrary primers (6-10 bp), which amplify anonymous, polymorphic DNA fragments (Meyer & Mitchell., 1995), is a technique for identification of polymorphism in genetic mapping, taxonomy and phylogenetic studies (Capece et al., 2003). This technique is wildly used for genotyping of yeast strains (Pfliegler et al., 2014). Cocolin et al. (2004) evaluated the impact of different wineries conditions on the diversity of *Saccharomyces* strains by using RAPD-PCR. In the study of Couto et al. (1996) random amplified polymorphic

DNA (RAPD) with the microsatellite oligonucleotide primers $(GAC)_5$ and $(GTG)_5$ and restriction fragment length polymorphism (RFLP) technique enabled discrimination between the strains isolated from spoiled wine. In an RAPD study by Tofalo et al. (2011) a high strain diversity was observed in the different species isolated from must of an organic vineyard of red Montepulciano d'Abruzzo and white Trebbiano grapes. Pfliegler et al. (2014), used 5 different RAPD and micro/minisatellite primers on strains of C. *zemplinina* isolated from various samples such as Botrytized grape, fermenting wine and soil of different locations. They found a relatively low diversity of different strains. A total of fifty *Hanseniaspora uvarum* were isolated from a spontaneous must fermentation of ''Negroamaro'' grape must in a study by De Benedictis et al. (2011). Totally nine primers were used to characterize this species at the strain level and nine different strains were found out of the 50 analyzed isolates.

#### 1.2.2.2.3 Mini- and Microsatellite Fingerprinting

Hypervariable repetitive sequences (mini- and microsatellite sequences) have been used for DNA fingerprinting analysis since 1985. Hypervariability of microsatellites and minisatellite loci made them ideal tools for genotyping analysis of closely related individuals, particularly by the use of PCR-based methods such as RAPD-PCR.

Minisatellite DNA is a sequence of DNA consisting of tandem repeat of 15-30 bp long sequence motifs at various loci (Lieckfeldt et al., 1993). On the other hand, microsatellites are tandem repeats of a short DNA sequence motif with the length of 2-10 bp multiplied more than 100 times and are distributed in the genome of most eukaryotes (Lieckfeldt et al., 1993; Walczak et al., 2007; Banilas et al., 2016).

Single primers of mini- and microsatellite oligonucleotides can be applied in the PCR to produce individual PCR fingerprints. In the PCR reaction, if tandem

repeats and one specific primer which are complementary became close to each other, the tandem repeat sequence can be amplified.

Mini- or microsatellite primer has the advantages over RAPD primers. There is a 100% homology between the sequence of the mini- or microsatellite primer and their binding site in DNA target. Furthermore, the size of mini- or microsatellite primers, are longer (15-17 bp), in comparison with RAPD primers that are shorter (6-10 bp) and arbitrary. This makes the binding of primer and the target to be stronger and the PCR fingerprinting technique to be more reliable than RAPD. PCR fingerprinting technique, which is rapid, sensitive, and reliable, has been reported to be useful in fungi studies such as taxonomy, epidemiology, and diagnosis of mycotic diseases (Meyer & Mitchell., 1995).

## 1.3 Alcoholic Fermentation (AF)

Alcoholic fermentation of grape juice into wine is a combined interaction between grapes and several microorganisms (fungi, yeasts, lactic acid bacteria, and acetic acid bacteria)(Raspor et al., 2006). In particular, yeasts play an essential role in conducting the AF by converting grape sugars into ethanol, carbon dioxide, and many other secondary compounds (Garofalo et al., 2016).

Non- *Saccharomyces* yeasts start the fermentation, and their populations can reach $10^7$ colony forming units (cfu) / mL or more until the concentration of alcohol exceeds 5–7% (Capece et al., 2003). As the number of non-*Saccharomyces* yeasts that are sensitive to alcohol begins to decrease they are very soon replaced by *Saccharomyces cerevisiae* which is more tolerant to ethanol (Di Maro et al., 2007) and some other environmental stresses conditions such as the presence of sulphur dioxide and anaerobiosis (ZHANG et al., 2010). *S. cerevisiae* is primarily responsible during alcoholic fermentation and completes the conversion of sugars to ethylic alcohol (Garofalo et al., 2016).

Under some conditions, such as low temperatures below 15–20 °C, the sensitivity of non-*Saccharomyces* yeasts to ethanol concentration can be decreased. Therefore, particularly *Hanseniaspora* and *Candida* species can be predominant along with *S. cerevisiae* until the end of the fermentation process. These species produce several secondary metabolites that positively influence the characteristics of the wine. They are capable of affecting the wine quality even in the fermentation process to which starter cultures of *S. cerevisiae* had been inoculated. Such an ability invalidates the studies indicating starter culture inoculation has a negative impact on the growth of non-*Saccharomyces* yeasts (Di Maro et al., 2007; Fleet, 2008).

### 1.3.1 Malolactic Fermentation

Malolactic fermentation (MLF) is a decarboxylation process converting l-malic acid into l-lactic acid and CO2, and similar to alcoholic fermentation the malolactic fermentation also is done through microbial activity. However, the lactic acid bacteria (LAB), *Oenococcus oeni,* and *Lactobacillus plantarum* species play the main role in this phase (Vittorio Capozzi et al., 2015).

### 1.3.2 Spontaneous Fermentation

Spontaneous fermentation is a process carried out by a great number of yeast species naturally present on the surface of grape berries and winemaking equipment without any inoculation of starter cultures (Nisiotou & Nychas., 2007; Di Maro et al., 2007).

#### 1.3.2.1 Advantages and Disadvantages of Spontaneous Fermentation

A high-quality wine with unique characteristics and commercial value can be produced under the conditions of spontaneous fermentation. As nature is not

always predictable and under control, it may sometimes lead to inconsistent quality of the wine (Fleet, 2008).

According to some studies, grape variety, climatic conditions, and geographical area of the vineyard affect the development of the yeast population and, consequently, influence the spontaneous fermentation. Besides, the type of soil, the use of antifungals, the age of the vineyard, and the harvest technique influence the yeast microbiota on grapes (Sabate et al., 2002). For these reasons, the outcome of spontaneous wine fermentation may not be the same from year to year and can be difficult to predict (Díaz et al., 2013). To address this problem, by using commercial strains in the fermentation process, wine producers can produce a wine with autochthonous character (Clemente-Jimenez et al., 2004). In fact, inoculation of a mixed starter culture of non- *Saccharomyces* yeast species together with *S. cerevisiae* is a method recently used to control the fermentation process and improve wine quality (De Benedictis et al., 2011).

### 1.3.3 Inoculation Fermentation

The fermentation process to which selected starter cultures are inoculated into the grape juice offers a more rapid and reliable fermentation resulting in a final wine with a more consistent quality that is a better choice for mass-market producers (Fleet, 2008).

## 1.4 Autochthonous Grape Variety and the 'Microbial Terroir'

Yeasts isolated from a grape-growing region or so-called ''terroir'' show more adaptation to particular conditions. Therefore, they are able to transfer a unique regional character to the wine (Liu et al., 2015; Clemente-Jimenez et al., 2004). Bokulich et al, (2014) studied the link between indigenous microbial community and environmental conditions of the vineyard and the role of "microbial terroir" on regional variation among grapes (Bokulich et al., 2014). From this point of view,

investigation of micro-biodiversity during spontaneous fermentation and the employment of autochthonous strains as a starter, can mimic the natural biodiversity and improve the technological properties of wine (Garofalo et al., 2016). This technique is an alternative to spontaneous fermentation (Capozzi et al., 2015).

Table 1.2 shows some studies that have been done to investigate microbial resources (yeasts or lactic acid bacteria and spoilage yeasts, such as *Brettanomyces bruxellensis*) for the production of wine with regional properties.

Table 1.2. Studies on autochthonous microbial resources for the oenological sector in the Apulian region, Southern Italy (Capozzi et al., 2015).

| | References |
|---|---|
| Yeast (non-*Saccharomyces* and *Saccharomyces* spp.) | (De Benedictis et al., 2011; Tristezza et al., 2012; Tristezza et al., 2013; Tristezza et al., 2014; Garofalo et al., 2016) |
| Lactic acid bacteria | (Cappello et al., 2008; Capozzi et al., 2010; Capozzi et al., 2012; Capozzi et al., 2014; Lamontanara et al., 2015; Garofalo et al., 2015) |
| Interaction between *Saccharomyces cerevisiae* and *Oenococcus oeni* | (Garofalo et al., 2015) |
| Spoilage yeast | (Di Toro et al., 2015) |

## 1.5 Most Important Yeasts Associated with Wine

In this section, frequently isolated yeasts in the wine environment are described and listed below;

- *Saccharomyces cerevisiae*
- *Candida*
- *Hanseniaspora*
- *Metschnikowia*
- *Lachancea*
- *Torulaspora*
- *Zygosaccharomyces*
- *Pichia*

### 1.5.1 *Saccharomyces cerevisiae*

The population of *Saccharomyces cerevisiae* species at the beginning of fermentation is low. However, when the alcohol concentration increases and non-*Saccharomyces* yeasts die, it leads AF until its end, often being the only species detectable at this last stage (Vittorio Capozzi et al., 2015).

### 1.5.2 *Candida*

*Candida* spp. has been isolated from different grape juice variety with high frequency and the associate with the production of high amounts of glycerol during AF (up to 14 g $l^{-1}$,) especially *C. stellata* and *Candida zemplinina* strains (Vittorio Capozzi et al., 2015).

### 1.5.3 *Hanseniaspora*

These apiculate yeasts are predominant on the grape surface (50-75%) (Raspor et al., 2006), and they have been identified at the beginning of AF in a percentage varying from 16-78%. In comparison with *Saccharomyces* strains, yeasts belong to *Hanseniaspora* species, such as *H. uvarum* and *H. guilliermondi*, produce a high amount of favorable volatile compounds and more extracellular enzymes, such as glucosidases or proteases. However, they have the poor fermentative ability (Capozzi et al., 2015).

### 1.5.4 *Metschnikowia*

The species belong to this genus such as *Metschnikowia pulcherrima*, that has been identified in the grape must in a high percentage, produce high concentrations of esters, in particular, ethyl caprylate (Medina et al., 2012) and several volatile compounds, such as volatile thiols and terpenes that improve the aroma and flavor of wines (Capozzi et al., 2015). They may cause slow fermentation as a result of their antimicrobial activity (Oro et al., 2014).

### 1.5.5 *Lachancea*

Inoculation of *Lachancea thermotolerans* (*Kluyveromyces thermotolerans*) strain in a mixed starter culture along with *S. cerevisiae*, resulted in increasing the total acidity, glycerol, and 2-phenylethanol, and reducing the pH and volatile acidity (Gobbi et al., 2013; Capozzi et al., 2015).

### 1.5.6 *Torulaspora*

In particular, *T. delbrueckii* shows the ability to produce less acetic acid than the *S. cerevisiae* strains. Therefore it is a suitable strain to use for the fermentation

process of high sugar musts made of botrytized grapes. Nevertheless, they are associated with the production of higher amounts of sulphur compounds in the wine (Capozzi et al., 2015).

### 1.5.7 *Zygosaccharomyces*

The use of *Zygosaccharomyces* spp. in a mixed culture with *S. cerevisiae* has shown several positive roles of this species during the grape must fermentation including, a high fermentation power, the ability to degrade malic acid, and the production of low concentrations of acetic acid, $SO_2$, and $H_2S$ (Capozzi et al., 2015).

### 1.5.8 *Pichia*

More aromatic wines were produced with the use of *Pichia* spp. and *S. cerevisiae* starter cultures due to the production of volatile compounds such as acetaldehyde, thiols, ethyl acetate, glycerol, and ethyl octanoate. In addition, *Pichia* spp. have shown antimicrobial activity associated with the production of killer toxin 'zymocins' (Capozzi et al., 2015; Domizio et al., 2011).

## 1.6 Aim of the Study

The main focus of this study was to use RFLP method in order to develop the knowledge of the yeast community associated with spontaneous wine fermentation. Furthermore, we aimed to use RAPD-PCR typing technique to investigate the biodiversity of wild yeast strains isolated from different vineyards in Turkey to investigate the influence of different grape-growing areas on wild yeast biodiversity. This study is the first step of a wider project aimed at patenting the important yeast strains that present unique fermentation characteristics and evaluate their biotechnological potential for using them as starter cultures.

# CHAPTER 2

# MATERIAL AND METHODS

In this chapter, the winemaking process is described first, then the molecular identification of wine yeast using molecular methods are explained.

## 2.1 Winemaking

Previously, traditional wines from five grape types Kalecik Karası, Öküzgözü, Boğazkere, Dimrit, and Emir were produced. The yeasts associated with the traditional wines were isolated and sequenced. Some biochemical tests were done in order to select the yeasts with high technical properties, such as ethanol and SO2 tolerance and H2S production. The selected yeasts were used as a starter culture to produce wine with desired characteristics through the inoculation fermentation process.

### 2.1.1 Grape Source and Varieties

Five grape varieties Öküzgözü, Kalecik karası, Boğazkere, Dimrit, Emir (Table and Figure 2.1) were used to produce traditional wine.

Table 2.1. Source and varieties of grapes and time of purchase

| Type of grape | Place | Date |
|---|---|---|
| öküzgözü | Elazığ | October 2017 |
| Kalecik karası | Ankara (Kalecik) | September 2017 |
| Boğazkere | Elazığ | October 2017 |
| Dimrit | Kapadokya, Ürgüp | September 2017 |
| Emir | Kapadokya, Ürgüp | September 2017 |


Figure 2.1. Five grape varieties from different vineyards in Turkey (Aktuna, 2019)

### 2.1.2 Destemming and Crushing

Öküzgözü grape type was destemmed and separated from the stalks. The grape berries were crushed gently in order to avoid damaging the seeds as they contain tannin that has a negative effect on wine flavor.

Sampling was done in this step to measure the specific gravity, pH, and Brix.

### 2.1.3 Maceration

Two types of maceration were applied. Cold maceration applied by keeping the musts at 4 ° C. After sampling in the fourth day samples were kept at normal maceration stage at 20 ° C. Air conditioning was used to keep the temperature at 20 ° C. During the maceration period sapmles should be supplied with oxygen through mixing it two times a day to avoid mildew (Figure 2.2) . The specific gravity of musts was measured every day until it reached to 1010-1020. At this gravity level, must is ready for pressing.

Figure 2.2. Must sample at maceration stage
(Aktuna, 2019)

### 2.1.4 Pressing

When the specific gravity reached an amount between 1.010 and 1.020, maceration is ended. At this stage, the seeds, pomace, and skins should be separated from syrup through pressing the must with using cheesecloth. The syrup was then poured back into glass jars and capped with an airlock to prevent oxygen diffusion. An oxygen-free condition, at 25 °C for two weeks, resulted in the completion of alcoholic fermentation.

The steps mentioned above are applied to produce red wine. For white wine with Emir grape type, after destemming and crushing the grapes, pressing was done directly. Alcoholic fermentation started at 18 °C after the glass jars were capped with airlocks and lasted for four weeks.

### 2.1.5 Filtration and Bottling

The wines were siphoned three times in a period of one month in order to clarify them from sediment and then poured into green bottles.

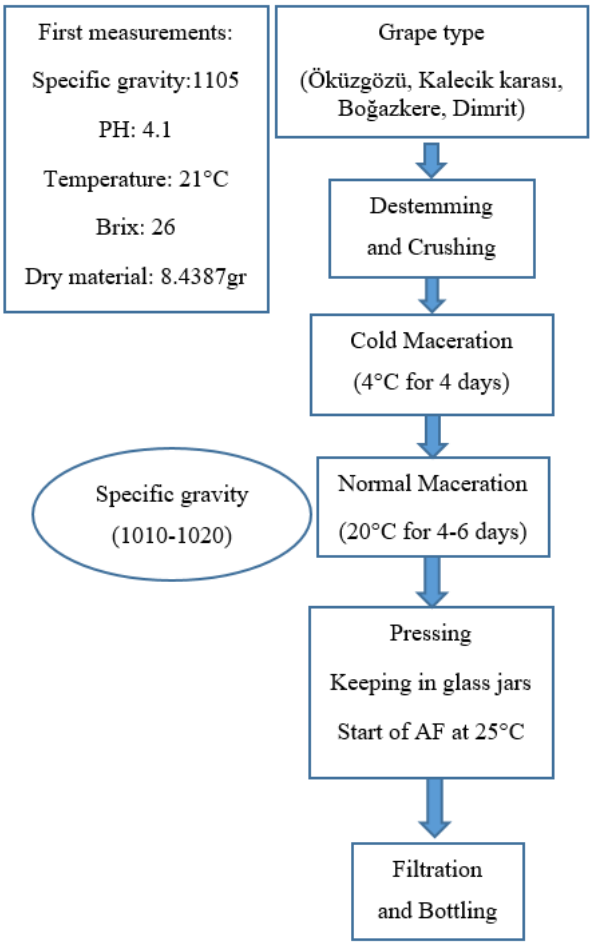


Figure 2.3. Schematic view of the winemaking process for red wine

## 2.2 ITS-RFLP

Totally eighteen NS isolates were objected to RFLP analysis (Table 2.2). All the isolates identified previously by ITS sequencing (Aktuna, 2019).

Table 2.2. Studied strains by RFLP analysis identified by ITS sequencing and their source

| **Strains** | **Lab Code** | **Lab Name** | **Source (City, Country, year)** |
|---|---|---|---|
| *Metschnikowia pulcherrima* | M. p | M. p | DSMZ |
| *Metschnikowia sinensis* | D3* | DA 0CM NS3 | Dimrit, Kapadokya, Türkiye, 2017 |
| *Metschnikowia pulcherrima* | B10 | BB 0CM NS2 | Boğazkere, Elazığ, Türkiye, 2017 |
| *Metschnikowia aff. pulcherrima* | B11* | BB 0CM NS3 | Boğazkere, Elazığ, Türkiye, 2017 |
| *Metschnikowia fructicola* | D15* | DB 4CM NS3 | Kapadokya, Türkiye, 2017 |
| *Metschnikowia aff. fructicola* | B33* | BB 4CM NS8 | Boğazkere Elazığ, Türkiye, 2017 |
| *Hanseniaspora uvarum* | - | Hu | DSMZ |
| *Hanseniaspora uvarum* | K13 | KA 4CM NS3 | Kalecik Karası Ankara, Türkiye, 2017 |
| *Hanseniaspora guilliermondii* | K1 | KA 0CM NS1 | Ankara, Türkiye, 2017 |
| *Hanseniaspora opuntiae* | K2 | KA 0CM NS2 | Ankara, Türkiye, 2017 |
| *Lachancea thermotolerans (Kluyveromyces thermotolerans)* | - | Lt | DSMZ |
| *Lachancea thermotolerans* | O9 | OB 4CM NS4 | Öküzgözü, Elazığ, Türkiye, 2017 |
| *Lachancea thermotolerans* | O16 | OA 0CM NS7 | Elazığ, Türkiye, 2017 |
| *Candida* spp. | - | C | DSMZ |
| *Starmerella bacillaris (Candida zemplinina)* | D10 | DA 4CM NS2 | Kapadokya, Türkiye, 2017 |
| *Rhodotorula mucilaginosa* | K9 | KB 0CM NS4 | Ankara, Türkiye, 2017 |
| *Wickerhamomyces anomalus (picha anomala)* | K41 | KA 0CM S1 | Ankara, Türkiye, 2017 |
| *Solicoccozyma aeria (Cryptococcus aerius)* | B4 | BA 0CM NS4 | Elazığ, Türkiye, 2017 |

DSMZ, German collection of microorganisms and cell cultures GmbH; DA, A Parallel of Wine Made from Dimrit Grape Variety; BB, B Parallel of Wine Made from Boğazkere Grape Variety; DB, B Parallel of Wine Made from Dimrit Grape Variety; KA, A Parallel of Wine Made from Kalecik Karası Grape Variety; ÖB, B Parallel of Wine Made from Öküzgözü Grape Variety; ÖA, A Parallel of Wine Made from Öküzgözü Grape Variety; KB, B Parallel of Wine Made from Kalecik Karası Grape Variety; BA, A Parallel of Wine Made from Boğazkere Grape Variety; 0CM, 0 Day of Cold Maceration; 4CM, 4th Day of Cold Maceration.

**Identified as different species by D1/D2 domain sequensing; D3, *Metschnikowia pulcherrima*; B11, *Metschnikowia aff. pulcherrima*; D15, *Metschnikowia fructicola*; B33, *Metschnikowia aff. fructicola.*

### 2.2.1 DNA Isolation

DNA was isolated using EurX Gene Matrix Bacterial & Yeast DNA isolation Kit (E3580) according to the instruction. Thermos Scientific Nanodrop 2000 (USA) was used to measure DNA purity.

### 2.2.2 ITS-PCR

DNA amplification was performed by preparing 50 µL reaction mix containing 5x FIREPol® Master Mix (Solis Bio Dyne), ITS1 and ITS4 primers (10 pmol / µl), water, and DNA sample. The PCR amplification was set up under the following conditions; 95°C for 12 min (initial denaturing), 30 cycles of 95°C for 1 min (denaturing), 56°C for 1 min (annealing), 72°C for 2 min (extension) followed by final extension step at 72°C for 7 min (Jeyaram et al., 2008).

### 2.2.3 Restriction Digestion

The ITS region amplified with PCR was digested by restriction enzymes (*Hae*III, *Hinf*I, *Cfo*I (*Hha*I), *Dde*I and *Dra*I) suitable for *Saccharomyces* and Non-*Saccharomyces* species. For this process, a 50 µL mixture was prepared using buffer solution (10X ONE Buffer), 100X BSA (Bovine Serum Albumin), restriction enzyme, water, and PCR product and incubated for 1-2 hours at the optimum temperature of the enzymes that is 37 ° C (Pham et al., 2011).

### 2.2.4 Gel Electrophoresis

Gel electrophoresis was used to analyze the PCR products and digested fragments, and the DNA fragments were run on the gel and separated by size. The PCR products run for 75 minutes at 100 V using a 2% (w/v) agarose gel. The digested fragments were separated for 85 minutes at 100 V using a 3% (w/v) agarose gel (Esteve-Zarzoso et al., 1999). 100 bp DNA Ladder (Solis BioDyne) was used as a reference. After gel electrophoresis, the gel was left in a mixture of ethidium bromide and water for 45 minutes and washed in pure water for 30 minutes. At the end of this period, the gel was visualized under UV light, and gel photos were taken with the help of the Quantity One program.

## 2.3 RAPD-PCR

Fortysix non-*Saccharomyces* yeasts (Table 2.3) were subjected to RAPD-PCR analysis in order to investigate the biodiversity between the strains isolated from different vineyards and also the same vineyards. All the studied species were identified previously by ITS sequencing and some of them by D1/D2 domain sequencing (Aktuna, 2019).

Table 2.3. Studied strains by RAPD analysis identified by ITS sequencing and their source

| **Strain** | **Lab code** | **Lab name** | **Source (city, country, date)** |
|---|---|---|---|
| *Lachancea thermotolerans* | - | L. t | DSMZ |
| *Lachancea thermotolerans* | O6 | OA 4CM NS3 | Elazığ, Turkey, 2017 |
| *Lachancea thermotolerans* | O9 | OB 4CM NS4 | Elazığ, Turkey, 2017 |
| *Lachancea thermotolerans* | O16 | OA 0CM NS7 | Elazığ, Turkey, 2017 |
| *Lachancea thermotolerans* | O22 | OB 0CM NS6 | Elazığ, Turkey, 2017 |
| *Hanseniaspora uvarum* | - | H. u | DSMZ |
| *Hanseniaspora uvarum* | K3 | KA 0-CM NS3 | Ankara, Turkey, 2017 |
| *Hanseniaspora uvarum* | K13 | KA 4CM NS3 | Ankara, Turkey, 2017 |
| *Hanseniaspora uvarum* | K25 | KA 2NM NS9 | Ankara, Turkey, 2017 |
| *Hanseniaspora uvarum* | K26 | KB 2-NM NS2 | Ankara, Turkey, 2017 |
| *Hanseniaspora uvarum* | K52 | KA 2-NM NS4 | Ankara, Turkey, 2017 |
| *Hanseniaspora uvarum* | K54 | KA 2-NM NS8 | Ankara, Turkey, 2017 |
| *Hanseniaspora uvarum* | K70 | KB 4-NM NS10 | Ankara, Turkey, 2017 |
| *Hanseniaspora uvarum* | E11 | EA 1W NS1 | Cappadocia, Turkey, 2017 |
| *Hanseniaspora uvarum* | E12 | EA 1.W NS2 | Cappadocia, Turkey, 2017 |
| *Hanseniaspora uvarum* | E13 | EA 1.W NS3 | Cappadocia, Turkey, 2017 |
| *Hanseniaspora uvarum* | E14 | EA 1W NS4 | Cappadocia, Turkey, 2017 |
| *Hanseniaspora opuntiae* | O5 | OA 4CM NS2 | Elazığ, Turkey, 2017 |
| *Hanseniaspora opuntiae* | O7 | OA 4CM NS4 | Elazığ, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K2 | KA 0CM NS2 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K7 | KB 0-CM NS2 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K10 | KB 0-CM NS5 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K12 | KA 4-CM NS2 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K15 | KA 4-CM NS5 | Ankara, Turkey, 2017 |

Table 2.3 (continued)

| | | | |
|---|---|---|---|
| *Hanseniaspora opuntiae* | K16 | KB 4-CM NS1 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K17 | KB 4-CM NS2 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K19 | KB 4-CM NS4 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K20 | KB 4-CM NS5 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K21 | KA 2-NM NS1 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K27 | KB 2-NM NS4 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K32 | KA 4-NM NS4 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K34 | KA 4-NM NS8 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K35 | KA 4-NM NS10 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K37 | KB 4-NM NS3 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K38 | KB 4-NM NS5 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K40 | KB 4-NM NS9 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K51 | KA 2NM NS2 | Ankara, Turkey, 2017 |
| *Hanseniaspora opuntiae* | K60 | KB 2NM NS9 | Ankara, Turkey, 2017 |
| *Metschnikowia pulcherrima* | M. p | Mp | DSMZ |
| *Metschnikowia pulcherrima* | B10 | BB 0CM NS2 | Diyarbakır, Turkey, 2017 |
| *Metschnikowia pulcherrima* | B11* | BB 0CM NS3 | Diyarbakır, Turkey, 2017 |
| *Metschnikowia pulcherrima* | B33* | BB 4CM NS8 | Diyarbakır, Turkey, 2017 |
| *Metschnikowia pulcherrima* | D4 | DA 0CM NS4 | Kapadokya, Turkey, 2017 |
| *Metschnikowia pulcherrima* | D12 | DA 4CM NS4 | Kapadokya, Turkey, 2017 |
| *Metschnikowia sinensis* | D3* | DA 0CM NS3 | Kapadokya, Turkey, 2017 |
| *Metschnikowia chrysoperlae* | D8* | DB 0CM NS4 | Kapadokya, Turkey, 2017 |

DSMZ, German collection of microorganisms and cell cultures GmbH (Germany); OA; A Parallel of Wine Made from Öküzgözü Grape, OB; B Parallel of Wine Made from Öküzgözü Grape, EA; A Parallel of Wine Made from Emir Grape, KA; A Parallel of Wine Made from Kalecik Karası Grape, KB; B Parallel of Wine Made from Kalecik Karası Grape, BB; B Parallel of Wine Made from Boğazkere Grape, DA; A Parallel of Wine Made from Dimrit Grape, DB; B Parallel of Wine Made from Dimrit. 0-CM; 0 Day of Cold Maceration, 4-CM; 4 Day of Cold Maceration, 1.W; 1 Week of Fermentatıon, 2-NM; 2 Day of Normal Maceration.
*Identified as different species by D1/D2 domain sequencing: B11, *Metschnikowia aff. pulcherrima*; B33, *Metschnikowia aff. fructicola*; D3, *Metschnikowia pulcherrima*; D8, *Metschnikowia pulcherrima*.

### 2.3.1 DNA Isolation

In this method, yeast DNA was isolated using EurX Gene Matrix Bacterial & Yeast DNA isolation Kit (E3580) according to the instruction (Guida et al., 2014; Šuranská et al., 2016).

### 2.3.2 PCR Reactions

RAPD-PCR using one 10-mer primer (OPA-03) (Bujdosó et al, 2001b), one minisatellite primer from wild-type M13 phage genome (Pfliegler et al., 2014), as well as two microsatellite primers $(ATG)_5$ and $(GTG)_5$ was performed (Table 2.4) (Walczak et al., 2007).

The PCR reactions were performed in 30 µL reaction mixtures containing 18.4 µL of distilled water, 0.5 µL of Taq DNA polymerase (5 U; Solis BioDyne), 0.6 µL of 100 mM primer, 3 µL of 10X reaction Buffer B (Solis BioDyne) [0.8 M Tris-HCl, 0.2 M (NH4)2SO4, 0.2% w/v Tween-20], 2 µL of 25 mM MgCl2 and 0.5 µL of 4 mM deoxynucleoside triphosphate (dNTP) mixture (Walczak et al. 2007; Bujdosó, et al., 2001b). The procedure used for Microsatellite-PCR primers was the same as that used for RAPD-PCR (Bujdosó et al., 2001a).

Table 2.4. Oligonucleotides used for RAPD-PCR analysis

| **Primer** | **Sequence** | **Tm (°C)** | **Application** | **Reference** |
|---|---|---|---|---|
| OPA03 | 5'AGTCAGCCAC3' | 32°C | RAPD-PCR | (Bujdosó G et al., 2001) |
| M13 | 5'-GAGGGTGGCGGTTCT-3' | 55°C | Minisatellite-PCR<br>RAPD-PCR | (Meyer and Mitchell 1995; De Benedictis et al., 2011; Bovo et al., 2009; Pfliegler et al., 2014) |
| $(ATG)_5$ | 5'-ATGATGATGATGATG-3' | 38°C | Microsatellite-PCR | (De Benedictis et al., 2011) |
| $(GTG)_5$ | 5'-GTGGTGGTGGTGGTG-3' | 52°C | Microsatellite-PCR | (De Benedictis et al., 2011) |

### 2.3.3 PCR Protocol

DNA amplification was done under the following protocol;

The initial step, 94°C for 5 min. 35 cycles denaturation at 94°C for 1 min, annealing at the primer-specific annealing temperature as the producer suggests (Table 2.2) for 1 min. Ramp to 72°C with 0.6°C/s, stay at 72°C for 2 min, according to Walczak et al. (2007) and Bujdosó et al. (2001) with some modifications. The PCR product then loaded to 2% agarose gel and stained with 0.5 mg/ml ethidium bromide and DNA bands were visualized under UV light. For testing the reproducibility, PCR reactions were performed at least twice in separate reactions and only strong and reproducible bands were used in the analysis.

### 2.3.4 Statistical Analysis

Unambiguous bands of amplification products based on combined RAPD-PCR, mini and microsatellite fingerprints (De Benedictis et al., 2011) were scored in a binary matrix in which the presence of a band scored as 1 and absence scored as 0. The similarity of the strains was estimated from pairwise comparisons of the strains based on similarity coefficients of DICE. This coefficient was used to clustering the strain using the unweighted pair-group method with arithmetic average (UPGMA) employing the SAHN (sequential, agglomerative, hierarchical and nested clustering) program from NTSYS-pc (De Benedictis et al., 2011; Javier Gallego et al., 2005).

# CHAPTER 3

## RESULTS AND DISCUSSIONS

In this section, the results of RFLP and RAPD assays are described. Figure 3.1 shows the experimental design of this study.

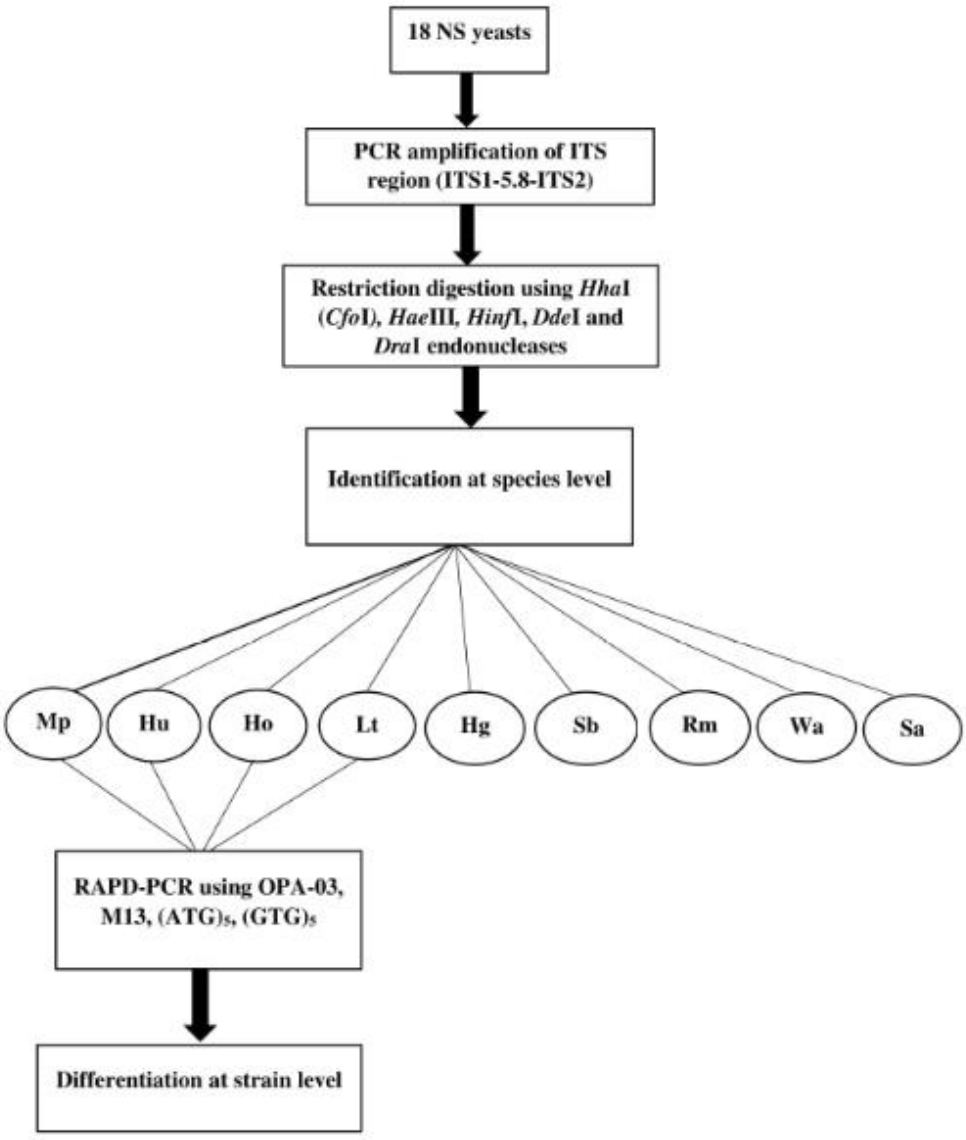


Figure 3.1. Experimental plan of the study

## 3.1 RFLP

RFLP studies with our NS isolates were carried out with the aim of confirming our results gained by ITS sequencing that was performed previously and providing differentiation at the species level. Our isolates selected for RFLP studies are shown in Table 2.2.

Figure 3.1 shows the bands generated after amplification of ITS region using ITS1 and ITS4 primers. PCR product separated on a 2% agarose gel electrophoresis ranging from 390 bp to 750 bp. Examined species of the same genus produced PCR products of the same size. So genus differences in *Metschnikowia*, *Hanseniaspora*, *Lachancea*, *Starmarella* (*Candida zemplinina*) can easily be observed, However, it was difficult to differentiate members of *Rhodotorula*, *Wickerhamomyces* and *Solicoccozyma* genera due to the similar size of the ITS-region bands (Fig. 3.2; Table 3.1), although they can be distinguished from the other examined genera (*Metschnikowia*, *Hanseniaspora*, *Rhodotorula*).

Furthermore, species of the same genus of *Metschnikowia*, *Hansenispora* such as *H. guilermondii* and *H. uvarium* cannot be distinguished from each other. The generated band sizes are compatible with the references.

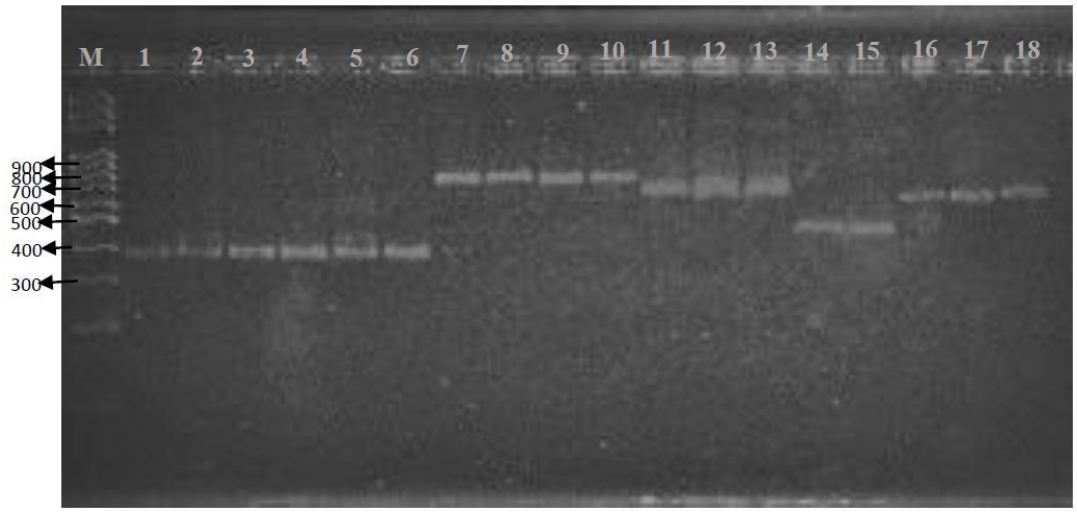


Figure 3.2. Non-*Saccharomyces* species PCR results

M, 100bp Marker; 1, referans strain *M. pulcherrima* (DSMZ); 2, *M. pulcherrima*(D1D2)/ *M. sinensis* (ITS) D3 (DA 0CM NS3); 3, *M. pulcherrima* B10 (BB 0CM NS2); 4, *M. aff. pulcherrima* B11 (BB 0CM NS3); 5, *M. fructicola* D15 (DB 4CM NS3); 6, *M. aff. fructicola* B33 (BB 4CM NS8); 7, referans suş *H. uvarum* (DSMZ); 8, *H. guilliermondii* K1 (KA 0CM NS1); 9, *H. opuntiae* K2 (KA 0CM NS2); 10, *H. uvarum* K13 (KA 4CM NS3); 11, referans strain *L. thermotolerans* (DSMZ); 12, *L. thermotolerans* O9 (OB 4CM NS4); 13, *L. thermotolerans* O16 (OA 0CM NS7); 14, reference strain *Candida* spp.(DSMZ); 15, *Candida zemplinina* (*Starmerella bacillaris*) D10 (DA 4CM NS2); 16, *Rhodotorula mucilaginosa* K9 (KB 0CM NS4); 17, *Wickerhamomyces anomalus* K41 (KA 0CM S1); 18, *Solicoccozyma aeria* B4 (BA 0CM NS4)

Table 3.1. ITS region PCR bands obtained by the use of ITS1 and ITS4 primers of yeast's genus.

| Yeast strain | Our work (bp) | Reference (bp) | References |
|---|---|---|---|
| *Metschnikowia* spp. | 390 | 400 | Sabate et al., 2002 |
| *Hanseniaspora* spp. | 750 | 750 | Garofalo et al., 2016 |
| *Lachancea* spp. | 690 | 700 | Garofalo et al., 2016 |
| *Candida* spp. | 475 | 475 | Garofalo et al., 2016 |
| *Rhodotorula* spp. | 620 | 640 | Sabate et al., 2002 |
| *Wickerhamomyces* spp. | 620 | 650 | Bautista-Gallego et al., 2011 |
| *Solicoccozyma* spp. | 620 | - | - |

On the other hand, when PCR products of ITS region are cut with REs, differences between some species were observed.

When the RFLP technique was applied with *Hha*I (*Cfo*I) restriction enzyme for our non-*Saccharomyces* isolates, results were obtained in accordance with the literature. Two genera of *Metschnikowia* and *Hanseniaspora* and five species of *Lachancea thermotolerans, Starmerella bacillaris*, *Rhodotorula mucilaginosa*, *Wickerhamomyces anomalus* and *Solicoccozyma aeria* could be distinguished from each other by *Hha*I (*Cfo*I) enzyme. As it was observed from Figure 3.3, this enzyme did not distinguish five *Metschnikowia* and three *Hanseniaspora* at species level. However, it can differentiate *Metschnikowia* and *Hanseniaspora* genera from other species *Lachancea thermotolerans, Starmerella bacillaris*, *Rhodotorula mucilaginosa*, *Wickerhamomyces anomalus* and *Solicoccozyma aeria*. In the case of *Metschnikowia* spp. all six isolates show a pattern similar to Mp (Table 3.4). However, based on ITS and D1/D2 sequencing, they belong to different species of *Metschnikowia* genus not only Mp. *L. thermotolerans* strains produced two bands close to each other (315+285 bp). All studied *Hanseniaspora* species produced two bands 320+105 bp, but from the reference in table 3.5 the bands are 320+310+105. The 310 bp band cannot be easily visualized and based on some other references pattern 320+110 are reported (Tofalo et al., 2011); Sometimes bands less than 100 bp were too faint and could not be seen as in the case of *Mestchikowia*. However, sometimes we could see them such as the 90 bp band generated with *Hae*Ⅲ enzyme in *L. thermotolerans* and the 95 bp band produced by *Dde*I in *Candida zemplinina* (Table 3.4). The reason can be either the gel concentration (in high concentrations small bands are better visualized) or the concentration of DNA template (when concentration is low, small bands cannot be visualized).

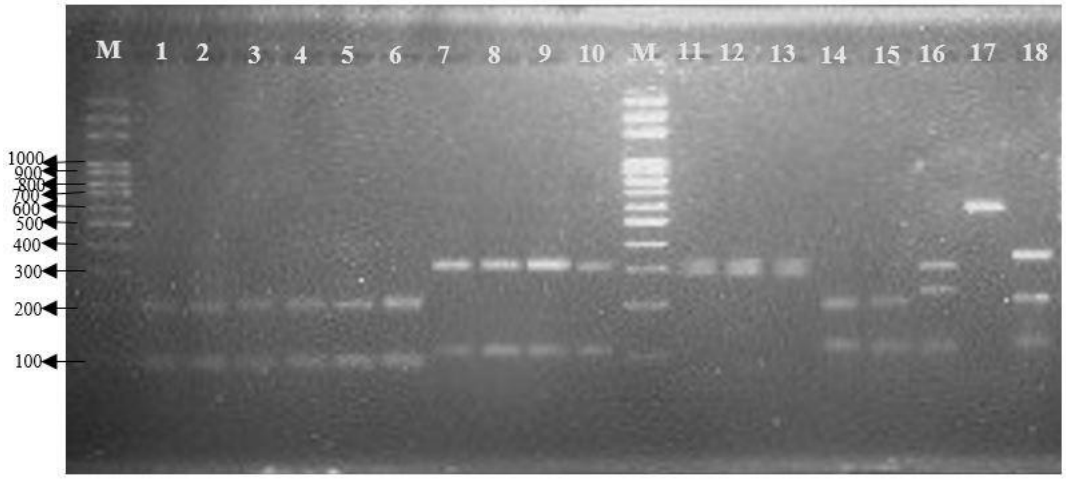


Figure 3.3. Gel image of PCR products cut with *Hha*I (CfoI) restriction enzyme
M, 100bp Marker; 1, reference strain *M. pulcherrima* (DSMZ); 2, *M. pulcherrima*(D1D2)/ *M. sinensis* (ITS) D3 (DA 0CM NS3); 3, *M. pulcherrima* B10 (BB 0CM NS2); 4, *M. aff. pulcherrima* B11 (BB 0CM NS3); 5, *M. fructicola* D15 (DB 4CM NS3); 6, *M. aff. fructicola* B33 (BB 4CM NS8); 7, reference strain *H. uvarum* (DSMZ); 8, *H. guilliermondii* K1 (KA 0CM NS1); 9, *H. opuntiae* K2 (KA 0CM NS2); 10, *H. uvarum* K13 (KA 4CM NS3); 11, reference strain *L. thermotolerans* (DSMZ); 12, *L. thermotolerans* O9 (OB 4CM NS4); 13, *L. thermotolerans* O16 (OA 0CM NS7); 14, reference strain *Candida* spp.(DSMZ); 15, *Candida zemplinina* (*Starmerella bacillaris*) D10 (DA 4CM NS2); 16, *Rhodotorula mucilaginosa* K9 (KB 0CM NS4); 17, *Wickerhamomyces anomalus* K41 (KA 0CM S1); 18, *Solicoccozyma aeria* B4 (BA 0CM NS4)

*Hinf*I enzyme was used for a total of 18 non-*Saccharomyces* containing four reference strains (*Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Lachancea thermotolerans* and *Candida* spp. purchased from DSMZ). As can be seen in Figure 3.4, *Metschnikowia* and *Hanseniaspora* genera, *Lachancea thermotolerans*, *Starmerella bacillaris*, *Rhodotorula mucilaginosa*, *Wickerhamomyces anomalus* and *Solicoccozyma aeria* species could be differentiated. All examined members of genera *Hanseniaspora* and *Metschnikowia* were differentiated from other genera at the genus level. However, this enzyme could not distinguish *Metschnikowia* and *Hanseniaspora* genera at species level. It is the same as *Hha*I (*Cfo*I) enzyme that was discussed previously. We examined three species of *Hanseniaspora* genus (Hu, Ho and Hg) but with *Hinf*I enzyme (and also *Hha*I (*Cfo*I) and *Hae*Ⅲ enzymes), these three species were not differentiated as we can see the same pattern for all three *Hanseniaspora.* The only enzyme that generated different

patterns for three species of *Hanseniaspora* was *Dde*I and *Dra*I which classified them as Hu, Ho and Hg. Moreover, we used six *Metschnikowia* belonging to five species (*M. pulcherrima*, *M. sinensis*, *M. fructicola*, *M. aff. fructicola*, *M. aff. pulcherrima*) but none of the enzymes could differentiate these species. All of them produced the same pattern as Mp according to references in Table 3.4.

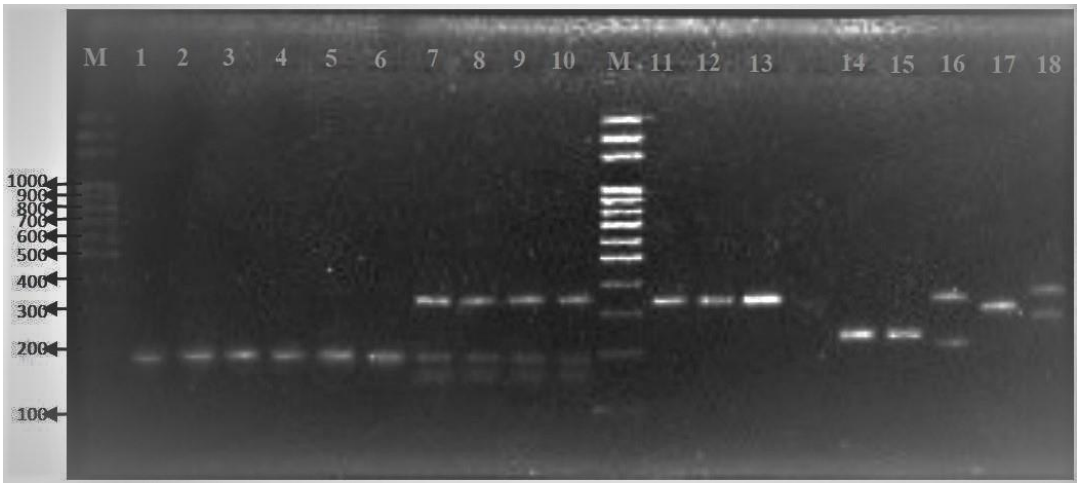


Figure 3.4. Gel image of PCR products cut with *Hinf*I restriction enzyme
M, 100bp Marker; 1, reference strain *M. pulcherrima* (DSMZ); 2- *M. pulcherrima*(D1D2)/ *M. sinensis* (ITS) D3 (DA 0CM NS3); 3, *M. pulcherrima* B10 (BB 0CM NS2); 4, *M. aff. pulcherrima* B11 (BB 0CM NS3); 5, *M. fructicola* D15 (DB 4CM NS3); 6, *M. aff. fructicola* B33 (BB 4CM NS8); 7, reference strain *H. uvarum* (DSMZ); 8, *H. guilliermondii* K1 (KA 0CM NS1); 9, *H. opuntiae* K2 (KA 0CM NS2); 10, *H. uvarum* K13 (KA 4CM NS3); 11, reference strain *L. thermotolerans* (DSMZ); 12, *L. thermotolerans* O9 (OB 4CM NS4); 13, *L. thermotolerans* O16 (OA 0CM NS7); 14, reference strain *Candida* spp.(DSMZ); 15, *Candida zemplinina* (*Starmerella bacillaris*) D10 (DA 4CM NS2); 16, *Rhodotorula mucilaginosa* K9 (KB 0CM NS4); 17, *Wickerhamomyces anomalus* K41 (KA 0CM S1); 18, *Solicoccozyma aeria* B4 (BA 0CM NS4)

Figure 3.5 shows the profile obtained by cutting non-*Saccharomyces* yeasts with *Hae*III restriction enzyme. The gel photograph illustrates seven different digestion profiles belonging to different species of 2 genera (*Metschnikowia*, *Hanseniaspora*) and five species of five different genera (*Lachancea thermotolerans*, *Starmerella bacillaris*, *Rhodotorula mucilaginosa*, *Wickerhamomyces anomalus* and *Solicoccozyma aeria*). *Metschnikowia* species were not identified by cutting with this enzyme. They all showed the same pattern with *M. pulcherima*, although they

were belonging to different species. But in the case of *Hanseniaspora*, based on references in Table 3.4, three species used in this study were not cut with *Hae*III enzyme. So they cannot be distinguished from each other, and we can say this enzyme can differentiate *Hanseniaspora* genus from other species of different genera. In the case of *Lachancea* we used three isolates of only one species "*Lachancea thermotolerans".* So they provided the same pattern in RFLP analysis as we expected. But in the case of *Hanseniaspora* and *Metschenikowia* genera, we used different species. However, we could not differentiate them between species except by using *Dde*I and *Dra*I enzymes. About *Candida*, one of them is the reference strain (*Candida spp*, number 14 on the gel) and the other one is the species *Candida zemplinina (Starmerella bacillaris)*, they also provide the same pattern with all four enzymes we used. In conclusion, it was possible to differentiate seven genera from each other also by *Hae*III enzyme.

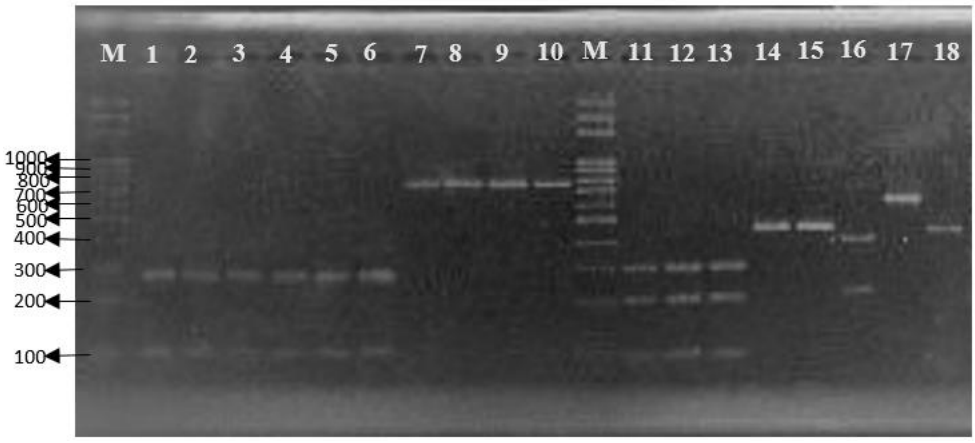


Figure 3.5. Gel image of PCR products cut with *Hae*III restriction enzyme

M, 100bp Marker; 1, reference strain *M. pulcherrima* (DSMZ); 2, *M. pulcherrima*(D1D2)/ *M. sinensis* (ITS) D3 (DA 0CM NS3); 3, *M. pulcherrima* B10 (BB 0CM NS2); 4, *M. aff. pulcherrima* B11 (BB 0CM NS3); 5, *M. fructicola* D15 (DB 4CM NS3); 6, *M. aff. fructicola* B33 (BB 4CM NS8); 7, reference strain *H. uvarum* (DSMZ); 8, *H. guilliermondii* K1 (KA 0CM NS1); 9, *H. opuntiae* K2 (KA 0CM NS2); 10, *H. uvarum* K13 (KA 4CM NS3); 11, reference strain *L. thermotolerans* (DSMZ); 12, *L. thermotolerans* O9 (OB 4CM NS4); 13, *L. thermotolerans* O16 (OA 0CM NS7); 14, reference strain *Candida* spp.(DSMZ); 15, *Candida zemplinina* (*Starmerella bacillaris*) D10 (DA 4CM NS2); 16, *Rhodotorula mucilaginosa* K9 (KB 0CM NS4); 17, *Wickerhamomyces anomalus* K41 (KA 0CM S1); 18, *Solicoccozyma aeria* B4 (BA 0CM NS4)

*Dde*I enzyme generated eight different restriction profiles belonging to *Metschnikowia* genus and species of *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii/ Hanseniaspora opuntiae*, *Lachancea thermotolerans*, *Starmerella bacillaris*, *Rhodotorula mucilaginosa*, *Wickerhamomyces anomalus*, and *Solicoccozyma aeria* (Figure 3.6). Similarly, seven examined genera were differentiated using *Dde*I restriction enzyme.

*Dde*I restriction enzyme differentiated yeasts species belonging to the *H. uvarum* (300 + 180 + 95 bp), from *H. guilliermondii* (360 + 180 + 100 bp) and *H. opuntiae* (360 + 180 + 100 bp) (Table 3.3).

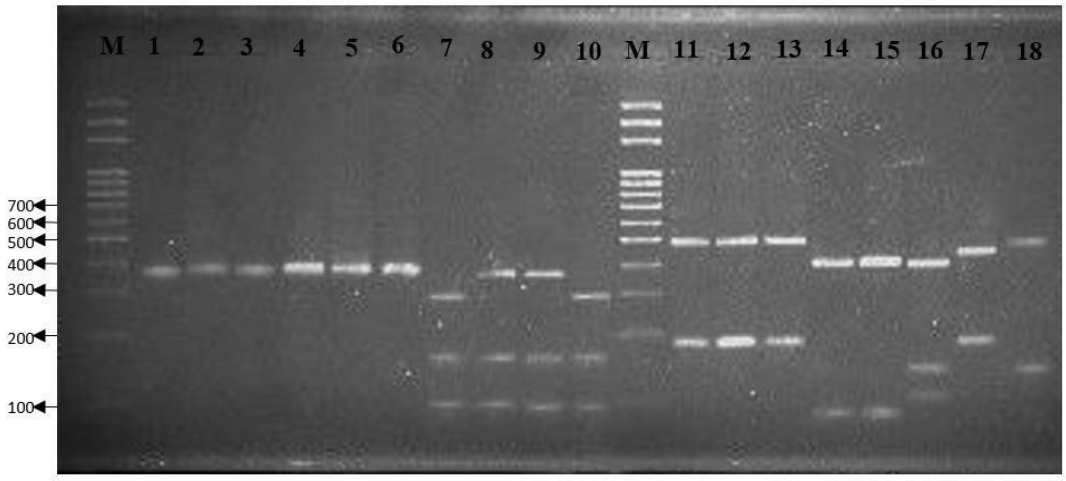


Figure 3.6. Gel image of PCR products cut with *Dde*I restriction enzyme

M, 100bp Marker; 1, reference strain *M. pulcherrima* (DSMZ); 2, *M. pulcherrima*(D1D2)/ *M. sinensis* (ITS) D3 (DA 0CM NS3); 3, *M. pulcherrima* B10 (BB 0CM NS2); 4, *M. aff. pulcherrima* B11 (BB 0CM NS3); 5, *M. fructicola* D15 (DB 4CM NS3); 6, *M. aff. fructicola* B33 (BB 4CM NS8); 7, reference strain *H. uvarum* (DSMZ); 8, *H. guilliermondii* K1 (KA 0CM NS1); 9, *H. opuntiae* K2 (KA 0CM NS2); 10, *H. uvarum* K13 (KA 4CM NS3); 11, reference strain *L. thermotolerans* (DSMZ); 12, *L. thermotolerans* O9 (OB 4CM NS4); 13, *L. thermotolerans* O16 (OA 0CM NS7); 14, reference strain *candida* spp.(DSMZ); 15, *Candida zemplinina* (*Starmerella bacillaris*) D10 (DA 4CM NS2); 16, *Rhodotorula mucilaginosa* K9 (KB 0CM NS4); 17, *Wickerhamomyces anomalus* K41 (KA 0CM S1); 18, *Solicoccozyma aeria* B4 (BA 0CM NS4)

Due to a high level of homology in two species *H. guilliermondii* and *H. opuntiae* they were not differentiated by *Dde*I (Nisiotou & Nychas, 2007). Nisiotou and Nychas (2007), suggested using *Dra*I enzyme to distinguish these two closely related organisms. According to sequence analysis of ITS region, these two organisms exhibited two nucleotide differences which are recognized by this enzyme. In our work, similarly, this enzyme generated a different pattern for the differentiation of *H. opuntiae* from *H. guilliermondii.*

In our study, as we can see in Figure 3.7, two different restriction profiles were obtained for three different species using *Dra*I enzyme. *H. guilliermondii* was differentiated from *H. opuntiae* and *H. uvarum*. Thus, it is possible to distinguish three different types in two stages (using two enzymes: *Dde*I and *Dra*I with a two by two comparisons) (Tables 3.2 and 3.3). Similarly, *H. opuntiae* can also be differentiated from *H. uvarum* and *H. guilliermondii* in two stages.

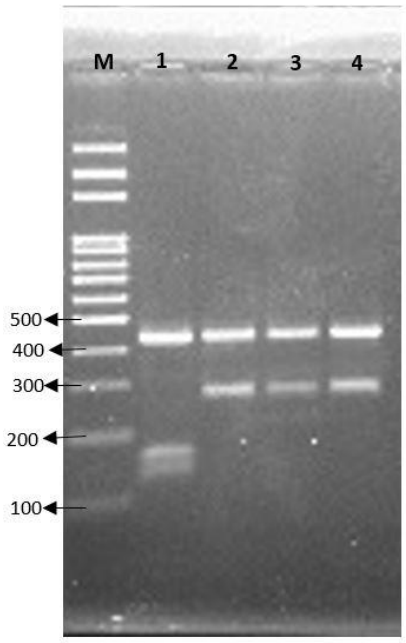


Figure 3.7. Gel image of PCR products cut with *Dra*I restriction enzyme
M,100 bp marker; 1, *H. guilliermondii* K1 (KA 0CM NS1); 2, *H. opuntiae* K2 (KA 0CM NS2); 3, reference strain *H. uvarum* (DSMZ); 4, *H. uvarum* K13 (KA 4CM NS3)

Table 3.2. *Dde*I enzyme restriction profile

| Strain | Strain code | AP* reference | AP | Restriction profile (bp) | | |
|---|---|---|---|---|---|---|
| | | | | Our results | Reference | Reference |
| *H. guilliermondii* | K1 | 750 | 750 | 360+180+100 | 360+180+85+70 +50 | Nisiotou ve Nychas., 2007 |
| *H. opuntiae* | K2 | 750 | 750 | 360+180+100 | 360+180+85+70 +50 | Nisiotou ve Nychas., 2007 |
| *H. uvarum* (DSMZ) | Hu | 750 | 750 | 300+180+100 | 300+180+95+90 +85 | Garofalo vd., 2016 |
| *H. uvarum* | K13 | 750 | 750 | 300+180+100 | 300+180+95+90 +85 | Garofalo vd., 2016 |

* Amplification Pattern

Table 3.3. *Dra*I enzyme restriction profile

| Strain | Strain code | AP* reference | AP | Restriksiyon Profili (bp) | | |
|---|---|---|---|---|---|---|
| | | | | Our results | Reference | Reference |
| *H. guilliermondii* | K1 | 750 | 750 | 420+150+130 | 420+150+130+30 | Nisiotou ve Nychas., 2007 |
| *H. opuntiae* | K2 | 750 | 750 | 420+300 | 420+300+30 | Nisiotou ve Nychas., 2007 |
| *H. uvarum* (DSMZ) | Hu | 750 | 750 | 420+300 | - | - |
| *H. uvarum* | K13 | 750 | 750 | 420+300 | - | - |

* Amplification Pattern

Table 3.4 illustrates the band size generated by ITS-PCR amplification, PCR products digested by restriction enzymes, and the references with similar results.

Table 3.4. PCR products of studied yeast species digested with restriction enzymes

| RE | Yeast species | Our results (bp) | According to references (bp) | References |
|---|---|---|---|---|
| ***Hae* Ⅲ** | | | | |
| | *Metschnikowia pulcherrima* | 280+100 | 280+100 | Sabate vd., 2002 |
| | *Metschnikowia sinensis* | 280+100 | - | - |
| | *Metschnikowia aff. pulcherrima* | 280+100 | - | - |
| | *Metschnikowia fructicola* | 280+100 | 300+100 | Tofalo vd., 2011 |
| | *Metschnikowia aff. fructicola* | 380+280+100 | - | - |
| | *Hanseniaspora uvarum* | 750 | 750 | Garofalo vd., 2016 |
| | *Hanseniaspora guilliermondii* | 750 | 750 | Garofalo vd., 2016 |
| | *Hanseniaspora opuntiae* | 750 | 750 | Garofalo vd., 2016 |
| | *Lachancea thermotolerans* | 310+215+90 | 310+215+90+90 | Garofalo vd., 2016 |
| | *Candida zemplinina* | 475 | 475 | Garofalo vd., 2016 |
| | *Rhodotorula mucilaginosa* | 425+215 | 425 +215 | Sabate vd., 2002 |
| | *Wickerhamomyces anomalus* | 620 | 625 | Gallego vd., 2011 |
| | *Solicoccozyma aeria* | 480 | 410 | Kwaśna vd., 2010 |
| ***Hha*I (*CfoI*)** | | | | |
| | *Metschnikowia pulcherrima* | 200+100 | 205+100+95 | Sabate vd., 2002 |
| | *Metschnikowia sinensis* | 200+100 | - | - |
| | *Metschnikowia aff. pulcherrima* | 200+100 | - | - |
| | *Metschnikowia fructicola* | 200+100 | 298+100 | Tofalo vd., 2011 |
| | *Metschnikowia aff. fructicola* | 200+100 | | - |
| | *Hanseniaspora uvarum* | 320+105 | 320+310+105 | Garofalo vd., 2016 |
| | *Hanseniaspora guilliermondii* | 320+105 | 320+310+105 | Garofalo vd., 2016 |
| | *Hanseniaspora opuntiae* | 320+105 | 320+310+120 | Garofalo vd., 2016 |
| | *Lachancea thermotolerans* | 315+285 | 315+285+95 | Garofalo vd., 2016 |
| | *Candida zemplinina* | 215+110 | 215+110+80+60 | Garofalo vd., 2016 |
| | *Rhodotorula mucilaginosa* | 320+240 | 320+240+80 | Sabate vd., 2002 |
| | *Wickerhamomyces anomalus* | 580 | 575 | Zarzoso vd., 1999 |
| | *Solicoccozyma aeria* | 380+250+180 | 420+200+150 | Kwaśna vd., 2010 |
| ***Hinf*I** | | | | |
| | *Metschnikowia pulcherrima* | 200 | 200 | Arroyo vd., 2010 |
| | *Metschnikowia sinensis* | 200 | - | - |
| | *Metschnikowia aff. pulcherrima* | 200 | - | - |
| | *Metschnikowia fructicola* | 200 | 200+200 | Tofalo vd., 2011 |
| | *Metschnikowia aff. fructicola* | 200 | - | - |
| | *Hanseniaspora uvarum* | 340 +190+170 | 340+190+170+60 | Nisiotou ve Nychas, 2007 |
| | *Hanseniaspora guilliermondii* | 340+190+170 | 340+190+170+60 | Nisiotou ve Nychas, 2007 |
| | *Hanseniaspora opuntiae* | 340+190+170 | 340+190+170+60 | Garofalo vd., 2016 |

Table 3.4 (continued)

| | | | | |
|---|---|---|---|---|
| | *Lachancea thermotolerans* | 355 | 355+354 | Garofalo vd., 2016 |
| | *Candida zemplinina* | 250 | 235+235 | Garofalo vd. 2016 |
| | *Rhodotorula mucilaginosa* | 340+220 | 340+225+75 | Garofalo vd., 2016 |
| | *Wickerhamomyces anomalus* | 320 | 325+325 | Sabate vd., 2002 |
| | *Solicoccozyma aeria* | 370+300 | - | Gallego vd., 2011 |
| ***Dde*I** | | | | |
| | *Metschnikowia pulcherrima* | 390 | - | - |
| | *Metschnikowia sinensis* | 390 | - | - |
| | *Metschnikowia aff. pulcherrima* | 390 | - | - |
| | *Metschnikowia fructicola* | 390 | - | - |
| | *Metschnikowia aff. fructicola* | 390 | - | - |
| | *Hanseniaspora uvarum* | 300+180+100 | 300+180+95+90+85 | Garofalo vd., 2016 |
| | *Hanseniaspora guilliermondii* | 360+180+100 | 360+180+85+70+50 | Nisiotou ve Nychas, 2007 |
| | *Hanseniaspora opuntiae* | 360+180+100 | 360+180+85+70+50 | Nisiotou ve Nychas, 2007 |
| | *Lachancea thermotolerans* | 500+190 | - | - |
| | *Candida zemplinina* | 410+95 | - | - |
| | *Rhodotorula mucilaginosa* | 410+150+110 | - | - |
| | *Wickerhamomyces anomalus* | 450+190 | - | - |
| | *Solicoccozyma aeria* | 500+150 | - | - |
| ***Dra*I** | | | | |
| | *Hanseniaspora guilliermondii* | 420+150+130 | 420+150+130+30 | Nisiotou ve Nychas, 2007 |
| | *Hanseniaspora opuntiae* | 420+300 | 420+300+30 | Nisiotou ve Nychas, 2007 |
| | *Hanseniaspora uvarum* | 420+300 | - | - |

* RE, restriction enzyme; *Candida zemplinina* (*Starmerella bacillaris*); *Solicoccozyma aeria* (*Cryptococcus aerius*).

## 3.2 RAPD Analysis

Table 2.3 shows the list and sources of forty-six non-*Saccharomyces* yeast used in this study. All the isolates were identified previously by ITS sequencing and some of them by D1/D2 domain sequencing as well.

RAPD reaction with different primers generated five to fourteen bands of amplified DNA, ranging from 150 to 2000 base pairs. A single primer was not sufficient to differentiate all the strains. Some strains show an identical pattern using one

primer. However, they can produce a different profile using a different primer. From this point of view, the information gained by a combination of four primers was used to separate strains (Echeverrigaray et al., 2000).

Four different primers (Table 2.4) were used to differentiate strains of 4 species (*Lachancea thermotolerans*, *Hanseniaspora uvarum*, *Hanseniaspora opuntia*, *Metschnikowia pulcherrima*). RAPD or Microsatellite fingerprint studies were conducted to investigate whether there are genotypic differences between the strains isolated from the same or different wines made of different grape types. RAPD-PCR was able to distinguish strains within species, based on the PCR product patterns generated by using a specific primer.

Four *Lachancea thermotolerans* samples were subjected to RAPD-PCR using four mentioned primers in order to determine the intraspecific genetic diversity of these strains.

As we can see from Figure 3.8, primer OPA-03 produced two different patterns. One pattern discriminates reference strain (DSMZ) from the other four isolated strains. The other unique pattern belongs to strain O6 with two extra upper bands (700 bp and 450 bp) which differentiates O6 from O9, O16 and O22.

- Pattern I: Lt (Ref)
- Pattern Ⅱ: O6
- Pattern Ⅲ: O9, O16, O22

The profile yielded by primer M13 was able to differentiate the reference strain from the other four strains as well. Using this primer, O9 showed a different pattern with a lack of two upper bands (1500 and 600 bp) in comparison with O6, O16 and O22. Therefore, OPA-03 differentiates O6 and, M13 differentiates O9 within four studied isolates of *L. thermotolerans* (Table. 3.8). Both of the primers show that DSMZ reference is different from our laboratory strains.

- Pattern I: Lt (Ref)
- Pattern Ⅱ: O9

- Pattern Ⅲ: O6, O16, O22

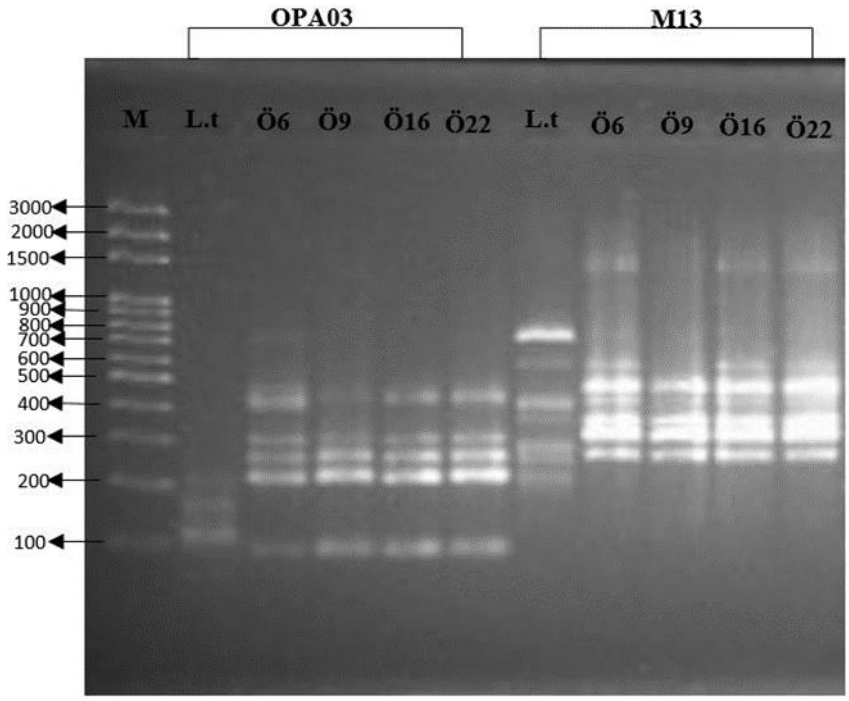


Figure 3.8. The amplification product of *L. thermotolerans* using primers OPA-03 and M13

Lane M, 100-bp molecular marker; lane Lt, reference strain *L. thermotolerans* (DSMZ); lane O6 (DA 0-CM NS3), *L. thermotolerans*; lane O9 (BB 0-CM NS2), *L. thermotolerans*; lane O16 (BB 0-CM NS3), *L. thermotolerans*; lane O22 (DB 4-CM NS3), *L. thermotolerans.*

Profiles generated using $(ATG)_5$ and $(GTG)_5$ microsatellite primers also confirms the difference between DSMZ strain and our strains. In Figure 3.9, we can see that there is no unique pattern produced by $(ATG)_5$ primer except DSMZ primer.

- Pattern I: Lt (Ref)
- Pattern Ⅱ: O9, O6, O16, O22

However, with $(GTG)_5$, O6 yielded a different pattern from O9, O16 and O22, showing two extra upper bands (420 and 500 bp) and approximately 250 bp band.

- Pattern I: Lt (Ref)

- Pattern Ⅱ: O6
- Pattern Ⅲ: O9, O16, O22

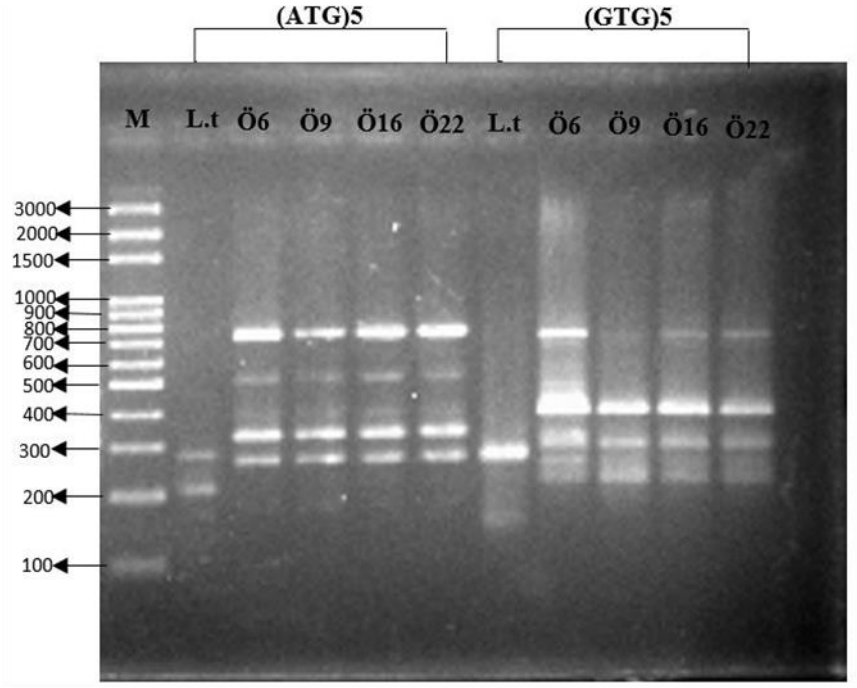


Figure 3.9. The amplification product of *L. thermotolerans* using $(ATG)_5$ and $(GTG)_5$ microsatellite primers

Lane M, 100-bp molecular marker; lane Lt, reference strain *L. thermotolerans* (DSMZ); lane O6 (DA 0-CM NS3), *L. thermotolerans*; lane O9 (BB 0-CM NS2), *L. thermotolerans*; lane O16 (BB 0-CM NS3), *L. thermotolerans*; lane O22 (DB 4-CM NS3), *L. thermotolerans*

Tables were prepared using the patterns obtained in the gel photographs. In Table 3.5 identical pattern types are represented by identical letter codes. The data in the last column shows the general pattern and was obtained by the combination of the results obtained with each primer. Different letters were given to the new combination of pattern types based on alphabetic order.

For example, in the column representing the primer OPA-03 results (column two), O6 starts with the letter A. However, O9 generated a different pattern with OPA-03, the letter B was given. On the other hand, both O16 and O22 produced the

same pattern as O9, after amplification of their DNA with OPA-03 primer. So the letter B was given to them as well.

The data in the last column, overall pattern, were obtained from combining the results of each primer. A different letter was given to a new combination of pattern types. As we can see, A was given to the first row, no matter what letter it is contained, we always start with A. In the second row, the combination of letters that were given to each primer is as follow; B, B, A, B. It is a different combination than the first row (A, A, A, A), so letter B was given to the second row of overall pattern column. The third row is a combination of B, A, A, B. This is a different combination than either the first and second row. So the letter C was given to it. Forth and the last row is combined with B, A, A, B, which is the same combination as the third row. Because they have a similar combination and C was given to the third row, a C was given to the fourth row as well.

Table 3.5. Patterns obtained with the use of four primers for *L. thermotolerance* strains

| Strain | RAPD primer | Mini- and Microsatellite primers | | | Overall Pattern |
|---|---|---|---|---|---|
| | OPA-03 | M13 | $(ATG)_5$ | $(GTG)_5$ | |
| O6 | A | A | A | A | A |
| O9 | B | B | A | B | B |
| O16 | B | A | A | B | C |
| O22 | B | A | A | B | C |

After PCR amplification of twelve *Hanseniaspora uvarum* species with four primers, results show that the reference strain purchased from DSMZ generates different patterns from *H. uvarum* strains isolated in our laboratory.

Results gained by OPA-03 primer (Figure 3.10) show ten different patterns. E11, E14 strains and E12, E13 strains produced a similar pattern respectively. The other eight studied strains, each show different patterns. For example, K3 has a 180 bp band which lacks in K13. Likewise, K52 has a 400 bp band that is not presented in

the K3 strain. However, the generation of minor differences in patterns does not always indicate the difference between strains. As an example, after calculation the similarity between a strain with the combination of results of four primers using the DICE coefficient (Figure 3.21), K2 and K52 showed 85.4 % similarity. Strains with a similarity percentage of more than 80% are considered as the same strain (Mateus et al., 2020).

We can classify isolates based on the generated patterns as follow;

- Pattern I; Hu (Ref)
- Pattern Ⅱ; K3
- Pattern Ⅲ; K13
- Pattern Ⅳ; K25
- Pattern Ⅴ; K26
- Pattern Ⅵ; K52
- Pattern Ⅶ; K54
- Pattern Ⅷ; K70
- Pattern Ⅸ; E11, E14
- Pattern Ⅹ; E12, E13

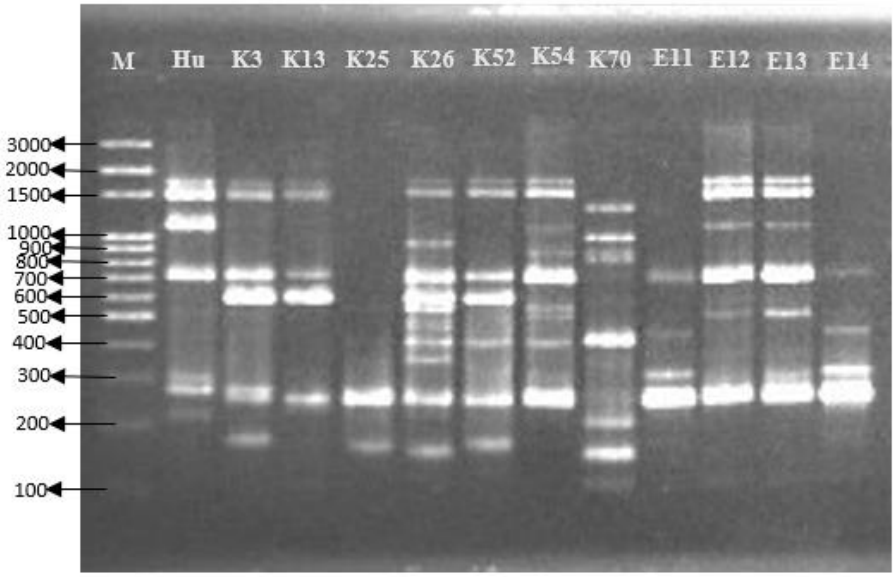


Figure 3.10. The amplification product of *H. uvarum* using primer OPA-03
Lane M, 100-bp molecular marker; lane Hu, reference strain *H. uvarum* (DSMZ); lane K3 (KA 0-CM NS3), *H. uvarum*; lane K13 (KA 4-CM NS3), *H. uvarum*; lane K25 (KA 2-NM NS9), *H. uvarum*; lane K26 (KB 2-NM NS2), *H. uvarum*; lane K52 (KA 2-NM NS4), *H. uvarum*; lane 54 (KA 2-NM NS8), *H. uvarum*; lane K70 (KB 4-NM NS10), *H. uvarum*; lane E11 (EA 1.W NS1), *H. uvarum*, lane E12 (EA 1.W NS2), *H. uvarum*; lane E13 (EA 1.W NS3), *H. uvarum*; lane E14 (EA 1.W NS4), *H. uvarum*

Figure 3.11 shows the amplicons produced by *H. uvarum* strains using M13 primer. M13 primer produced eleven different patterns ranging from 150-2000 bp. Two strains, K25 and K26 show a similar pattern. Although other strains each have a unique pattern, the differences are sometimes minor. For example, E11 has two bands (180 and 150 bp) which E14 lacks. On the other hand, E14 has a 500 bp band that is not presented in the E11 pattern. Different patterns produced by M13 primer belonging to isolates as follow;

- Pattern I; Hu (Ref)
- Pattern Ⅱ; K3
- Pattern Ⅲ; K13
- Pattern Ⅳ; K25/K26
- Pattern Ⅴ; K52

- Pattern Ⅵ; K54
- Pattern Ⅶ; K70
- Pattern Ⅷ; E11
- Pattern Ⅸ; E12
- Pattern Ⅹ; E13
- Pattern Ⅺ; E14

The similarity coefficient is discussed in section 3.2.1.

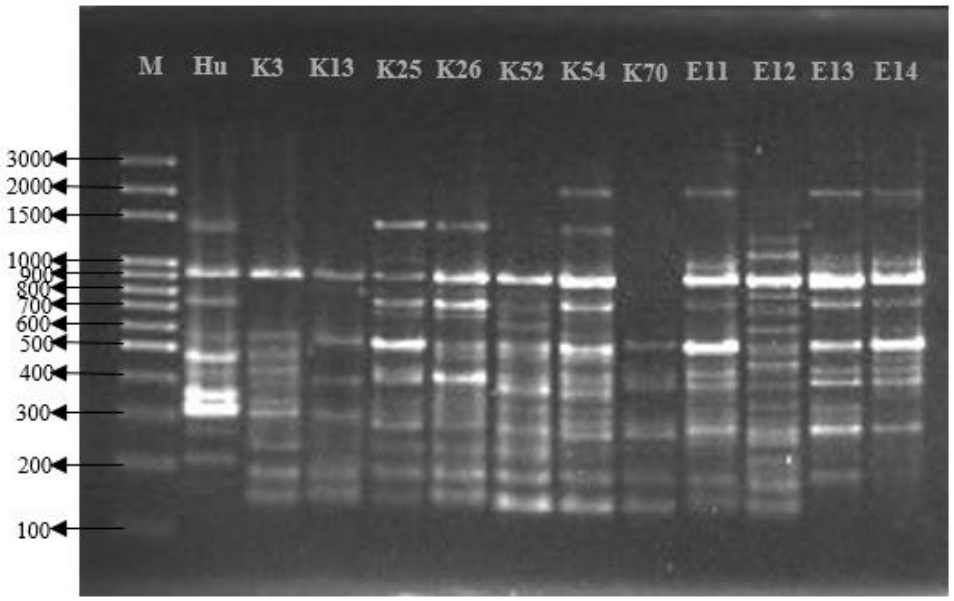


Figure 3.11. The amplification product of *H. uvarum* using M13 primer
Lane M, 100-bp molecular marker; lane Hu, reference strain *H. uvarum* (DSMZ); lane K3 (KA 0-CM NS3), *H. uvarum*; lane K13 (KA 4-CM NS3), *H. uvarum*; lane K25 (KA 2-NM NS9), *H. uvarum*; lane K26 (KB 2-NM NS2), *H. uvarum*; lane K52 (KA 2-NM NS4), *H. uvarum*; lane 54 (KA 2-NM NS8), *H. uvarum*; lane K70 (KB 4-NM NS10), *H. uvarum*; lane E11 (EA 1.W NS1), *H. uvarum*, lane E12 (EA 1.W NS2), *H. uvarum*; lane E13 (EA 1.W NS3), *H. uvarum*; lane E14 (EA 1.W NS4), *H. uvarum*

Figure 3.12 shows the results of the amplification of studied *H. uvarum* strains by $(ATG)_5$ primer. As we can see from the gel picture, ten different patterns are generated. K3 and K52 have the same pattern. K26 and K54 also generated the same profile. Differences in the patterns also are indicated in table 3.12. For

example, a comparison of K3 and K26 shows that the K3 strain has one 600 bp band that is not produced in the K26 strain. Also, the only difference between K52 and K54 is a 600 bp band in K52 that lacks in K54.

Isolates can be classified according to the patterns produced with $(ATG)_5$ primer as follow;

- Pattern I; Hu (Ref)
- Pattern Ⅱ; K3, K52
- Pattern Ⅲ; K13
- Pattern Ⅳ; K25
- Pattern Ⅴ; K26, K54
- Pattern Ⅵ; K70
- Pattern Ⅶ; E11, E14
- Pattern Ⅷ; E12
- Pattern Ⅸ; E13

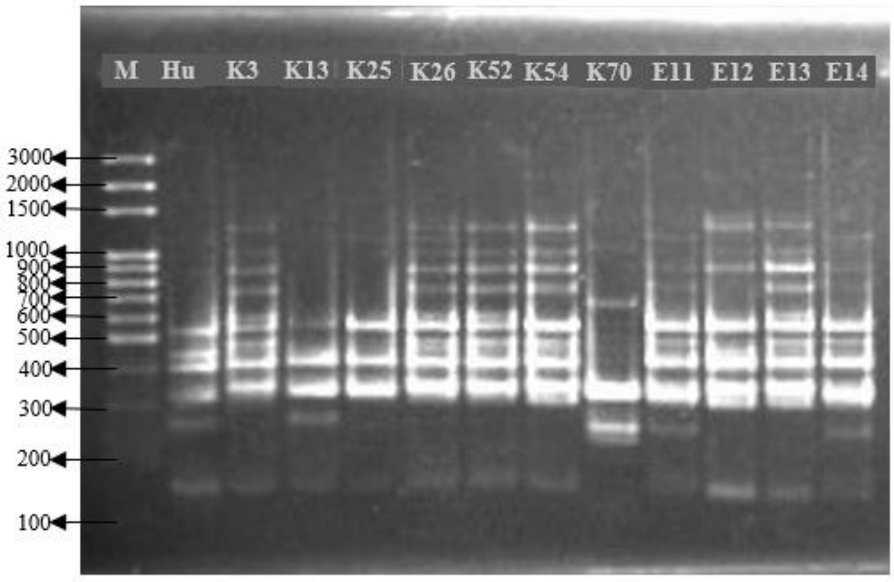


Figure 3.12. The amplification product of *H. uvarum* using $(ATG)_5$ primer
Lane M, 100-bp molecular marker; lane Hu, reference strain *H. uvarum* (DSMZ); lane K3 (KA 0-CM NS3), *H. uvarum*; lane K13 (KA 4-CM NS3), *H. uvarum*; lane K25 (KA 2-NM NS9), *H. uvarum*; lane K26 (KB 2-NM NS2), *H. uvarum*; lane K52 (KA 2-NM NS4), *H.*

*uvarum*; lane 54 (KA 2-NM NS8), *H. uvarum*; lane K70 (KB 4-NM NS10), *H. uvarum*; lane E11 (EA 1.W NS1), *H. uvarum*, lane E12 (EA 1.W NS2), *H. uvarum*; lane E13 (EA 1.W NS3), *H. uvarum*; lane E14 (EA 1.W NS4), *H. uvarum*.

Amplification results of twelve *H. uvarum* strains using $(GTG)_5$ primer are shown in Figure 3.13. There are eight different patterns in which five of the strains produced a similar pattern (K26, K52, K54, E11, E14). Other strains generated a unique pattern. As an example, K3 was differentiated from K13 for lacking an 850 bp band. And K13 can be distinguished from K26 through the absence of an upper 1000 bp band in K13.

Isolates can be classified based on the pattern produced by $(GTG)_5$ primer as follow;

- Pattern I; Hu (Ref)
- Pattern Ⅱ; K3
- Pattern Ⅲ; K13
- Pattern Ⅳ; K25
- Pattern Ⅴ; K26, K52, K54, E11, E14
- Pattern; Ⅵ; K70
- Pattern Ⅶ; E12
- Pattern Ⅷ; E13

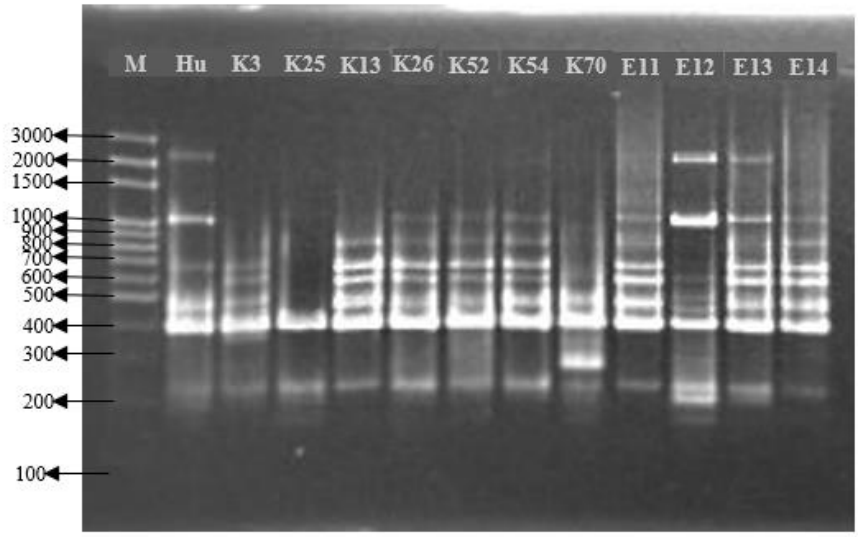


Figure 3.13. The amplification product of *H. uvarum* using $(GTG)_5$ primer
Lane M, 100-bp molecular marker; lane Hu, reference strain *H. uvarum* (DSMZ); lane K3 (KA 0-CM NS3), *H. uvarum*; lane K25 (KA 2-NM NS9), *H. uvarum*; lane K13 (KA 4-CM NS3), *H. uvarum*; lane K26 (KB 2-NM NS2), *H. uvarum*; lane K52 (KA 2-NM NS4), *H. uvarum*; lane 54 (KA 2-NM NS8), *H. uvarum*; lane K70 (KB 4-NM NS10), *H. uvarum*; lane E11 (EA 1.W NS1), *H. uvarum*, lane E12 (EA 1.W NS2), *H. uvarum*; lane E13 (EA 1.W NS3), *H. uvarum*; lane E14 (EA 1.W NS4), *H. uvarum*.

In table 3.6 each column with a specific primer starts with A (according to alphabetic order) as the first pattern for the first examined strain (Couto et al., 1996). If the next strain shows the same pattern as the previous one, the same letter was given to it. If they show a different pattern, different letters were given to them. For example, in the column that indicates OPA-03 primer patterns, K3 has a different letter (B) in comparison with Hu (A). It means they produced different profiles using OPA-03. K13 has the C letter. It means the pattern produced by this strain is not the same as Hu and K3. The letters were given based on alphabetic order. If some strains produced the same pattern, the same letter was given to them. For example, the letter I was given to both E11 and E14. And the letter J was given to E12 and E13. Those strains with the same letter show the same pattern in the gel picture. The same assessment method was applied to other columns representing primers M13, $(ATG)_5$, and $(GTG)_5$. The overall pattern column is the combination

of each primer used for amplification of each strain. It shows the similarity or differentiation between strains after amplification by all four primers. In the table above, the first row in the last column indicates the combination results of four primers for Hu strain. The letter A was given to the first row. It is because we start with alphabetic order. If The second row was the same as the first row (A A A A) the letter A was given to it. But as we can see from the table, the second row is combined with four B letter. So we need to go through alphabetic order and give a B to the overall pattern of the second row. In the table above twelve different letters were given to the overall pattern of twelve studied strains. It shows, non of the studied strains show the same pattern after combining the results of four primers. However, some of them produced a similar pattern using only one primer, such as K26, K52, K54, E11 when using $(GTG)_5$ primer.

Table 3.6. Patterns obtained with the use of four primers for *H. uvarum* strains

| Strain | RAPD Primer | Mini and Microsatellite primers | | | Overal pattern |
|---|---|---|---|---|---|
| | OPA-03 | M13 | $(ATG)_5$ | $(GTG)_5$ | |
| Hu | A | A | A | A | A |
| K3 | B | B | B | B | B |
| K13 | C | C | C | C | C |
| K25 | D | D | D | D | D |
| K26 | E | D | E | E | E |
| K52 | F | E | B | E | F |
| K54 | G | F | E | E | G |
| K70 | H | G | F | F | H |
| E11 | I | H | G | E | I |
| E12 | J | I | H | G | J |
| E13 | J | J | I | H | K |
| E14 | I | K | G | E | L |

Although several molecular techniques used for the determination of biodiversity of different yeast species, to our knowledge, no one has been developed so far for *H. opuntiae*. In this study, 21 isolates of *H. opuntiae* were subjected to characterization by RAPD-PCR.

As we can see from the gel picture in Figure 3.14, by using RAPD primer OPA-03, six different patterns generated;

- Pattern I; O5
- Pattern Ⅱ; O7
- Pattern Ⅲ; K2
- Pattern Ⅳ; K7, K10, K12, K17, K19, K32, K34, K38, K40, K51, K60
- Pattern Ⅴ; K15, K16, K20, K27, K35, K37
- Pattern Ⅵ; K21

Pattern Ⅵ is differentiated from pattern Ⅴ with an extra 300 bp band that lacks in pattern Ⅴ.

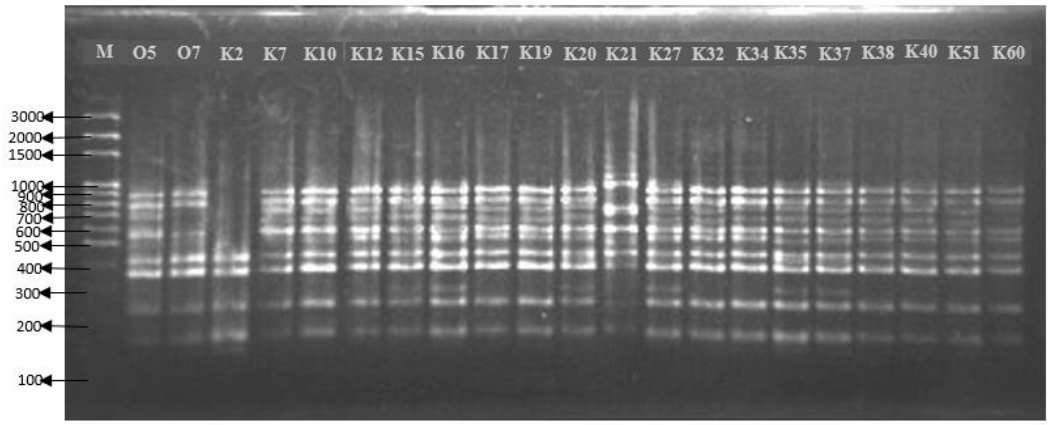


Figure 3.14. The amplification product of *H. opuntiae* using OPA-03 primer
Lane M, 100-bp molecular marker; lane O5 (OA 4-CM NS2), *H. opuntiae*; lane O7 (OA 4-CM NS4), *H. opuntiae*; lane K2 (KA 0-CM NS2), *H. opuntiae*; lane K7 (KB 0-CM NS2), *H. opuntiae*; lane K10 (KB 0-CM NS5), *H. opuntiae*; lane K12 (KA 4-CM NS2), *H. opuntiae*; lane K15 (KA 4-CM NS5), *H. opuntiae*; lane K16 (KB 4-CM NS1), *H. opuntiae*; lane K17 (KB 4-CM NS2), *H. opuntiae*; lane K19 (KB 4-CM NS4), *H. opuntiae*; lane K20 (KB 4-CM NS5), *H. opuntiae*; lane K21 (KA 2-NM NS1), *H. opuntiae*; lane K27 (KB 2-NM NS4), *H. opuntiae*; lane K32 (KA 4-NM NS4), *H. opuntiae*; lane K34 (KA 4-NM NS8), *H. opuntiae*; lane K35 (KA 4-NM NS10), *H. opuntiae*; lane K37 (KB 4-NM NS3), *H. opuntiae*; lane K38 (KB 4-NM NS5), *H. opuntiae*; lane K40 (KB 4-NM NS9), *H. opuntiae*; lane K51 (KA 2NM NS2), *H. opuntiae*; lane K60 (KB 2-NM NS9), *H. opuntiae*.

Amplification of 21 strains by minisatellite primer M13, produced ten different patterns (Figure 3.15).

- Pattern I; O5
- Pattern Ⅱ; O7
- Pattern Ⅲ; K2
- Pattern Ⅳ; K7, K10
- Pattern Ⅴ; K12, K17, K19
- Pattern Ⅵ; K15, K16, K20
- Pattern Ⅶ; K21
- Pattern Ⅷ; K27, K51
- Pattern Ⅸ; K32, K34, K35, K38, K60
- Pattern Ⅹ; K37, K40

The discriminatory power of M13 was more than OPA-03, as it generated more different patterns. The similarity percentage of the strains was calculated by the Dice coefficient and the results are presented in Figure 3.22.

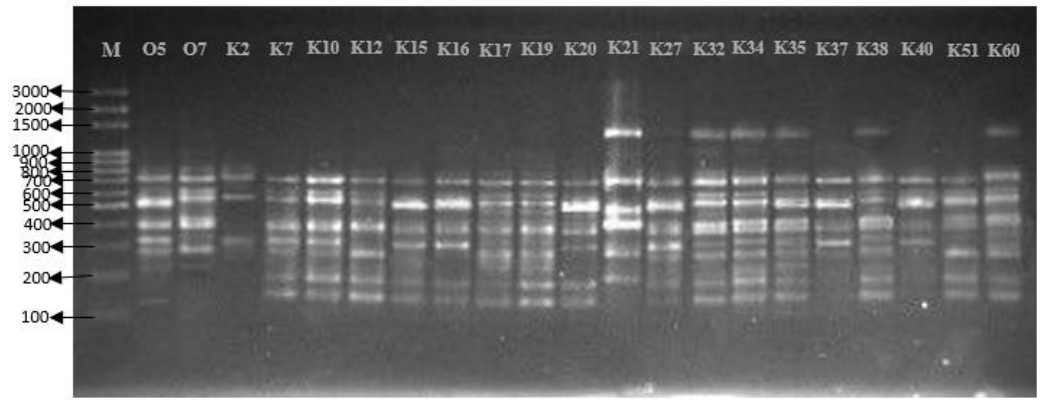


Figure 3.15. The amplification product of *H. opuntiae* using M13 primer
Lane M, 100-bp molecular marker; lane O5 (OA 4-CM NS2), *H. opuntiae*; lane O7 (OA 4-CM NS4), *H. opuntiae*; lane K2 (KA 0-CM NS2), *H. opuntiae*; lane K7 (KB 0-CM NS2), *H. opuntiae*; lane K10 (KB 0-CM NS5), *H. opuntiae*; lane K12 (KA 4-CM NS2), *H. opuntiae*; lane K15 (KA 4-CM NS5), *H. opuntiae*; lane K16 (KB 4-CM NS1), *H. opuntiae*; lane K17 (KB 4-CM NS2), *H. opuntiae*; lane K19 (KB 4-CM NS4), *H. opuntiae*; lane K20 (KB 4-CM NS5), *H. opuntiae*; lane K21 (KA 2-NM NS1), *H. opuntiae*; lane K27 (KB 2-NM NS4), *H. opuntiae*; lane K32 (KA 4-NM NS4), *H. opuntiae*; lane K34 (KA 4-NM NS8), *H. opuntiae*; lane K35 (KA 4-NM NS10), *H. opuntiae*; lane K37 (KB 4-NM NS3), *H. opuntiae*; lane K38 (KB 4-NM NS5), *H. opuntiae*; lane K40 (KB 4-NM NS9), *H. opuntiae*; lane K51 (KA 2NM NS2), *H. opuntiae*; lane K60 (KB 2-NM NS9), *H. opuntiae*.

RAPD analysis using microsatellite primer $(ATG)_5$ discriminated eight patterns (Figure 3.16; Table 3.9).

- Pattern Ⅰ; O5
- Pattern Ⅱ; O7
- Pattern Ⅲ; K2
- Pattern Ⅳ; K7, K10, K20
- Pattern Ⅴ; K12, K15, K16, K17, K19
- Pattern Ⅵ; K21
- Pattern Ⅶ; K27, K32, K34, K35, K37, K38, K51, K60
- Pattern Ⅷ; K40

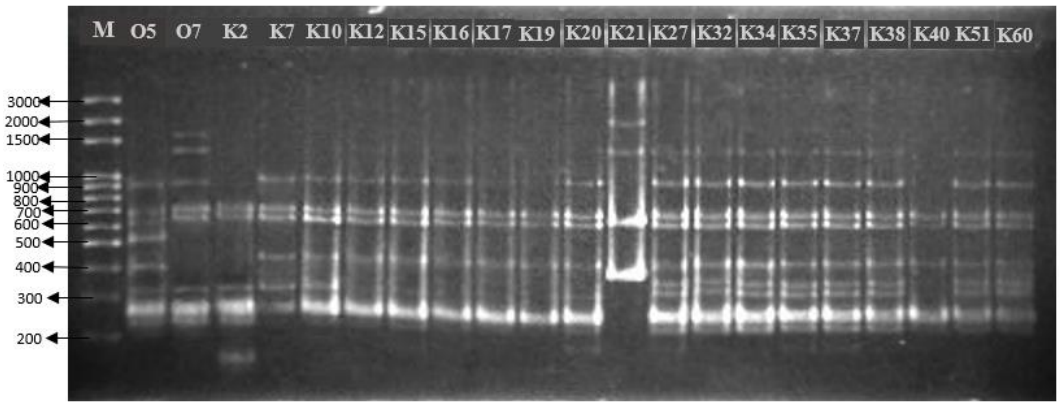


Figure 3.16. The amplification product of *H. opuntiae* using $(ATG)_5$ primer
Lane M, 100-bp molecular marker; lane O5 (OA 4-CM NS2), *H. opuntiae*; lane O7 (OA 4-CM NS4), *H. opuntiae*; lane K2 (KA 0-CM NS2), *H. opuntiae*; lane K7 (KB 0-CM NS2), *H. opuntiae*; lane K10 (KB 0-CM NS5), *H. opuntiae*; lane K12 (KA 4-CM NS2), *H. opuntiae*; lane K15 (KA 4-CM NS5), *H. opuntiae*; lane K16 (KB 4-CM NS1), *H. opuntiae*; lane K17 (KB 4-CM NS2), *H. opuntiae*; lane K19 (KB 4-CM NS4), *H. opuntiae*; lane K20 (KB 4-CM NS5), *H. opuntiae*; lane K21 (KA 2-NM NS1), *H. opuntiae*; lane K27 (KB 2-NM NS4), *H. opuntiae*; lane K32 (KA 4-NM NS4), *H. opuntiae*; lane K34 (KA 4-NM NS8), *H. opuntiae*; lane K35 (KA 4-NM NS10), *H. opuntiae*; lane K37 (KB 4-NM NS3), *H. opuntiae*; lane K38 (KB 4-NM NS5), *H. opuntiae*; lane K40 (KB 4-NM NS9), *H. opuntiae*; lane K51 (KA 2NM NS2), *H. opuntiae*; lane K60 (KB 2-NM NS9), *H. opuntiae*

Figure 3.17 illustrates the results gained after amplification by using $(GTG)_5$ primer. For twenty-one strains that were used, seven different patterns were produced.

- Pattern I; O5
- Pattern Ⅱ; O7
- Pattern Ⅲ; K2
- Pattern Ⅳ; K7, K10, K17, K19, K20
- Pattern Ⅴ; K12, K15, K16
- Pattern Ⅵ; K21
- Pattern Ⅶ; K27, K32, K34, K35, K37, K38, K40, K51, K60

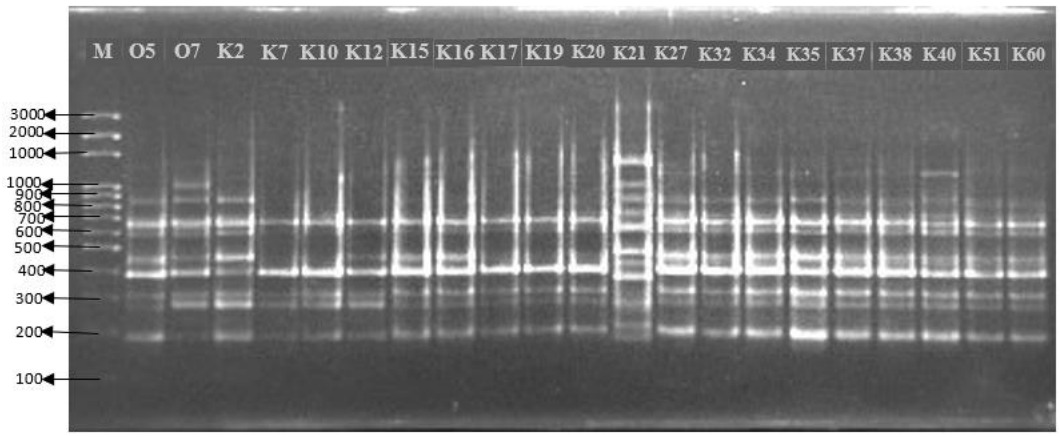


Figure 3.17. The amplification product of *H. opuntiae* using $(GTG)_5$ primer
Lane M, 100-bp molecular marker; lane O5 (OA 4-CM NS2), *H. opuntiae*; lane O7 (OA 4-CM NS4), *H. opuntiae*; lane K2 (KA 0-CM NS2), *H. opuntiae*; lane K7 (KB 0-CM NS2), *H. opuntiae*; lane K10 (KB 0-CM NS5), *H. opuntiae*; lane K12 (KA 4-CM NS2), *H. opuntiae*; lane K15 (KA 4-CM NS5), *H. opuntiae*; lane K16 (KB 4-CM NS1), *H. opuntiae*; lane K17 (KB 4-CM NS2), *H. opuntiae*; lane K19 (KB 4-CM NS4), *H. opuntiae*; lane K20 (KB 4-CM NS5), *H. opuntiae*; lane K21 (KA 2-NM NS1), *H. opuntiae*; lane K27 (KB 2-NM NS4), *H. opuntiae*; lane K32 (KA 4-NM NS4), *H. opuntiae*; lane K34 (KA 4-NM NS8), *H. opuntiae*; lane K35 (KA 4-NM NS10), *H. opuntiae*; lane K37 (KB 4-NM NS3), *H. opuntiae*; lane K38 (KB 4-NM NS5), *H. opuntiae*; lane K40 (KB 4-NM NS9), *H. opuntiae*; lane K51 (KA 2NM NS2), *H. opuntiae*; lane K60 (KB 2-NM NS9), *H. opuntiae*

Among primers used in this study, M13 could distinguish more isolates of *H. Opuntiae* by producing more different patterns (ten patterns). Primer $(ATG)_5$ produced eight different patterns and $(GTG)_5$ generated seven different profiles. Primer OPA-03 had the least discriminatory power with producing six patterns out of twenty-one isolates. It was noticed that strains from the same grape samples were differentiated. The biodiversity between strains isolated from different grape types is not surprising. For example, O5 and O7 isolated from öküzgözü grape type are different strains than K2 and K21 which are isolated from kalecik karası grape variety. However, wide biodiversity is reported by González-Arenzana et al (2017) between those isolates that belonged to the same winery or those isolated from the same medium (González-Arenzana et al., 2017).

Table 3.7 shows the patterns produced by four primers used and also the overall pattern of combining the results of primers for each strain. Identical letters are

given to those strains that have identical patterns. For example, in the second column that indicates results gained by OPA-03 primer, K15, K16, K20, K27, K35, K37 isolates have the letter E. It means they generated the same pattern using OPA-03 (Figure 3.13). The isolates K7 and K10 show the same overall pattern as both of them represents the D letter. It means these two strains produced the same pattern with all four primers. Also, K32, K34, K38, K60 represent the letter K in the overall pattern column that means they generated the same profile using all four primers.

Table 3.7 Patterns obtained with the use of 4 primers for *H. opuntia* strains

| Strain | RAPD Primer | Mini and Microsatellite Primers | | | Overall Pattern |
|---|---|---|---|---|---|
| | OPA-03 | M13 | $(ATG)_5$ | $(GTG)_5$ | |
| O5 | A | A | A | A | A |
| O7 | B | B | B | B | B |
| K2 | C | C | C | C | C |
| K7 | D | D | D | D | D |
| K10 | D | D | D | D | D |
| K12 | D | E | E | E | E |
| K15 | E | F | E | E | F |
| K16 | E | F | E | E | F |
| K17 | D | E | E | D | G |
| K19 | D | E | E | D | G |
| K20 | E | F | D | D | H |
| K21 | F | G | F | F | I |
| K27 | E | H | G | G | J |
| K32 | D | I | G | G | K |
| K34 | D | I | G | G | K |
| K35 | E | I | G | G | L |
| K37 | E | J | G | G | M |
| K38 | D | I | G | G | K |
| K40 | D | J | H | G | N |
| K51 | D | H | G | G | O |
| K60 | D | I | G | G | K |

Seven strains of *Metschnikowia pulcherrima* were studied in order to determine diversity among them. Based on ITS sequencing B10, B11, B33, D4, and D12 are

*M. pulcherrima.* On the other hand, D3 and D8 are *M. pulcherrima* based on the sequencing of D1/D2 domain. However, strain D3 was identified as *M. sinensis* and D8 as *M. chrysoperlae* according to the ITS region sequence.

The results of RAPD-PCR by using all four primers show the same pattern for B10 and B33. D3 and D8 also generated a similar profile. The reference *M. pulcherrima* (DSMZ) yielded a different pattern from all seven strains isolated in our laboratory (Table 3.8). As an interesting finding, according to our sequence results; DSMZ *M. pulcherrima* strain was identified as *M. aff pullcherrima* according to the ITS region sequence, and as *M. pulcherrima* according to the D1 / D2 sequence analysis. OPA-03 generated a total of five different patterns between 5 strains identified as *M. pulcherrima,* according to ITS and Mp reference strain (Figure 3.18).

- Pattern I; Mp (Ref)
- Pattern Ⅱ; B10 and B33
- Pattern Ⅲ; D4
- Pattern Ⅳ; B11
- Pattern Ⅴ; D12

D3, D8 also produced a pattern similar to pattern Ⅲ. M13 primer generated four different profiles as follow;

- Pattern I; Mp (Ref)
- Pattern Ⅱ; B10, B11, B33
- Pattern Ⅲ; D4
- Pattern Ⅳ; D12

The other two *M. pulcherrima* identified by D1/D2 domain sequencing, D3 and D8, produced the same profile that is different from the other isolates' patterns.

None of the primers could differentiate two D1/D2 identified strains (D3 and D8) (Figure 3.18; Table 3.8).

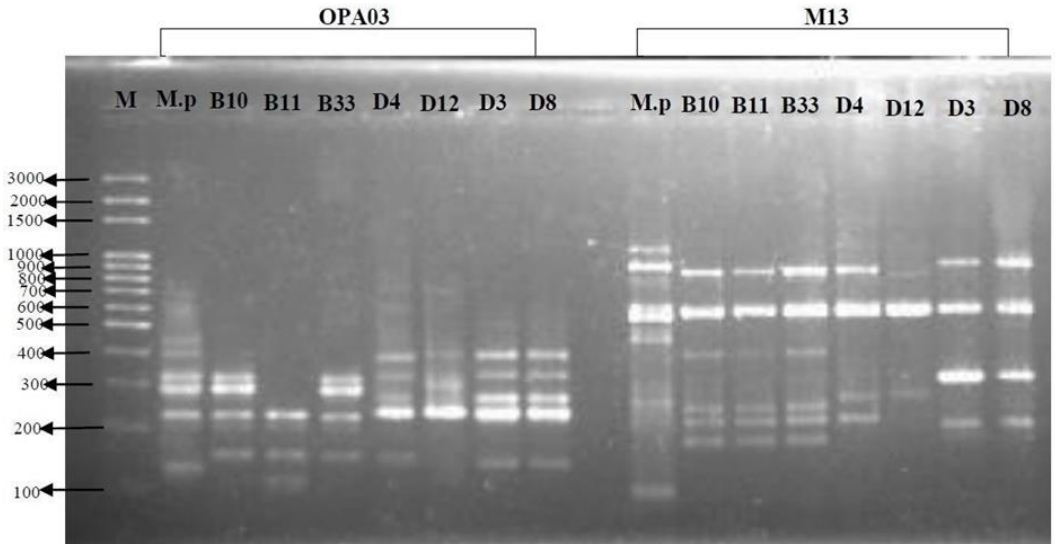


Figure 3.18. The amplification product of *M. pulcherrima* strains using OPA-03 and M13 primers

Lane M, 100-bp molecular marker; lane Mp reference strain of *M. pulcherrima* (DSMZ); lane B10 (BB 0-CM NS2), *M. pulcherrima* (ITS); lane B11 (BB 0-CM NS3), *M. pulcherrima* (ITS), *M. off. pulcherrima* (D1/D2); lane B33 (BB 4-CM NS8), *M. pulcherrima* (ITS), *M. aff. fructicola* (D1/D2); lane D4 (DA 0-CM NS4), *M. pulcherrima* (ITS); lane D12 (DA 4-CM NS4), *M. pulcherrima* (ITS); lane D3 (DA 0-CM NS3), *M. sinensis* (ITS), *M. pulcherrima* (D1/D2); lane D8 (DB 0-CM NS4), *M. chrysoperlae* (ITS), *M. pulcherrima* (D1/D2).

Microsatellite primers $(ATG)_5$ and $(GTG)_5$ both discriminated five different strains of *M. pulcherima* based on ITS sequencing among five studied strains plus one reference strain (Figure 3.19).

- Pattern I: Mp (Ref)
- Pattern Ⅱ: B10 and B33
- Pattern Ⅲ: B11
- Pattern Ⅳ: D4
- Pattern Ⅴ: D12

Based on D1/D2 domains D3 and D8 strains which were identified as *M. pulcherima*, showed a similar profile that is different from the other isolates. Therefore, primers OPA-03, $(ATG)_5$ and $(GTG)_5$ have more discriminatory power than primer M13 on *Metschnikowia pulcherrima* strains with discriminating five

strains among six. Using all four primers, two strains identified by D1/D2 domain sequencing (D3 and D8) produced a similar pattern (Table 3.8).

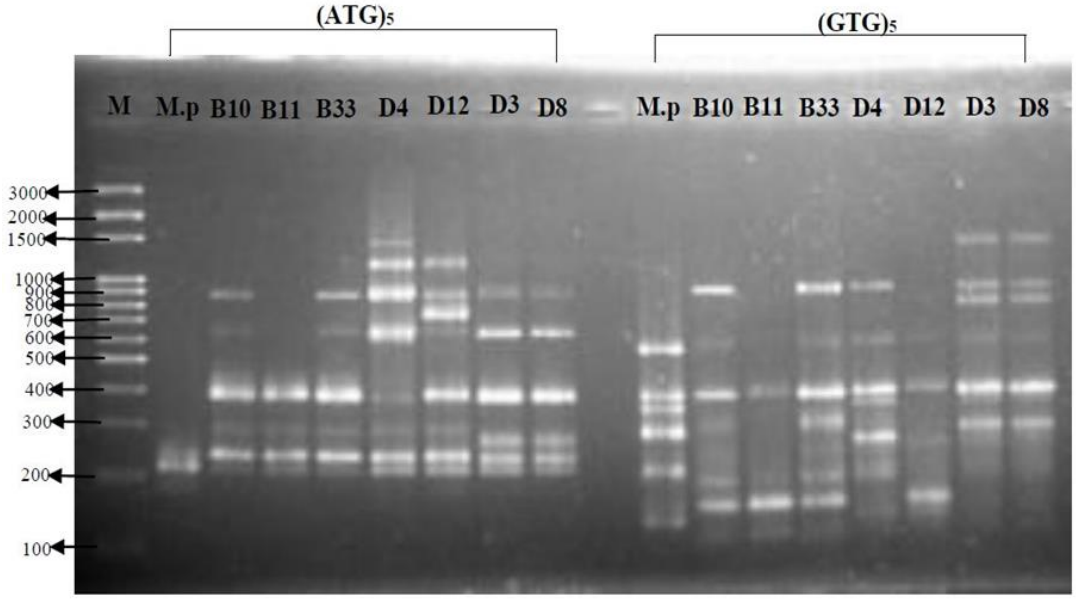


Figure 3.19. The amplification product of *M. pulcherrima* strains using $(ATG)_5$ and $(GTG)_5$ microsatellite primers

Lane M, 100-bp molecular marker; lane Mp reference strain of *M. pulcherrima* (DSMZ); lane B10 (BB 0-CM NS2), *M. pulcherrima* (ITS); lane B11 (BB 0-CM NS3), *M. pulcherrima* (ITS), *M. off. pulcherrima* (D1/D2); lane B33 (BB 4-CM NS8), *M. pulcherrima* (ITS), *M. aff. fructicola* (D1/D2); lane D4 (DA 0-CM NS4), *M. pulcherrima* (ITS); lane D12 (DA 4-CM NS4), *M. pulcherrima* (ITS); lane D3 (DA 0-CM NS3), *M. sinensis* (ITS), *M. pulcherrima* (D1/D2); lane D8 (DB 0-CM NS4), *M. chrysoperlae* (ITS), *M. pulcherrima* (D1/D2).

Table 3.8. Patterns obtained with the use of 4 primers for *M. Pulcherrima* strains

| Strain | RAPD primer | Mini and Microsatellite primers | | | Overal pattern |
|---|---|---|---|---|---|
| | OPA03 | M13 | $(ATG)_5$ | $(GTG)_5$ | |
| B10 | A | A | A | A | A |
| B11 | B | A | B | B | B |
| B33 | A | A | A | A | A |
| D4 | C | B | C | C | C |
| D12 | D | C | D | D | D |
| D3* | C | D | E | E | E |
| D8* | C | D | E | E | E |

*Identified as *M. Pulcherrima* by D1/D2 domain sequencing and *M. sinensis* (D3) and *M. chrysoperlae* (D8) by ITS sequencing.

Table 3.9. Molecular profiles of yeast species produced with the use of different primers

| Strain | Code | RAPD Primer | Microsatellite Primes | | |
|---|---|---|---|---|---|
| | | OPA03 | M13 | $(ATG)_5$ | $(GTG)_5$ |
| *L. thermotolerans* | | | | | |
| | DSMZ | 215+170+120 | 730+600+390+ 350+260+240+215 | 280+220 | 270+170 |
| | Ö6 | 700+450+400+ 270+250+215+95 | 1500+600+500+400+390 +320+280 | 750+510+400+ 350+270 | 750+500+4 50+400+32 0+260+250 |
| | Ö9 | 400+270+250+ 215+95 | 500+400+390+320+280 | 750+510+400+ 350+270 | 750+400+3 20+260+ 250 |
| | Ö16 | 400+270+250+ 215+95 | 1500+600+500+400+390 +320+280 | 750+510+400+ 350+270 | 750+400+3 20+260+ 250 |
| | Ö22 | 400+270+250+ 215+95 | 1500+600+500+400+390 +320+280 | 750+510+400+ 350+270 | 750+400+3 20+260+ 250 |
| *H. uvarum* | | | | | |
| | DSMZ | 1700+1500+1200 +700+300+280+2 20 | 1400+900+750+ 550+480+420+350+300+ 280+210 | 550+450+400+3 20+270+150 | 2500+1000 650+480+ 400+220 |
| | K3 | 1700+1500+700+ 600+280+180 | 900+550+480+420+400+ 350+300+250+180+150 | 1300+1100+900 +750+600+550 +500+400+350 +150 | 650+600+ 480+400+ 220 |
| | K13 | 1700+1500+700+ 600+280 | 900+550+400+300+180+ 150 | 550+400+350+2 70+150 | 850+650+6 00+480+40 0+220 |
| | K25 | 280+180 | 1400+900+750+700+ 550+400+280+250+180+ 150 | 550+400+350+1 50 | 400+220 |
| | K26 | 1700+1500+900+ 700+600+550+49 0+450+400+350+ 280+160 | 1400+900+750+700+ 550+400+280+250+180+ 150 | 1300+1100+900 +750+550+500 +400+350+150 | 1000+850+ 650+600+4 80+400+22 0 |
| | K52 | 1700+1500+700+ 600+400+280+ 180 | 900+750+650+550+380+ 300+280+250+180+150 | 1300+1100+900 +750+600+550 +500+400+350 +150 | 1000+850+ 650+600+4 80+400+ 220 |
| | K54 | 1700+1500+1200 +900+700+550+ 500+400+280 | 2000+1400+900+750+550 +400+380+350+300+280 +180+150 | 1300+1100+900 +750+550+500 +400+350+150 | 1000+850+ 650+600+4 80+400+22 0 |
| | K70 | 1400+1000+900+ 400+200+160+ 100 | 550+400+280+180+150 | 700+350+270+2 50 | 480+400+3 00+220 |
| | E11 | 700+420+300+ 280 | 2000+1000+900+750+550 +400+380+280+180+150 | 1200+950+800+ 550+500+400+3 50+270+150 | 1000+850+ 650+600+4 80+400+22 0 |
| | E12 | 1700+1500+1200 +700+500+280 | 1300+1100+900+800+750 +600+550+480+380+350 +280+210+180+150 | 1600+1500+110 0+550+500+400 +350+300+150 | 2500+1000 +600+500+ 450+400+2 20+200+ 180 |
| | E13 | 1700+1500+1200 +700+500+280 | 2000+1000+900+750+550 +400+380+350+280+190 | 1600+1500+110 0+800+600+550 +500+400+350 +300+150 | 2500+1000 +850+650+ 600+480+4 00+220 |

Table 3.9 (continued)

| | | | | | |
|---|---|---|---|---|---|
| | E14 | 700+420+300+280 | 2000+1000+900+750+550+500+400+380+280 | 1200+950+550+500+400+350+270+150 | 1000+850+650+600+480+400+220 |
| *H. opuntiae* | | | | | |
| | Ö5 | 900+800+700+590+420+380+250+180 | 850+750+600+400+350+300+220+150 | 950+700+650+530+410+320+280+240 | 850+650+600+450+400+320+290+200 |
| | Ö7 | 900+800+590+420+380+250+180 | 850+750+600+400+350+300+220 | 1700+1400+950++700+650+320+280+240 | 1100+850+650+600+450+400+320+290+200 |
| | K2 | 420+380+250+180 | 850+750+600+400+320 | 700+650+320+280+240+150 | 850+650+600+450+400+320+290+200 |
| | K7 | 900+800+780+700+590+550+420+380+250+180 | 750+650+600+400+320+280+200+150 | 950+700+650+450+320+280 | 650+400+320+290+200 |
| | K10 | 900+800+780+700+590+550+420+380+250+180 | 750+650+600+400+320+280+200+150 | 950+700+650+450+320+280 | 650+400+320+290+200 |
| | K12 | 900+800+780+700+590+550+420+380+250+180 | 850+750+650+600+400+280+200+150 | 950+700+650+450+280 | 650+450+400+320+290+200 |
| | K15 | 900+800+780+700+590+550+420+380+300+250+180 | 750+500+400+320+200+150 | 950+700+650+450+280 | 650+450+400+320+290+200 |
| | K16 | 900+800+780+700+590+550+420+380+300+250+180 | 750+500+400+320+200+150 | 950+700+650+450+280 | 650+450+400+320+290+200 |
| | K17 | 900+800+780+700+590+550+420+380+250+180 | 850+750+650+600+400+280+200+150 | 950+700+650+450+280 | 650+400+320+290+200 |
| | K19 | 900+800+780+700+590+550+420+380+250+180 | 850+750+650+600+400+280+200+150 | 950+700+650+450+280 | 650+400+320+290+200 |
| | K20 | 900+800+780+700+590+550+420+380+300+250+180 | 750+500+400+320++200+150 | 950+700+650+450+320+280 | 650+400+320+290+200 |
| | K21 | 1100+1000+780+590+550+420+380+250+180 | 1300+750+500+420+300+200 | 2000+1400+650+410 | 1400+850+750+650+600+500+450+400+290+220+180 |
| | K27 | 900+800+780+700+590+550+420+380+300+250+180 | 750+650+600+400+280+200+150 | 1400+950+700+650+450+380+320+280+240 | 1200+850+650+600+450+400+320+290+200 |
| | K32 | 900+800+780+700+590+550+420+380+250+180 | 1300+850+750+650+600+420+400+280+200+150 | 1400+950+700+650+450+380+320+280+240 | 1200+850+650+600+450+400+320+290+200 |
| | K34 | 900+800+780+700+590+550+420+380+250+180 | 1300+850+750+650+600+420+400+280+200+150 | 1400+950+700+650+450+380+320+280+240 | 1200+850+650+600+450+400+320+290+200 |

Table 3.9 (continued)

| | | | | | |
|---|---|---|---|---|---|
| | K35 | 900+800+780+700+590+550+420+380+300+250+180 | 1300+850+750+650+600+420+400+280+200+150 | 1400+950+700+650+450+380+320+280+240 | 1200+850+650+600+450+400+320+290+200 |
| | K37 | 900+800+780+700+590+550+420+380+300+250+180 | 750+600+420+400+320 | 1400+950+700+650+450+380+320+280+240 | 1200+850+650+600+450+400+320+290+200 |
| | K38 | 900+800+780+700+590+550+420+380+250+180 | 1300+850+750+650+600+420+400+280+200+150 | 1400+950+700+650+450+380+320+280+240 | 1200+850+650+600+450+400+320+290+200 |
| | K40 | 900+800+780+700+590+550+420+380+250+180 | 750+600+420+400+320 | 700+650+450+280+240 | 1200+850+750+650+600+420+400+320+290+200 |
| | K51 | 900+800+780+700+590+550+420+380+250+180 | 750+650+600+400+280+200+150 | 1400+950+700+650+450+380+320+280+240 | 1200+850+650+600+450+400+320+290+200 |
| | K60 | 900+800+780+700+590+550+420+380+250+180 | 1300+850+750+650+600+420+400+280+200+150 | 1400+950+700+650+450+380+320+280+240 | 1200+850+650+600+450+400+320+290+200 |
| *Metschnikowia spp.* | | | | | |
| *M. pulcherrima* | DSMZ | 450+400+310+280+220+200+130 | 1100+900+800+600+590+450+250+100 | 250+220+180 | 550+400+350+280+250+200+120 |
| *M. pulcherrima* | B10 | 310+280+220+200+150 | 850+600+400+220+210+180 | 900+650+400+300+250+220 | 900+550+400+300+180+150 |
| *M. pulcherrima* | B11* | 220+200+150+120 | 850+600+400+220+210+180 | 400+300+250+220 | 400+150+110 |
| *M. pulcherrima* | B33* | 310+280+220+200+150 | 850+600+400+220+210+180 | 900+650+400+300+250+220 | 900+550+400+300+180+150 |
| *M. pulcherrima* | D4 | 400+320+270+220+150 | 850+600+270+210 | 1500+1200+900+650+400+300+250+220 | 900+550+400+350+270+180+130 |
| *M. pulcherrima* | D12 | 400+290+220 | 850+600+270 | 1200+900+700+650+400+300+250+220 | 550+400+270+150 |
| *M. sinensis* | D3* | 400+320+270+220+150 | 900+600+300+210 | 900+650+400+270+250+200 | 1500+900+800+550+400+300 |
| *M. chrysoperlae* | D8* | 400+320+270+220+150 | 900+600+300+210 | 900+650+400+270+250+200 | 1500+900+800+550+400+300 |

DSMZ; German collection of microorganisms and cell cultures GmbH (Germany); * Identified as different strains based on D1/D2 domain sequencing: B11, *Metschnikowia aff. pulcherrima*; B33, *Metschnikowia aff. fructicola*; D3, *Metschnikowia pulcherrima*; D8, *Metschnikowia pulcherrima.*

### 3.2.1 The Similarity of Strains on UPGMA Dendrogram

The results of the similarity calculation between combined fingerprints using the DICE coefficient are described in this section. According to Mateus et al. (2020), when the similarity between strains of the same species is more than 80%, they are considered as the same strain.

Figure 3.20 shows the dendrogram drived after cluster analysis of *L. thermotolerans* in which two distinct groups could be recognized. Group I comprised a single strain Lt (Reference strain) differed from group Ⅱ by a similarity value of 36%. In group Ⅱ, the strains O9, O6, O16 and O22 were similar in the range of more than 91%. A 100% similarity was recognized between strains O16 and O22.

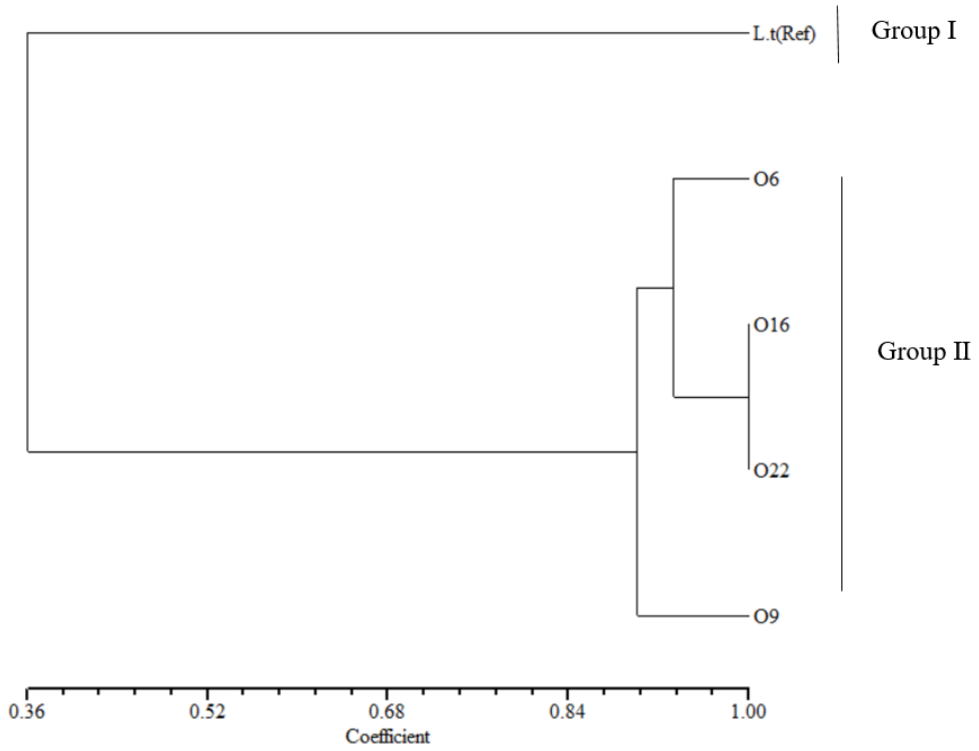


Figure 3.20. UPGMA cluster analysis of five *L. thermotolerans* strains
Lt (Ref) is *L. thermotolerans* reference strain purchased from German collection of microorganisms and cell cultures GmbH (Germany)

UPGMA clustering of *H. uvarum* strains is depicted in Figure 3.21. The 12 studied isolates segregated into seven groups. The strains K25 and K70 were found to be the most distantly related to the other six groups branching at a similarity level of 61%. The two mentioned strains also segregated into two different groups at a similarity level of 76%. The type strain of the species Hu (Ref), clustered at the similarity level of 68.7% (Group I). Group Ⅱ formed by four strains, K3, K52, K54 and K26 differed from group Ⅲ (K13) at the similarity level of 76.5%. Group Ⅳ comprised two strains E12 and E13 that clustered at 74.8% from group Ⅱ and group Ⅲ. Isolates E11 and E14 (group Ⅴ) clustered at a similarity level of 72.2%. Group Ⅵ comprised a single strain K25 with 76% of similarity. Group Ⅶ also formed by a single strain K70 branching at a similarity value of 76%. A 100% similarity could be observed between E11 and E14 strains.

Variability between strains of *H. uvarum* was observed and was high at the primary stages of AF that is in agreement with a study reported by Mateus et al (2020). In that study, they could discriminate seventeen groups out of forty studied isolates from one grape variety, Touriga Franca.

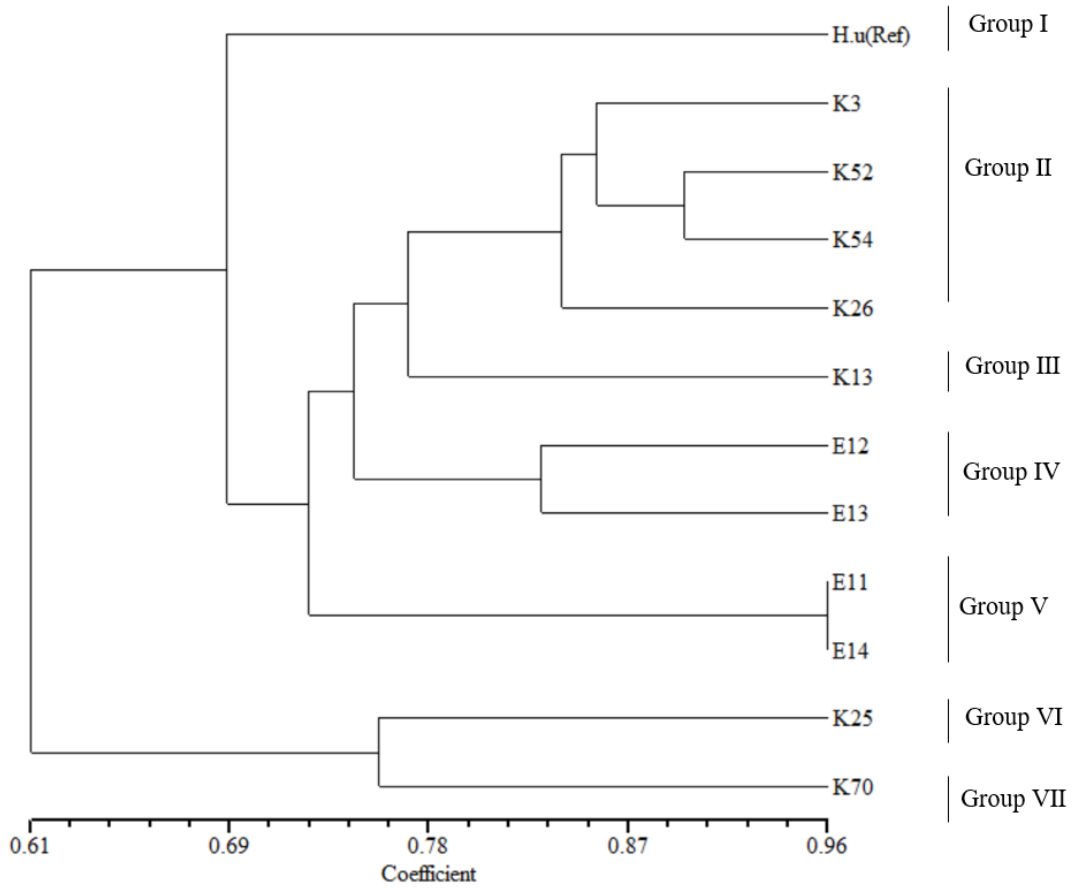


Figure 3.21. UPGMA cluster analysis of five *H. uvarum* strains
Hu (Ref) is *H. uvarum* reference strain purchased from German collection of microorganisms and cell cultures GmbH (Germany)

The similarity coefficient of twenty-one *H. opuntiae* strains is illustrated in the UPGMA dendrogram in Figure 3.22. Four major groups could be observed. Two strains (O5 and O7) isolated from Öküzgözü grape type clustered in Group I branching at 79.8% of similarity from Group Ⅱ. Group Ⅱ comprised seventeen isolates. The strain K2 differed from group I and Ⅱ by 78.5% of similarity. Group Ⅳ formed by a single strain K21 clustered at a similarity value of 67%.

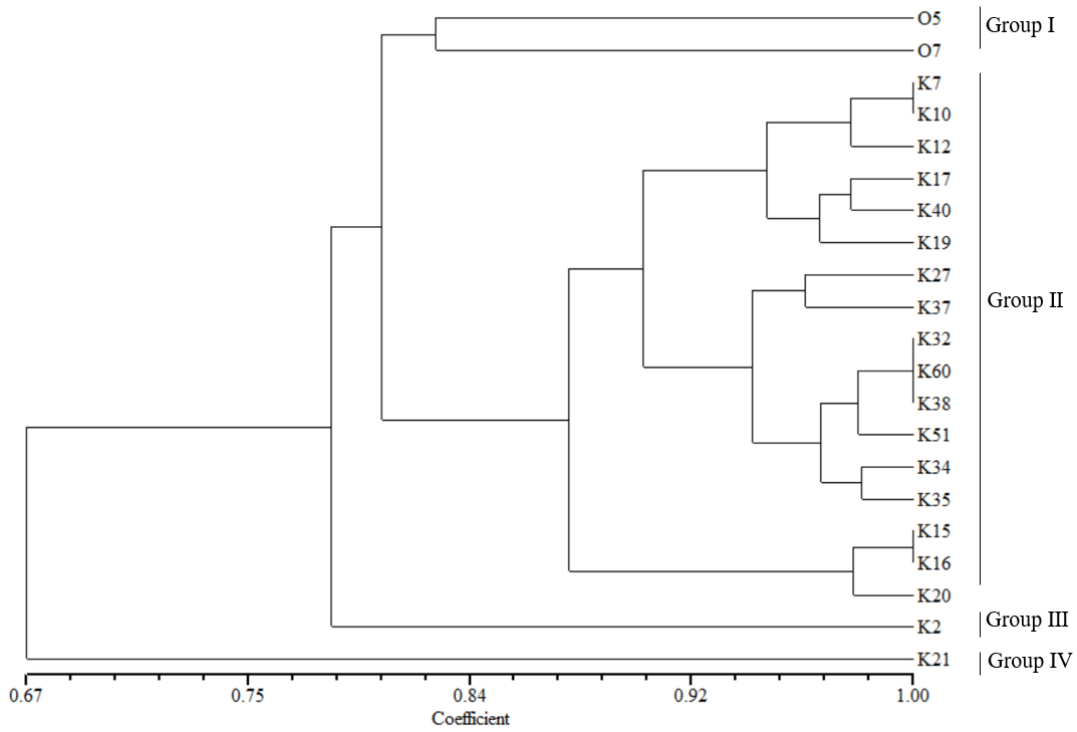


Figure 3.22. UPGMA cluster analysis of five *H. opuntiae* strains

UPGAMA cluster analysis of *M. pulcherima* divided strains of this species into six groups (Figure 3.23). The type strain Mp (Ref) differed from five other groups by 50% of similarity. Group Ⅱ comprised two strains B10 and B33 differed from group Ⅲ by 74% of similarity. Group Ⅳ formed by a single strain D4 that differed from group Ⅴ (D3 and D8) by 78.6% of similarity. Finally, group Ⅵ that comprised one strain D12, separated from groups Ⅱ, Ⅲ, Ⅳ and Ⅴ at a similarity value of 62%. A 100% similarity level observed between D3 and D8 strains.

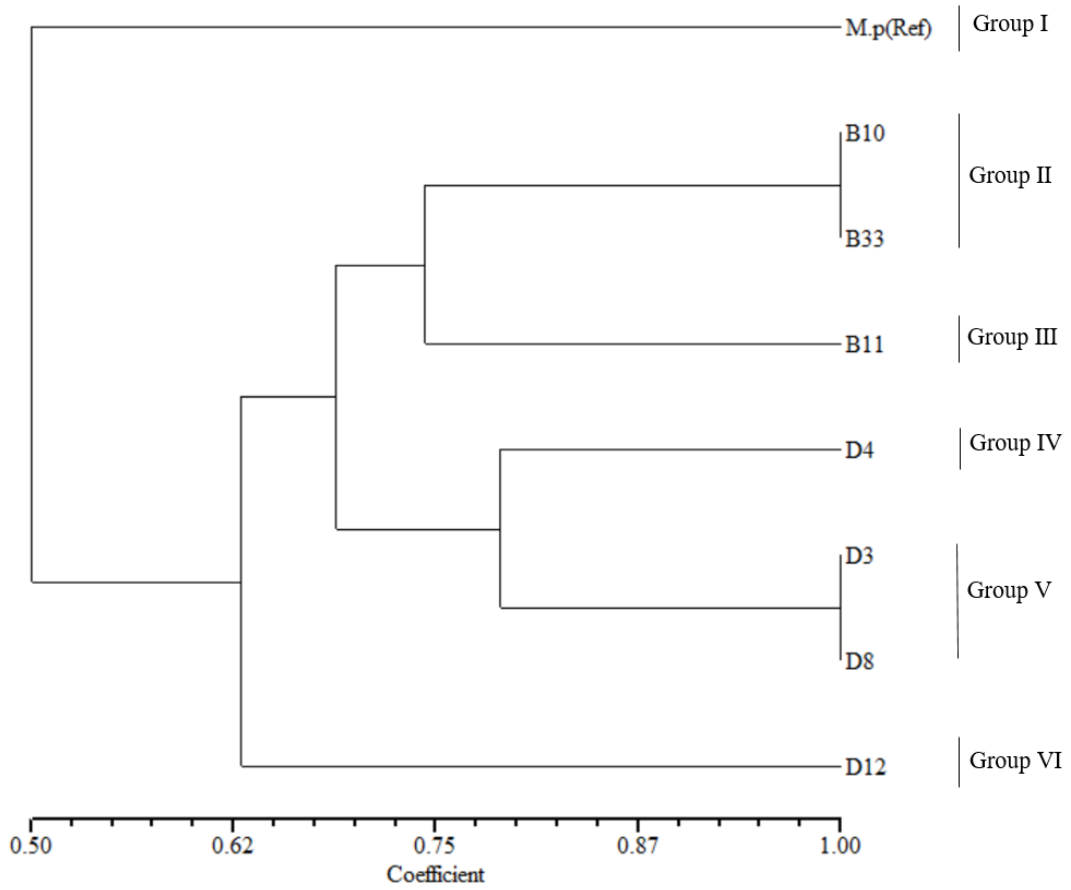


Figure 3.23. UPGMA cluster analysis of five *M. pulcherima* strains
Mp (Ref) is *M. pulcherima* reference strain purchased from German collection of microorganisms and cell cultures GmbH (Germany)

### 3.2.2 Correlation Between Biochemical Properties and Genotypic Results

Aktuna. (2019) studied the biochemical properties of all the isolates examined in this study. The biochemical tests that were done by Aktuna. (2019) were ethanol tolerance test, sulfur dioxid resistance test, hydrogen sulfide production test. The table 3.10, 3.11, 3.12 and 3.13 show the results of the biochemical examinations.

As it can be observed from the Table 3.10, O5 and O7 that were isolated from the öküzgözü grape type showed a different alcohol tolerance in comparison to other isolates from Kalecik karası. They tolerated to 10, 13, and 15% of alcohol. These

two isolates showed a different pattern in the genotyping analysis as well. They differed from other isolates by an 80 % similarity level.

K2 that was separated at 78.5% after cluster analysis, showed almost the same biochemical properties as isolates of group Ⅱ.

Strain K21 that showed a different pattern in RAPD-PCR analysis and separated from other isolates at 67% similarity, indicated some different biochemical traits as well. As it can be seen from the Table 3.10, this isolate could tolerate to 10% alcohol and could not grow at 200 mg/l $SO_2$. Also, it produced $H_2S$ less than other studied isolates (2).

Table 3.10. The results of biochemical tests for *H. opuntiae*

| Species | | Alcohol Tolerance (%) | | | SO2 Tolerance (mg/l) | | | | H2S production |
|---|---|---|---|---|---|---|---|---|---|
| *H. opuntiae* | | 10 | 13 | 15 | 50 | 100 | 150 | 200 | |
| Group I | | | | | | | | | |
| | O5 | vw | vw | vw | + | + | + | + | 3 |
| | O7 | + | + | + | + | + | + | + | 4 |
| Group Ⅱ | | | | | | | | | |
| | K7 | | | | | | | | 4 |
| | K10 | vw | vw | vw | + | + | + | + | 3 |
| | K12 | - | - | - | + | + | + | + | 3 |
| | K17 | - | - | - | + | + | + | + | 3 |
| | K40 | + | - | - | + | + | + | + | 4 |
| | K19 | - | - | - | + | + | + | + | 3 |
| | K27 | | | | | | | | 4 |
| | K37 | | | | | | | | 4 |
| | K32 | - | - | - | + | + | + | + | 5 |
| | K60 | | | | | | | | 4 |
| | K38 | | | | | | | | 4 |
| | K51 | - | - | - | + | + | + | + | 4 |
| | K34 | w | | | + | + | + | + | 5 |
| | K35 | | | | | | | | 5 |
| | K15 | w | - | - | + | + | + | + | ng |
| | K16 | | | | | | | | ng |
| | K20 | + | - | - | + | + | + | + | 4 |
| Group Ⅲ | | | | | | | | | |
| | K2 | - | - | - | + | + | + | + | 3 |
| Group Ⅳ | | | | | | | | | |
| | K21 | + | - | - | + | + | + | - | 2 |

+positive growth, w weak growth, - negative growth; 1: white, 2: cream, 3: light brown, 4: brown, 5: dark brown, 6: black; ng: not grown

*H. uvarum* isolates were clustered into seven groups after UPGMA analysis. As it can be seen from the Table 3.11, K25 isolate in group Ⅵ (61% similarity) shows a

high level of $H_2S$ production (6) in comparison with the other isolates belonging to the other groups.

K70 was separated at 61% similarity and clustered in group Ⅶ. From the table above, it was seen that this isolate could not tolerate 200 mg/l of $SO_2$ whereas the other isolates were able to grow at this level of $SO_2$.

Table 3.11. The results of biochemical tests for *H. uvarum*

| Species | | Alcohol Tolerance (%) | | | SO2 Tolerance (mg/l) | | | | H2S production |
|---|---|---|---|---|---|---|---|---|---|
| *H. uvarum* | | 10 | 13 | 15 | 50 | 100 | 150 | 200 | |
| Group I | | | | | | | | | |
| | Hu | | | | | | | | |
| Group Ⅱ | | | | | | | | | |
| | K3 | - | - | - | + | + | + | + | 5 |
| | K52 | - | - | - | + | + | + | + | 3 |
| | K54 | w | | | + | + | + | + | 5 |
| | K26 | + | w | - | + | + | + | + | 5 |
| Group Ⅲ | | | | | | | | | |
| | K13 | | | | | | | | ng |
| Group Ⅳ | | | | | | | | | |
| | E12 | | | | | | | | 4 |
| | E13 | + | vw | - | + | + | + | + | 3 |
| Group Ⅴ | | | | | | | | | |
| | E11 | - | - | - | + | + | + | + | 3 |
| | E14 | vw | vw | vw | + | + | + | + | 3 |
| Group Ⅵ | | | | | | | | | |
| | K25 | w | | | + | + | + | + | 6 |
| Group Ⅶ | | | | | | | | | |
| | K70 | + | - | - | + | + | + | - | ng |

+positive growth, w weak growth, - negative growth; 1: white, 2: cream, 3: light brown, 4: brown, 5: dark brown, 6: black; ng: not grown

Although *M. pulcherima* isolates showed different patterns in RAPD analysis and they were clustered into six groups, they showed almost the same biochemical results (Table 3.12).

Table 3.12. The results of biochemical tests for *M. pulcherima*

| Species | | Alcohol Tolerance (%) | | | SO2 Tolerance (mg/l) | | | | H2S production |
|---|---|---|---|---|---|---|---|---|---|
| *M. pulcherima* | | 10 | 13 | 15 | 50 | 100 | 150 | 200 | |
| Group I | | | | | | | | | |
| | Mp | | | | | | | | |
| Group Ⅱ | | | | | | | | | |
| | B10 | w | w | - | + | + | + | + | 3 |
| | B33 | - | - | - | + | + | + | + | 3 |
| Group Ⅲ | | | | | | | | | |
| | B11 | | | | | | | | 5 |
| Group Ⅳ | | | | | | | | | |
| | D4 | | | | | | | | 4 |
| Group Ⅴ | | | | | | | | | |
| | D3 | - | - | - | + | + | + | + | 3 |
| | D8 | | | | | | | | 4 |
| Group Ⅵ | | | | | | | | | |
| | D12 | - | - | - | + | + | + | + | 3 |

+positive growth, w weak growth, - negative growth; 1: white, 2: cream, 3: light brown, 4: brown, 5: dark brown, 6: black; ng: not grown

All the biochemical tests were not done for *L. thermotolerans* isolates (Table 3.13)

Table 3.13. The results of biochemical tests for *L. thermotolerans*

| Species | | Alcohol Tolerance (%) | | | SO2 Tolerance (mg/l) | | | | H2S production |
|---|---|---|---|---|---|---|---|---|---|
| *L. thermotolerans* | | 10 | 13 | 15 | 50 | 100 | 150 | 200 | |
| Group I | | | | | | | | | |
| | Lt | | | | | | | | |
| Group II | | | | | | | | | |
| | O6 | | | | | | | | 4 |
| | O9 | - | - | - | + | + | + | + | 3 |
| | O16 | | | | | | | | 5 |
| | O22 | | | | | | | | 5 |

+positive growth, w weak growth, - negative growth; 1: white, 2: cream, 3: light brown, 4: brown, 5: dark brown, 6: black; ng: not grown

# CHAPTER 4

# DISCUSSIONS

In this study, the composition of wild yeast populations, isolated during spontaneous fermentation of musts from five wine-producing areas of Turkey (öküzgözü, Kalecik karası, Boğazkere, Dimrit, Emir) were studied using RFLP technique. This method proved to be reliable and useful for the rapid identification of yeast populations at the species level. Furthermore, in order to investigate the influence of different grape-growing areas on the distribution of wild wine yeast strains, RAPD-PCR was performed using four different primers. Determination of composition and investigation of the biodiversity of wild yeast strains would help to control winemaking practice and improve the final quality of the wine. From this point of view, Pramateftaki et al. (2000), evaluated the biochemical and organoleptic potential of *S. cerevisiae* after typing the strains with molecular methods. Those strains characterized with desirable oenological properties were patented to use as dry active yeast starters in the wine-making industry (Pramateftaki et al., 2000). Šuranská et al. (2016) also studied the biodiversity of the *S. cerevisiae* strains and selected some indigenous strains for using them as starter cultures as they proved promising oenological characteristics (Šuranská et al., 2016). In the last decade, the design of new species/strains of wild non-*Saccharomyces* yeasts for the selection of new starter cultures to control alcoholic fermentation has been attracting increasing attention. Moreover, study the intraspecific diversity of non-*Saccharomyces* yeasts associated with wine is a subject that more recently has been highlighted. For this purpose number of molecular techniques such as RAPD-PCR and microsatellite genotyping were examined and showed to be useful for study the biodiversity of oenological yeasts (Roudil et al., 2019).

In our study, a total of eighteen non-*Saccharomyces* isolates were subjected to PCR-RFLP analysis, including four reference yeasts (DSM's *Metschnikowia Pulcherrima, Hanseniaspora uvarum, Lachancea thermotolerans,* and *Candida spp.)* by using five different restriction enzymes *Hae*III, *Hha*I, *Hinf*I, *Dde*I and *Dra*I. PCR products of the 5.8S ITS region was different in size ranging from 390 bp to 750 bp (Table 3.2). After digestion by enzymes, the yielded banding patterns could distinguish *Metschnikowia* genus and eight species of *Hanseniaspora uvarum, Hanseniaspora guilliermondii, Hanseniaspora opuntiae, Lachancea thermotolerans, Starmerella bacillaris, Rhodotorula mucilaginosa, Wickerhamomyces anomalus, Solicoccozyma aeria.* These results were in agreement with the result of the sequencing of the 5.8S ITS rRNA, which was done in the previous study.

*Dde*I enzyme could distinguish *Hanseniaspora uvarum* (300+180+95) from *H. guilliermondii* (360+180+100) and *H. opuntiae* (360+180+100). As *H. guilliermondii* and *H. opuntiae* are closely related organisms, they did not differentiate by *Dde*I. However, another enzyme, *Dra*I generated different RFLP patterns for these two organisms.

None of the enzymes used in this study could differentiate five *Metschnikowia* at the species level, although they discriminated *Metschnikowia* genus from the other genera. So further molecular conformation is needed.

Totally forty-six non-*Saccharomyces* yeasts belonging to four different species (*L. thermotolerans, H. uvarum, H. opuntiae* and *M. pulcherrima*) analyzed by RAPD-PCR technique to investigate the biodiversity between the isolates of each species. RAPD and microsatellite primers were able to determine the biodiversity between yeast strains isolated from grapes of different vineyards and even the same vineyard. The ability of primers to distinguish different strains was species-specific. UPGMA dendrograms were generated through a combination of band patterns produced by all the primers used in RAPD assay and applied for estimating the similarity of the strains by the Dice coefficient.

For *Lachancea thermotolerans* strains, the least discriminating primer was $(ATG)_5$ with producing no unique profile. The other three primers differentiated either O6 or O9 from other strains. M13 primer discriminatory power was studied by González-Arenzana et al. (2017**)** for differentiation between *L. thermotolerans* species. After similarity calculation, the results showed a 91% similarity between *L. thermotolerans* strains dividing them into two main groups. Four isolates of *L. thermotolerans* (O6, O9, O16, O22) were classified into one group and Lt reference strain was classified into another distinct group. Therefore, all four isolates of öküzgözü grape type are belonging to the same strain.

The best primer for differentiation of *H. uvarum* strains was M13 that was able to produce eleven different patterns, and $(GTG)_5$ had the least discriminating power with producing eight unique patterns. $(ATG)_5$, $(GTG)_5$, OPA-03, and M13 primers were used by Cadez et al. (2002) for the cluster analysis of *Hanseniaspora (Kloeckera)* strains. Bujdosó et al. (2001b) also reported that RAPD primers OPA-03 and M13 were appropriate for discriminating *H. uvarumn* isolated from wine at the strain level. However, the PCR results generated in their study were different from the results gained in our work. For example, the range of PCR products using OPA-03 was between 676-2645 bp in the study done by Bujdosó et al. (2001b) and it was between 180-1700 bp in our work. The same result was gained by using M13 primer. Bujdosó et al. (2001b) reported the PCR products ranging between 396-2645 bp and it was between 150 to 2000 in our work. Cluster analysis of *H. uvarum* using UPGAMA method, by considering more than 80% similarity as the same strain, showed seven distinct strains among twelve studied strains. This indicated a wide diversity between *H. uvarum* strains either among those isolated from the same grape type and different grape types. From this point of view, five strains were classified among seven isolates from Kalecik Karası grape variety and two strains were classified among four isolated from Emir grape variety.

There are no studies regarding the differentiation between *Hanseniaspora opuntiae* species in the literature. However, in our research, by using the four enzymes, the

clonal characterization of *H. opuntiae* strains showed four clusters after UPGAMA analysis. Among nineteen isolates from Kalecik Karası grape type, three clusters were identified. Two isolated from Öküzgözü grape variety clustered in one group.

M13 and OPA-03 primers could produce five patterns of eight *M. pulcherrima* studied strains. $(ATG)_5$ and $(GTG)_5$ primers separated six of eight *M. pulcherrima* strains. Using M13 primer for discriminating *M. pulcherrima* was reported by González-Arenzana et al. (2017). However, cluster analysis with UPGAMA method showed six different groups in which Mp reference strain was in one group individually. Three isolates from Boğazkere grape variety are classified into two distinct groups and four isolates from Dimrit grape type classified into three different groups.

In a study done by Capece et al. (2003), *M. pulcherrima* strains isolated from wine were subjected to RAPD using $(GTG)_5$ primer. The PCR amplification resulted in producing six bands (1150+860+760+600+460+330) which were similar to the reference strain that they purchased from Centraalbureau voor Schimmelcultures (*M. pulcherrima* (CBS5833)). In our work using the same primer, D3 and D8 isolates almost showed the same profile (1500+900+800+550+400+300). The related groups of species *H. opuntiae* that clustered at diferent similarity levels after UPGMA analysis, showed similar biochemical properties. However, *Metschnikowia spps* did not show any correlation. Two strains of *H. uvarum* (K25 and K70) that were clustered in different groups showed nearly different biochemical properties as well.

# CHAPTER 5

# CONCLUSION

In this study, non-*Saccharomyces* yeasts isolated from wine made of 5 grape types in Turkey were analyzed to evaluate the diversity among them based on two molecular methods, RAPD-PCR and ITS-RFLP. All of the studied species were identified before by the ITS sequencing method and some of them by sequencing the D1/D2 domains, which gave controversial results in the case of *Metschnikowia* genus.

Analysis of the 5.8S-ITS-RFLP of fourteen isolates by five restriction enzymes (*Hha*I (*Cfo*I*), Hae*III, *Hinf*I, *Dde*I, *Dra*I) generated different patterns belonging to different genera. Species belonging to *Metschnikowia* genus were not differentiated with the enzymes used in this study. However, *Dde*I and *Dra*I enzymes in two steps could differentiate *H. uvarum, H. opuntiae* and *H. guilliermondii.* These results are in agreement with the sequence analysis of the 5.8S-ITS that was performed previously.

In addition, RAPD analysis of four species was performed using different RAPD, mini- and microsatellite primers, which could discriminate isolates at the species level and were able to determine the biodiversity between yeast strains isolated from a grape of different vineyards and even the same vineyard.

The discriminatory power of primers depends on specific species. For example, $(ATG)_5$ primer did not produce any distinct pattern between four strains of *L. thermotolerans.* However, the other three enzymes were able to generate different patterns by which either O6 or O9 could be differentiated from other strains. $(GTG)_5$ primer produced eight different patterns for twelve *H. uvarum* strains. However, M13 primer could produce eleven distinct patterns for twelve *H. uvarum* isolates. M13 primer had the most discriminatory power for *H. opuntiae* isolates as

it generated ten different patterns for twentyone isolates. In contrast, OPA-03 had the least differentiating ability with producing six distinct patterns. $(ATG)_5$ and $(GTG)_5$ had the best discriminatory power for *Metschnikowia pulcherrima* strains. Seven studied isolates identified as *Metschnikowia pulcherrima* by ITS sequencing could be differentiated into five groups using the two mentioned primers. Finally, cluster analysis of isolates was performed using a combination of band patterns produced by all the primers used in RAPD assay. The similarity of the strains was estimated by the Dice coefficient and strains classified into different groups. In conclusion, molecular techniques used in this study showed to be easy, fast and accurate for investigation of yeast populations at species and strain level.

## REFERENCES


Abliz, P., Fukushima, K., Takizawa, K., & Nishimura, K. (2004). Identification of pathogenic dematiaceous fungi and related taxa based on large subunit ribosomal DNA D1/D2 domain sequence analysis. *FEMS Immunology and Medical Microbiology*, *40*(1), 41–49. https://doi.org/10.1016/S0928-8244(03)00275-X

Aktuna, İ. (2019). *Isolation, Identification and Characterization of non-Saccharomyces and Saccharomyces Yeasts in Traditional Wines Made from Five Different National Grapes in Turkey*. Middle East Technical University, Ankara, Turkey.

Alves, M., Gonçalves, T., & Quintas, C. (2012). Microbial quality and yeast population dynamics in cracked green table olives' fermentations. *Food Control*, *23*(2), 363–368. https://doi.org/10.1016/j.foodcont.2011.07.033

Arroyo-López, F. N., Durán-Quintana, M. C., Ruiz-Barba, J. L., Querol, A., & Garrido-Fernández, A. (2006). Use of molecular methods for the identification of yeast associated with table olives. *Food Microbiology*, *23*(8), 791–796. https://doi.org/10.1016/j.fm.2006.02.008

B. Esteve-Zarzoso, lt2 C. Belloch, l F. U. and A. Q. (1999). Identification of yeasts by RFLP analysis of the 5.85 rRNA gene and the two ribosomal internal transcribed spacers. *International Journal of Systematic Bacteriology*, *49*, 329–337.

Banilas, G., Sgouros, G., & Nisiotou, A. (2016). Development of microsatellite markers for Lachancea thermotolerans typing and population structure of wine-associated isolates. *Microbiological Research*, *193*, 1–10. https://doi.org/10.1016/j.micres.2016.08.010

Bautista-Gallego, J., Rodríguez-Gómez, F., Barrio, E., Querol, A., Garrido-Fernández, A., & Arroyo-Lopez, F. N. (2011). Exploring the yeast biodiversity of green table olive industrial fermentations for technological applications. *International Journal of Food Microbiology*, *147*(2), 89–96. https://doi.org/10.1016/j.ijfoodmicro.2011.03.013

Bernardi, T. L., De Melo Pereira, G. V., Cardoso, P. G., Dias, E. S., & Schwan, R. F. (2008). Saccharomyces cerevisiae strains associated with the production of cachaça: Identification and characterization by traditional and molecular methods (PCR, PFGE and mtDNA-RFLP). *World Journal of Microbiology and Biotechnology*, *24*(11), 2705–2712. https://doi.org/10.1007/s11274-008-9799-y

Bokulich, N. A., Thorngate, J. H., Richardson, P. M., & Mills, D. A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(1), 139–148. https://doi.org/10.1073/pnas.1317377110

Bujdosó, G., Egli, C. M., & Henick-Kling, T. (2001). Inter- and Intra-Specific Differentiation of Natural Wine Strains of Hanseniaspora (Kloeckera) by Physiological and Molecular Methods. *Food Technology and Biotechnology*, *39*(1), 19–28.

Capece, A., Salzano, G., & Romano, P. (2003). Molecular typing techniques as a tool to differentiate non-Saccharomyces wine species. *International Journal of Food Microbiology*, *84*(1), 33–39. https://doi.org/10.1016/S0168-1605(02)00392-6

Capozzi, V., Russo, P., Beneduce, L., Weidmann, S., Grieco, F., Guzzo, J., & Spano, G. (2010). Technological properties of Oenococcus oeni strains isolated from typical southern Italian wines. *Letters in Applied Microbiology*, *50*(3), 327–334. https://doi.org/10.1111/j.1472-765X.2010.02795.x

Capozzi, Vittorio, Garofalo, C., Chiriatti, M. A., Grieco, F., & Spano, G. (2015). Microbial terroir and food innovation: The case of yeast biodiversity in wine. *Microbiological Research*, *181*, 75–83. https://doi.org/10.1016/j.micres.2015.10.005

Capozzi, Vittorio, Russo, P., Ladero, V., Fernández, M., Fiocco, D., Alvarez, M. A., … Spano, G. (2012). Biogenic amines degradation by Lactobacillus plantarum: Toward a potential application in wine. *Frontiers in Microbiology*, *3*(APR), 1–6. https://doi.org/10.3389/fmicb.2012.00122

Capozzi, Vittorio, Russo, P., Lamontanara, A., Orrù, L., Cattivelli, L., & Spano, G. (2014). Genome sequences of five Oenococcus oeni strains isolated from Nero di Troia wine from the same terroir in Apulia, southern Italy. *Genome Announcements*, *2*(5), 1–2. https://doi.org/10.1128/genomeA.01077-14

Cappello, M. S., Stefani, D., Grieco, F., Logrieco, A., & Zapparoli, G. (2008). Genotyping by Amplified Fragment Length Polymorphism and malate metabolism performances of indigenous Oenococcus oeni strains isolated from Primitivo wine. *International Journal of Food Microbiology*, *127*(3), 241–245. https://doi.org/10.1016/j.ijfoodmicro.2008.07.009

Christine, L. J., Marc, L., Catherine, D., Claude, E., Jean-Luc, L., Michel, A., & Isabelle, M. P. (2007). Characterization of natural hybrids of Saccharomyces cerevisiae and Saccharomyces bayanus var. uvarum. *FEMS Yeast Research*, *7*, 540–549. https://doi.org/10.1111/j.1567-1364.2007.00207.x

Clemente-Jimenez, J. M., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Las Heras-Vázquez, F. J., & Rodríguez-Vico, F. (2004). Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. *Food Microbiology*, *21*(2), 149–155. https://doi.org/10.1016/S0740-0020(03)00063-7

Cocolin, L., Pepe, V., Comitini, F., Comi, G., & Ciani, M. (2004). Enological and genetic traits of Saccharomyces cerevisiae isolated from former and modern wineries. *FEMS Yeast Research*, *5*(3), 237–245. https://doi.org/10.1016/j.femsyr.2004.08.005

Couto, M. M. B., Eijsma, B., Hofstra, H., Huis In'T Veld, J. H. J., & Van Der Vossen, J. M. B. M. (1996). Evaluation of molecular typing techniques to assign genetic diversity among Saccharomyces cerevisiae strains. *Applied and Environmental Microbiology*, *62*(1), 41–46. https://doi.org/10.1128/aem.62.1.41-46.1996

Dagar, S. S., Kumar, S., Mudgil, P., Singh, R., & Puniya, A. K. (2011). D1/D2 domain of large-subunit ribosomal DNA for differentiation of Orpinomyces spp. *Applied and Environmental Microbiology*, *77*(18), 6722–6725. https://doi.org/10.1128/AEM.05441-11

De Benedictis, M., Bleve, G., Tristezza, M., Tufariello, M., & Grieco, F. (2011). An optimized procedure for the enological selection of non-Saccharomyces starter cultures. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *99*(2), 189–200. https://doi.org/10.1007/s10482-010-9475-8

Di Maro, E., Ercolini, D., & Coppola, S. (2007). Yeast dynamics during spontaneous wine fermentation of the Catalanesca grape. *International Journal of Food Microbiology*, *117*(2), 201–210. https://doi.org/10.1016/j.ijfoodmicro.2007.04.007

Di Toro, M. R., Capozzi, V., Beneduce, L., Alexandre, H., Tristezza, M., Durante, M., … Spano, G. (2015). Intraspecific biodiversity and "spoilage potential" of Brettanomyces bruxellensis in Apulian wines. *LWT - Food Science and Technology*, *60*(1), 102–108. https://doi.org/10.1016/j.lwt.2014.06.059

Díaz, C., Molina, A. M., Nähring, J., & Fischer, R. (2013). Characterization and dynamic behavior of wild yeast during spontaneous wine fermentation in steel tanks and amphorae. *BioMed Research International*, *2013*. https://doi.org/10.1155/2013/540465

Domizio, P., Romani, C., Lencioni, L., Comitini, F., Gobbi, M., Mannazzu, I., & Ciani, M. (2011). Outlining a future for non-Saccharomyces yeasts: Selection of putative spoilage wine strains to be used in association with Saccharomyces cerevisiae for grape juice fermentation. *International Journal of Food Microbiology*, *147*(3), 170–180. https://doi.org/10.1016/j.ijfoodmicro.2011.03.020

Echeverrigaray, S., Paese-Toresan, S., & Carrau, J. L. (2000). RAPD marker polymorphism among commercial winery yeast strains. *World Journal of Microbiology and Biotechnology*, *16*(2), 143–146. https://doi.org/10.1023/A:1008993129713

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., & Querol, A. (1999). Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *International Journal of Systematic Bacteriology*, *49*(1), 329–337. https://doi.org/10.1099/00207713-49-1-329

Esteve-Zarzoso, Braulio, Manzanares, P., Ramön, D., & Quero, A. (1998). The role of non-Saccharomyces yeasts in industrial winemaking. *International Microbiology*, *1*(2), 143–148. https://doi.org/10.2436/im.v1i2.59

Esteve-Zarzoso, Braulio, Peris-Torán, M. J., Ramón, D., & Querol, A. (2001). Molecular characterisation of Hanseniaspora species. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *80*(1), 85–92. https://doi.org/10.1023/A:1012268931569

Fernández-Espinar, M. T., López, V., Ramón, D., Bartra, E., & Querol, A. (2001). Study of the authenticity of commercial wine yeast strains by molecular techniques. *International Journal of Food Microbiology*, *70*(1–2), 1–10. https://doi.org/10.1016/S0168-1605(01)00502-5

Fleet, G. H. (2008). Wine yeasts for the future. *FEMS Yeast Research*, *8*(7), 979–995. https://doi.org/10.1111/j.1567-1364.2008.00427.x

Garofalo, C., El Khoury, M., Lucas, P., Bely, M., Russo, P., Spano, G., & Capozzi, V. (2015). Autochthonous starter cultures and indigenous grape variety for regional wine production. *Journal of Applied Microbiology*, *118*(6), 1395–1408. https://doi.org/10.1111/jam.12789

Garofalo, Carmela, Russo, P., Beneduce, L., Massa, S., Spano, G., & Capozzi, V. (2016). Non-Saccharomyces biodiversity in wine and the 'microbial terroir': a survey on Nero di Troia wine from the Apulian region, Italy. *Annals of Microbiology*, *66*(1), 143–150. https://doi.org/10.1007/s13213-015-1090-5

Garofalo, Carmela, Tristezza, M., Grieco, F., Spano, G., & Capozzi, V. (2016). From grape berries to wine: population dynamics of cultivable yeasts associated to "Nero di Troia" autochthonous grape cultivar. *World Journal of Microbiology and Biotechnology*, *32*(4), 1–10. https://doi.org/10.1007/s11274-016-2017-4

Gil-Lamaignere, C., Roilides, E., Hacker, J., & Müller, F.-M. C. (2003). Molecular typing for fungi--a critical review of the possibilities and limitations of currently and future methods. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *9*, 172–185. https://doi.org/10.1046/j.1469-0691.2003.00649.x

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., & Ciani, M. (2013). Lachancea thermotolerans and Saccharomyces cerevisiae in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. *Food Microbiology*, *33*(2), 271–281. https://doi.org/10.1016/j.fm.2012.10.004

González-Arenzana, L., Garijo, P., Berlanas, C., López-Alfaro, I., López, R., Santamaría, P., & Gutiérrez, A. R. (2017). Genetic and phenotypic intraspecific variability of non-Saccharomyces yeasts populations from La Rioja winegrowing region (Spain). *Journal of Applied Microbiology*, *122*(2), 378–388. https://doi.org/10.1111/jam.13341

Guida, M., Cannavacciuolo, P. L., Cesarano, M., Borra, M., Biffali, E., D'Alessandro, R., & De Felice, B. (2014). Microbial diversity of landslide soils assessed by RFLP and SSCP fingerprints: Microbial fingerprint of landslide soil. *Journal of Applied Genetics*, *55*(3), 403–415. https://doi.org/10.1007/s13353-014-0208-y

Hierro, N., González, Á., Mas, A., & Guillamón, J. M. (2004). New PCR-based methods for yeast identification. *Journal of Applied Microbiology*, *97*(4), 792–801. https://doi.org/10.1111/j.1365-2672.2004.02369.x

Javier Gallego, F., Angeles Pérez, M., Núñez, Y., & Hidalgo, P. (2005). Comparison of RAPDs, AFLPs and SSR markers for the genetic analysis of yeast strains of Saccharomyces cerevisiae. *Food Microbiology*, *22*(6), 561–568. https://doi.org/10.1016/j.fm.2004.11.019

Jeyaram, K., Singh, W. M., Capece, A., & Romano, P. (2008). Molecular identification of yeast species associated with "Hamei" - A traditional starter used for rice wine production in Manipur, India. *International Journal of Food Microbiology*, *124*(2), 115–125. https://doi.org/10.1016/j.ijfoodmicro.2008.02.029

Kállai, Z., Pfliegler, W. P., Mitercsák, J., Szendei, G., & Sipiczki, M. (2019). Preservation of diversity and oenological properties of wine yeasts during long-term laboratory maintenance: A study of strains of a century-old Tokaj wine yeast collection. *Lwt*, *101*(November 2018), 789–798. https://doi.org/10.1016/j.lwt.2018.12.002

Kurtzman, Cletus., Fell, J. W., Boekhout, T. (2011). *The Yeasts : A Taxonomic Study* (5th ed.). Elsevier Science & Technology.

Kwaśna, H., Bateman, G. L., & Ward, E. (2010). Microbiota in wheat roots evaluated by cloning of ITS1/2 rDNA and sequencing. *Journal of Phytopathology*, *158*(4), 278–287. https://doi.org/10.1111/j.1439-0434.2009.01595.x

Lamontanara, A., Caggianiello, G., Orrù, L., Capozzi, V., Michelotti, V., Bayjanov, J. R., … Spano, G. (2015). Draft Genome Sequence of Lactobacillus plantarum Lp90 Isolated from Wine. *Genome Announcements*, *3*(6), e00097-15. https://doi.org/10.1128/genomeA.00097-15

Las Heras-Vazquez, F. J., Mingorance-Cazorla, L., Clemente-Jimenez, J. M., & Rodriguez-Vico, F. (2003). Identification of yeast species from orange fruit and juice by RFLP and sequence analysis of the 5.8S rRNA gene and the two internal transcribed spacers. *FEMS Yeast Research*, *3*(1), 3–9. https://doi.org/10.1016/S1567-1356(02)00134-4

Leaw, S., Chang, H., Sun, H., Barton, R., Bouchara, J., & Chang, T. (2006). Identification of Medically Important Yeast Species by Sequence. *J Clin Microbiol*, *44*(3), 693–699. https://doi.org/10.1128/JCM.44.3.693

Lewis, L. A. (1996). Deep-Level Diagnostic Value of the rDNA-ITS Region.

Lieckfeldt, E., Meyer, W., & Börner, T. (1993). Rapid identification and differentiation of yeasts by DNA and PCR fingerprinting. *Journal of Basic Microbiology*, *33*(6), 413–425. https://doi.org/10.1002/jobm.3620330609

Liu, R., Zhang, Q., Chen, F., & Zhang, X. (2015). Analysis of culturable yeast diversity in spontaneously fermented orange wine, orange peel and orangery soil of a Ponkan plantation in China. *Annals of Microbiology*, *65*(4), 2387–2391. https://doi.org/10.1007/s13213-015-1081-6

Madani, M., Vovlas, N., Castillo, P., Subbotin, S. A., & Moens, M. (2004). Molecular characterization of cyst nematode species (Heterodera spp.) from the Mediterranean Basin using RFLPs and sequences of ITS-rDNA. *Journal of Phytopathology*, *152*(4), 229–234. https://doi.org/10.1111/j.1439-0434.2004.00835.x

Martin, K. J., & Rygiewicz, P. T. (2005). Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology*, *5*. https://doi.org/10.1186/1471-2180-5-28

Martorell, P., Barata, A., Malfeito-Ferreira, M., Fernández-Espinar, M. T., Loureiro, V., & Querol, A. (2006). Molecular typing of the yeast species Dekkera bruxellensis and Pichia guilliermondii recovered from wine related sources. *International Journal of Food Microbiology*, *106*(1), 79–84. https://doi.org/10.1016/j.ijfoodmicro.2005.05.014

Mateus, D., Sousa, S., & Rogerson, F. S. (2020). Identification and Characterization of Non-Saccharomyces Species Isolated from Port Wine Spontaneous Fermentations. *Foods*.

Medina, K., Boido, E., Dellacassa, E., & Carrau, F. (2012). Growth of non-Saccharomyces yeasts affects nutrient availability for Saccharomyces cerevisiae during wine fermentation. *International Journal of Food Microbiology*, *157*(2), 245–250. https://doi.org/10.1016/j.ijfoodmicro.2012.05.012

Meyer, W., & Mitchell, T. G. (1995). Polymerase chain reaction fingerprinting in fungi using single primers specific to minisatellites and simple repetitive DNA sequences: Strain variation in Cryptococcus neoformans. *Electrophoresis*, *16*(1), 1648–1656. https://doi.org/10.1002/elps.11501601273

Mitterdorfer, G., Mayer, H. K., Kneifel, W., & Viernstein, H. (2002). Clustering of Saccharomyces boulardii strains within the species S. cerevisiae using molecular typing techniques. *Journal of Applied Microbiology*, *93*(4), 521–530. https://doi.org/10.1046/j.1365-2672.2002.01710.x

Neza Cadez a;b, Peter Raspor a, Arthur W.A.M. de Cock b, T. B. b, & B;*, M. T. S. (2002). Molecular identi¢cation and genetic diversity within species of the genera Hanseniaspora and Kloeckera. *FEMS Yeast Research*, 279–289.

Nisiotou, A. A., & Nychas, G. J. E. (2007). Yeast populations residing on healthy or Botrytis-infected grapes from a vineyard in Attica, Greece. *Applied and Environmental Microbiology*, *73*(8), 2765–2768. https://doi.org/10.1128/AEM.01864-06

Oro, L., Ciani, M., & Comitini, F. (2014). Antimicrobial activity of Metschnikowia pulcherrima on wine yeasts. *Journal of Applied Microbiology*, *116*(5), 1209–1217. https://doi.org/10.1111/jam.12446

Osumi, M. (1998). The Ultrastructure of Yeast : Cell Wall Structure and. *Micron*, *29*(2), 207–233. Retrieved from https://doi.org/10.1016/S0968-4328(97)00072-3

Pfliegler, W. P., Horváth, E., Kállai, Z., & Sipiczki, M. (2014). Diversity of Candida zemplinina isolates inferred from RAPD, micro/minisatellite and physiological analysis. *Microbiological Research*, *169*(5–6), 402–410. https://doi.org/10.1016/j.micres.2013.09.006

Pham, T., Wimalasena, T., Box, W. G., Koivuranta, K., Storgårds, E., Smart, K. A., & Gibson, B. R. (2011). Evaluation of ITS PCR and RFLP for differentiation and identification of brewing yeast and brewery “wild” yeast contaminants. *Journal of the Institute of Brewing*, *117*(4), 556–568. https://doi.org/10.1002/j.2050-0416.2011.tb00504.x

Pincus, D. H., Orenga, S., & Chatellier, S. (2007). Yeast identification - Past, present, and future methods. *Medical Mycology*, *45*(2), 97–121. https://doi.org/10.1080/13693780601059936

Pramateftaki, P. V, Lanaridis, P., & Typas, M. A. (2000). Molecular identification of wine yeasts at species or strain level : a case study with strains from two vine-growing areas of, 236–248.

Raja, H. A., Miller, A. N., Pearce, C. J., & Oberlies, N. H. (2017). Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *Journal of Natural Products*, *80*(3), 756–770. https://doi.org/10.1021/acs.jnatprod.6b01085

Raspor, P., Milek, D. M., Polanc, J., Smole Možina, S., & Čadež, N. (2006). Yeasts isolated from three varieties of grapes cultivated in different locations of the Dolenjska vine-growing region, Slovenia. *International Journal of Food Microbiology*, *109*(1–2), 97–102. https://doi.org/10.1016/j.ijfoodmicro.2006.01.017

Romanelli, A. M., Fu, J., Herrera, M. L., & Wickes, B. L. (2014). A universal DNA extraction and PCR amplification method for fungal rDNA sequence-based identification, 612–622. https://doi.org/10.1111/myc.12208

Roudil, L., Russo, P., Berbegal, C., Albertin, W., Spano, G., & Capozzi, V. (2019). Non-Saccharomyces Commercial Starter Cultures : Scientific Trends , Recent Patents and Innovation in the Wine Sector Non- Saccharomyces Commercial Starter Cultures : Scientific Trends , Recent Patents and Innovation in the Wine Sector, (January). https://doi.org/10.2174/2212798410666190131103713

Sabate, J., Cano, J., Esteve-Zarzoso, B., & Guillamón, J. M. (2002). Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. *Microbiological Research*, *157*(4), 267–274. https://doi.org/10.1078/0944-5013-00163

Spencer, J., Ragout de Spencer, A., & Laluce, C. (2002). Non-conventional yeasts. *Applied Microbiology and Biotechnology*, *58*(2), 147–156. https://doi.org/10.1007/s00253-001-0834-2

Šuranská, H., Vránová, D., & Omelková, J. (2016). Isolation, identification and characterization of regional indigenous Saccharomyces cerevisiae strains. *Brazilian Journal of Microbiology*, *47*(1), 181–190. https://doi.org/10.1016/j.bjm.2015.11.010

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., & Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Australian Journal of Grape and Wine Research*, *11*(2), 139–173. https://doi.org/10.1111/j.1755-0238.2005.tb00285.x

T. ARROYO1, G. Cordero1, A. Serrano1, and E. V. (2010). β-GLUCOSIDASE PRODUCTION BY NON-SACCHAROMYCES YEASTS ISOLATED FROM VINEYARD T. *Expression of Multidisciplinary Flavour Science*.

Tofalo, R., Schirone, M., Telera, G. C., Manetta, A. C., Corsetti, A., & Suzzi, G. (2011). Influence of organic viticulture on non-Saccharomyces wine yeast populations. *Annals of Microbiology*, *61*(1), 57–66. https://doi.org/10.1007/s13213-010-0102-8

Tristezza, M., Vetrano, C., Bleve, G., Grieco, F., Tufariello, M., Quarta, A., … Spano, G. (2012). Autochthonous fermentation starters for the industrial production of Negroamaro wines. *Journal of Industrial Microbiology and Biotechnology*, *39*(1), 81–92. https://doi.org/10.1007/s10295-011-1002-z

Tristezza, M., Vetrano, C., Bleve, G., Spano, G., Capozzi, V., Logrieco, A., … Grieco, F. (2013). Biodiversity and safety aspects of yeast strains characterized from vineyards and spontaneous fermentations in the Apulia Region, Italy. *Food Microbiology*, *36*(2), 335–342. https://doi.org/10.1016/j.fm.2013.07.001

Van Der Aa Kühle, A., & Jespersen, L. (2003). The Taxonomic Position of Saccharomyces boulardii as Evaluated by Sequence Analysis of the D1/D2 Domain of 26S rDNA, the ITS1-5.8S rDNA-ITS2 Region and the Mitochondrial Cytochrome-c Oxidase II Gene. *Systematic and Applied Microbiology*, *26*(4), 564–571. https://doi.org/10.1078/072320203770865873

Viana, F., Belloch, C., Vallés, S., & Manzanares, P. (2011). Monitoring a mixed starter of Hanseniaspora vineae-Saccharomyces cerevisiae in natural must: Impact on 2-phenylethyl acetate production. *International Journal of Food Microbiology*, *151*(2), 235–240. https://doi.org/10.1016/j.ijfoodmicro.2011.09.005

Walczak, E., Czaplińska, A., Barszczewski, W., Wilgosz, M., Wojtatowicz, M., & Robak, M. (2007). RAPD with microsatellite as a tool for differentiation of Candida genus yeasts isolated in brewing. *Food Microbiology*, *24*(3), 305–312. https://doi.org/10.1016/j.fm.2006.04.012

Walker, G. M., & White, N. A. (2005). Introduction to Fungal Physiology. *Fungi: Biology and Applications*, 1–34. https://doi.org/10.1002/0470015330.ch1

ZHANG, H. Y., LEE, S. A., BRADBURY, J. E., WARREN, R. N., SHETH, H., HOOKS, D. O., … GARDNER, R. C. (2010). Yeasts isolated from New Zealand vineyards and wineries. *Australian Journal of Grape and Wine Research*, *16*(3), 491–496. https://doi.org/10.1111/j.1755-0238.2010.00111.x